

Creatine and Creatinine Metabolism

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Wyss, Markus, and Rima Kaddurah-Daouk. Creatine and Creatinine Metabolism. *Physiol Rev* 80: 1107–1213, 2000.—The goal of this review is to present a comprehensive survey of the many intriguing facets of creatine (Cr) and creatinine metabolism, encompassing the pathways and regulation of Cr biosynthesis and degradation, species and tissue distribution of the enzymes and metabolites involved, and of the inherent implications for physiology and human pathology. Very recently, a series of new discoveries have been made that are bound to have distinguished implications for bioenergetics, physiology, human pathology, and clinical diagnosis and that suggest that deregula-

tion of the creatine kinase (CK) system is associated with a variety of diseases. Disturbances of the CK system have been observed in muscle, brain, cardiac, and renal diseases as well as in cancer. On the other hand, Cr and Cr analogs such as cyclocreatine were found to have antitumor, antiviral, and antidiabetic effects and to protect tissues from hypoxic, ischemic, neurodegenerative, or muscle damage. Oral Cr ingestion is used in sports as an ergogenic aid, and some data suggest that Cr and creatinine may be precursors of food mutagens and uremic toxins. These findings are discussed in depth, the interrelationships are outlined, and all is put into a broader context to provide a more detailed understanding of the biological functions of Cr and of the CK system.

I. INTRODUCTION

Ever since the discovery of phosphorylcreatine (PCr) in 1927 and of the creatine kinase (CK; EC 2.7.3.2) reaction in 1934 (see Refs. 140, 833), research efforts focused mainly on biochemical, physiological, and pathological aspects of the CK reaction itself and on its involvement in "high-energy phosphate" metabolism of cells and tissues with high-energy demands. In contrast, Cr (from greek *kreas*, flesh) metabolism in general has attracted considerably less attention. In recent years, however, a series of fascinating new discoveries have been made. For instance, Cr analogs have proven to be potent anticancer agents that act synergistically with currently used chemotherapeutics. Cyclocreatine, one of the Cr analogs, as well as PCr protect tissues from ischemic damage and may therefore have an impact on organ transplantation. Circumstantial evidence suggests a link between disturbances in Cr metabolism and muscle diseases as well as neurological disorders, and beneficial effects of oral Cr supplementation in such diseases have in fact been reported. Oral Cr ingestion has also been shown to increase athletic performance, and it therefore comes as no surprise that Cr is currently used by many athletes as a performance-boosting supplement. Some data suggest that Cr and creatinine (Crn) may act as precursors of food mutagens and uremic toxins. Finally, the recent identification, purification, and cloning of many of the enzymes involved in Cr metabolism have just opened the door to a wide variety of biochemical, physiological, as well as clinical investigations and applications.

The goal of this article is to provide a comprehensive overview on the physiology and pathology of Cr and Crn metabolism. Because some of these aspects have already been covered by earlier reviews (e.g., Refs. 55, 669, 1056, 1077), preference will be given to more recent developments in the field. The text is written in a modular fashion, i.e., despite the obvious fact that complex interrelationships exist between different parts of the text, every section should, by and large, be self-explanatory. It is our hope that this review will stimulate future multidisciplinary research on the physiological functions of the CK system, on the pathways and regulation of Cr metabolism, and on the relationships between disturbances in Cr metabolism and human disease.

II. ABBREVIATIONS

Cr	Creatine
Crn	Creatinine
PCr	Phosphorylcreatine
CK	Creatine kinase
Mi-CK	Mitochondrial CK isoenzyme
B-CK	Cytosolic brain-type CK isoenzyme
M-CK	Cytosolic muscle-type CK isoenzyme
AGAT	L-Arginine:glycine amidinotransferase
GAMT	S-adenosyl-L-methionine:N-guanidinoacetate methyltransferase
GPA	Guanidinopropionate, if not otherwise mentioned, the 3-guanidinopropionate or β -guanidinopropionate isomer
GBA	Guanidinobutyrate
cCr	Cyclocreatine = 1-carboxymethyl-2-iminoimidazolidine
hcCr	Homocyclocreatine = 1-carboxyethyl-2-iminoimidazolidine
Gc	Glycocyamine = guanidinoacetate
Tc	Taurocyamine
L	Lombricine
PCrn, PGPA, PcCr, PhcCr, PArg, PGc, PTc, PL	N-phosphorylated forms of the respective guanidino compounds
ArgK	Arginine kinase
DNFB	2,4-Dinitrofluorobenzene
AdoMet	S-adenosyl-L-methionine
GSH	Reduced glutathione
GSSG	Oxidized glutathione
OAT	L-Ornithine:2-oxoacid aminotransferase

III. THE PHYSIOLOGICAL RELEVANCE OF CREATINE: THE CREATINE KINASE REACTION

To understand why nature has "developed" reaction pathways for the biosynthesis of PCr and of other phosphagens, one must briefly explain the main functions proposed for the CK/PCr/Cr system (for a detailed discus-

sion and for references, see Refs. 837, 838, 1084, 1124). In textbooks of biochemistry, the participation of the CK/PCr/Cr system in energy metabolism is often neglected, and it is tacitly assumed that high-energy phosphate transport between sites of ATP production (mitochondria, glycolysis) and ATP consumption (all sorts of cellular ATPases) relies on diffusion of ATP and ADP alone. This concept may reflect the situation in tissues devoid of CK and PCr, like liver, but is clearly inadequate for CK-containing tissues with high and fluctuating energy demands like skeletal or cardiac muscle, brain, retina, and spermatozoa. In these latter tissues of mammals and birds, four distinct types of CK subunits are expressed species specifically, developmental stage specifically, and tissue specifically. The cytosolic M-CK (M for muscle) and B-CK (B for brain) subunits form dimeric molecules and thus give rise to the MM-, MB-, and BB-CK isoenzymes. The two mitochondrial CK isoforms, ubiquitous Mi-CK and sarcomeric Mi-CK, are located in the mitochondrial intermembrane space and form both homodimeric and homooctameric molecules that are readily interconvertible. All CK isoenzymes catalyze the reversible transfer of the γ -phosphate group of ATP to the guanidino group of Cr to yield ADP and PCr (Fig. 1).

In fast-twitch skeletal muscles, a large pool of PCr is available for immediate regeneration of ATP hydrolyzed during short periods of intense work. Because of the high cytosolic CK activity in these muscles, the CK reaction remains in a near-equilibrium state, keeps [ADP] and [ATP] almost constant (over several seconds), and thus "buffers" the cytosolic phosphorylation potential that seems to be crucial for the proper functioning of a variety of cellular ATPases.

Heart, slow-twitch skeletal muscles, or spermatozoa, on the other hand, depend on a more continuous delivery

of high-energy phosphates to the sites of ATP utilization. According to the "transport" ("shuttle") hypothesis for the CK system, distinct CK isoenzymes are associated with sites of ATP production (e.g., Mi-CK in the mitochondrial intermembrane space) and ATP consumption [e.g., cytosolic CK bound to the myofibrillar M line, the sarcoplasmic reticulum (SR), or the plasma membrane] and fulfill the function of a "transport device" for high-energy phosphates. The γ -phosphate group of ATP, synthesized within the mitochondrial matrix, is transferred by Mi-CK in the mitochondrial intermembrane space to Cr to yield ADP plus PCr. ADP liberated by the Mi-CK reaction may directly be transported back to the matrix where it is rephosphorylated to ATP. PCr leaves the mitochondria and diffuses through the cytosol to the sites of ATP consumption. There cytosolic CK isoenzymes locally regenerate ATP and thus warrant a high phosphorylation potential in the intimate vicinity of the respective ATPases. Cr thus liberated diffuses back to the mitochondria, thereby closing the cycle. According to this hypothesis, transport of high-energy phosphates between sites of ATP production and ATP consumption is achieved mainly (but not exclusively) by PCr and Cr. Whereas for the buffer function, no Mi-CK isoenzyme is required, Mi-CK may be a prerequisite for efficient transport of high-energy phosphates, especially if diffusion of adenine nucleotides across the outer mitochondrial membrane were limited (see sect. IVB). In accordance with these ideas, the proportion of Mi-CK seems to correlate with the oxidative capacity of striated muscles. It is by far higher in heart (up to 35% of total CK activity) than in fast-twitch skeletal muscles (0.5–2%).

Although the shuttle hypothesis seems logical and intelligible on first sight, there is an ongoing debate on whether it accurately describes the function of the CK

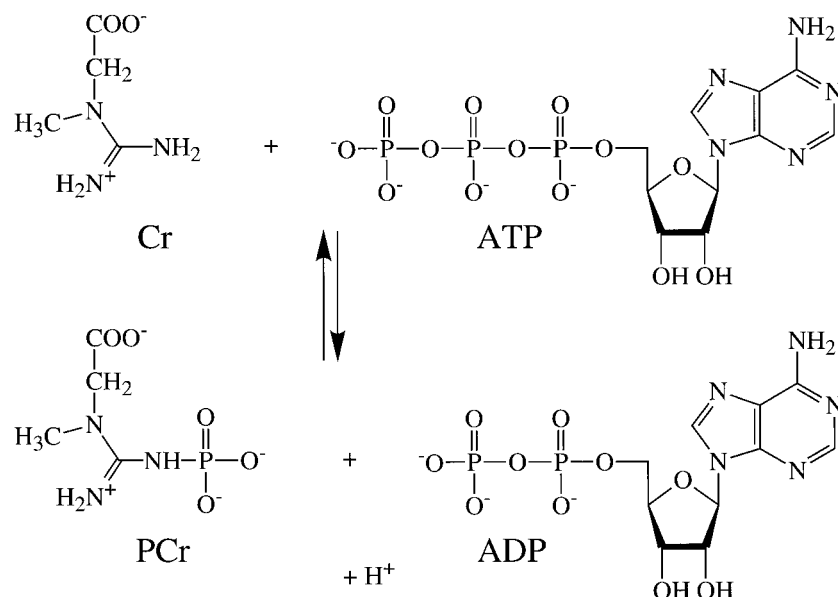


FIG. 1. The creatine kinase (CK) reaction. PCr, phosphorylcreatine; Cr, creatine.

system in endurance-type tissues (524, 837, 964). Therefore, the buffer and transport models for CK function should be regarded neither as strictly true nor as static views that can be applied directly to any one tissue; rather, the CK system displays a high degree of flexibility and is able to adapt to the peculiar physiological requirements of a given tissue. In skeletal muscle, for example, an adaptation of the CK system from a more buffer to a more transport type can be induced by endurance training or by chronic electrical stimulation (26, 861).

PCr and Cr, relative to ATP and ADP, are smaller and less negatively charged molecules and can build up to much higher concentrations in most CK-containing cells and tissues, thereby allowing for a higher intracellular flux of high-energy phosphates. Furthermore, the change in free energy ($\Delta G^{\circ'}$) (pH 7.0) for the hydrolysis of PCr is

−45.0 kJ/mol compared with −31.8 kJ/mol for ATP, implying that in tissues with an active CK system, the cytosolic phosphorylation potential can be buffered at a higher level than in tissues devoid of the CK system. This factor may, again, be essential for the proper functioning of at least some cellular ATPases, e.g., the Ca^{2+} -ATPase of the SR (see Ref. 646). Finally, by keeping [ADP] low, the CK/PCr/Cr system may also protect the cell from a net loss of adenine nucleotides via adenylate kinase, AMP deaminase, and 5'-nucleotidase.

IV. CREATINE METABOLISM IN VERTEBRATES

Although the pathways of Cr metabolism in vertebrates seem simple (Fig. 2), the situation is complicated

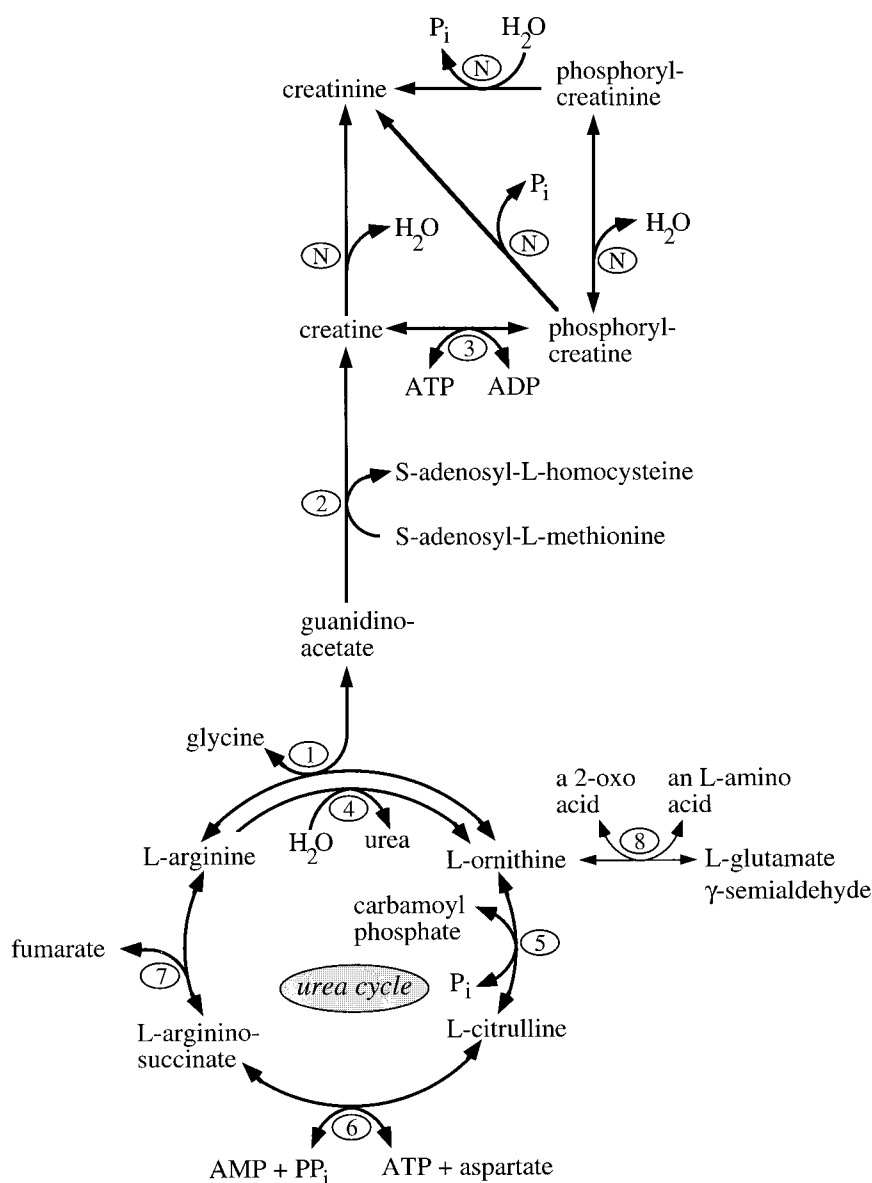


FIG. 2. Schematic representation of the reactions and enzymes involved in vertebrate creatine and creatinine metabolism. The respective enzymes are denoted by numbers: 1) L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1); 2) S-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2); 3) creatine kinase (CK; EC 2.7.3.2); 4) arginase (L-arginine amidinohydrolase; EC 3.5.3.1); 5) ornithine carbamoyltransferase (EC 2.1.3.3); 6) argininosuccinate synthase (EC 6.3.4.5); 7) argininosuccinate lyase (EC 4.3.2.1); 8) L-ornithine:2-oxo-acid aminotransferase (OAT; EC 2.6.1.13); N) nonenzymatic reaction.

by the fact that most tissues lack several of the enzymes required, thus necessitating transport of intermediates between the tissues through the blood to allow the whole cascade of reactions to proceed. In mammals, for instance, a complete urea cycle operates actively only in liver. The main site of Arg biosynthesis for other bodily tissues is, however, the kidney. Citrulline, synthesized in the liver or small intestine and transported through the blood, is taken up by the kidney and converted into Arg mainly by the proximal tubule of the nephron (273, 553). Arg formed within the kidney is then either released into the blood and consumed by other tissues or used within the kidney itself for guanidinoacetate synthesis.

A. Biosynthesis and Tissue Uptake of Cr

The transfer of the amidino group of Arg to Gly to yield L-ornithine and guanidinoacetic acid (GAA) represents the first of two steps in the biosynthesis of Cr (Fig. 3) and is catalyzed by L-arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1). GAA, by the action of *S*-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), is then methylated at the amidino group to give Cr (M_r 131.1). In the course of evolution, both AGAT and GAMT seem to have evolved with the appearance of the lampreys (1056). These enzyme activities were not detected in invertebrates, whereas they are found in most but not all vertebrates examined. Some invertebrate species (e.g., some annelids, echinoderms, hemichordates, and urochordates) nevertheless contain significant amounts of Cr, PCr, and CK, particularly in spermatozoa (226, 811, 1056, 1092). This implies that these species either accumulate Cr from the environment or from the feed, or that the enzymes for Cr biosynthesis in these animals escaped detection so far.

Many of the lower vertebrates (fish, frogs, and birds) have both AGAT and GAMT in their livers and often also in the kidneys (see Refs. 634, 1056, 1077). In mammals, pancreas contains high levels of both enzymes, whereas

kidneys express fairly high amounts of AGAT but relatively lower levels of GAMT. On the contrary, livers of all mammalian species tested so far contain high amounts of GAMT but display only low levels of Cr and almost completely lack CK activity. Although livers of cow, pig, monkey, and human also have high amounts of AGAT, livers of common laboratory mammals such as the rat, mouse, dog, cat, and rabbit were reported to lack AGAT activity. On the basis mostly of these latter findings and of the fact that the rate of Cr biosynthesis is considerably reduced in nephrectomized animals (248, 291, 554), it was postulated, and is still largely accepted, that the main route of Cr biosynthesis in mammals involves formation of guanidinoacetate in the kidney, its transport through the blood, and its methylation to Cr in the liver (Fig. 4). Cr exported from the liver and transported through the blood may then be taken up by the Cr-requiring tissues.

Several lines of experimental evidence, however, demonstrate that this concept of the organization of Cr metabolism is too simplistic. Pyridoxine-deficient rats, despite a 65% decrease in kidney AGAT activity relative to controls, displayed increased liver and skeletal muscle concentrations of Cr (572). In another study, extrarenal synthesis was suggested to account for 40–60% of total Cr (290). Similarly, comparison of the hepatic and renal venous levels with the arterial levels of Arg, GAA, and Cr suggested that in humans, the liver is the most important organ contributing to biosynthesis of both GAA and Cr, whereas the kidney plays only a secondary role (842). In accordance with these observations, immunofluorescence microscopy revealed significant amounts of AGAT not only in rat kidney and pancreas, but also in liver (623). The apparent discrepancy from earlier investigations may be explained by the high levels of liver arginase interfering with AGAT activity assays. Furthermore, AGAT activity was detected in heart, lung, spleen, muscle, brain, testis, and thymus, and it has been estimated that the total amount of AGAT in these tissues approaches that found in kidney and pancreas (769, 1055). Although AGAT is absent from human placenta, the decidua of pregnant females displayed the highest specific AGAT activity of all rat tissues examined (1077), implying a major involvement of this tissue in Cr biosynthesis during early stages of development. In line with this conclusion, maternofetal transport of Cr was demonstrated in the rat (157).

On the other hand, GAMT mRNA and protein levels higher than those observed in male liver were detected in mouse testis, caput epididymis, and ovary (see Ref. 543). Likewise, Sertoli cells of rat seminiferous tubules, in contrast to germ cells and interstitial cells, were shown to synthesize guanidinoacetate and Cr from Arg and Gly (664). It may therefore be hypothesized that the Cr-synthesizing machinery in reproductive organs plays a role in the development or function of germ cells. GAMT activity was also detected in rat spleen, heart, and skeletal mus-

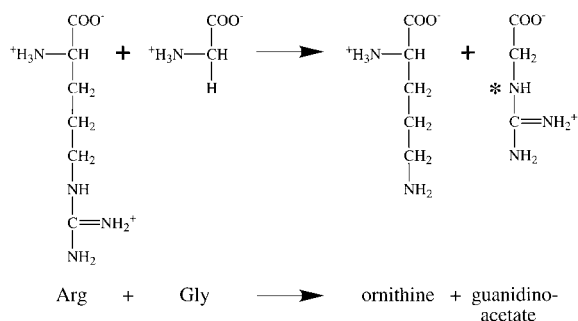


FIG. 3. The AGAT reaction. The asterisk indicates the nitrogen atom to which a methyl group from *S*-adenosyl-L-methionine is transferred by GAMT to yield Cr.

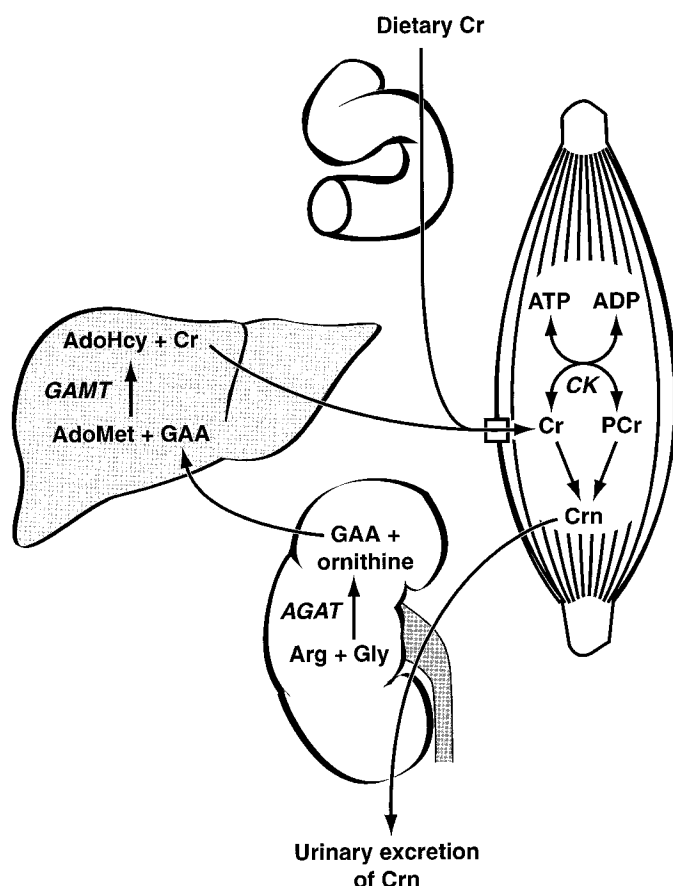


FIG. 4. Major routes of Cr metabolism in the mammalian body. The most part (up to 94%) of Cr is found in muscular tissues. Because muscle has virtually no Cr-synthesizing capacity, Cr has to be taken up from the blood against a large concentration gradient by a saturable, Na^+ - and Cl^- -dependent Cr transporter that spans the plasma membrane (\square). The daily demand for Cr is met either by intestinal absorption of dietary Cr or by de novo Cr biosynthesis. The first step of Cr biosynthesis probably occurs mainly in the kidney, whereas the liver is likely to be the principal organ accomplishing the subsequent methylation of guanidinoacetic acid (GAA) to Cr. It must be stressed that the detailed contribution of different bodily tissues (pancreas, kidney, liver, testis) to total Cr synthesis is still rather unclear and may vary between species (see text). The muscular Cr and PCr are nonenzymatically converted at an almost steady rate ($\sim 2\%$ of total Cr per day) to creatinine (Crn), which diffuses out of the cells and is excreted by the kidneys into the urine.

cle, in sheep muscle, as well as in human fetal lung fibroblasts and mouse neuroblastoma cells (149, 1130, 1135, 1136). Although the specific activities in these tissues are rather low, the GAMT activity in skeletal muscle was calculated to have the potential to synthesize all Cr needed in this tissue (149). Finally, feeding of rats and mice with 3-guanidinopropionic acid (GPA), a competitive inhibitor of Cr entry into cells, progressively decreased the concentrations of Cr and PCr in heart and skeletal muscle but had only little influence on the Cr and PCr contents of brain (372). One possible explanation is that the brain contains its own Cr-synthesizing machinery (171). To conclude, the detailed contribution of the various tissues of the body to total Cr biosynthesis as well as

the relevance of guanidinoacetate and Cr transport between the tissues are still rather unclear; this is due both to a lack of thorough investigations and to the pronounced species differences observed so far.

A specific, saturable, Na^+ - and Cl^- -dependent Cr transporter responsible for Cr uptake across the plasma membrane has been described for skeletal muscle, heart, smooth muscle, fibroblasts, neuroblastoma and astroglia cells, as well as for red blood cells and macrophages (149, 150, 250, 570, 659, 711, 876, 965). These findings have recently been corroborated by cDNA cloning and Northern blot analysis of the rabbit, rat, mouse, and human Cr transporters (295, 319, 415, 543, 691, 697, 840, 860, 927). Although the quantitative results of these latter studies differ to some extent, the highest amounts of Cr transporter mRNA seem to be expressed in kidney, heart, and skeletal muscle; somewhat lower amounts in brain, small and large intestine, vas deferens, seminal vesicles, epididymis, testis, ovary, oviduct, uterus, prostate, and adrenal gland; and only very low amounts or no Cr transporter mRNA at all in placenta, liver, lung, spleen, pancreas, and thymus.

An important aspect of Cr biosynthesis to add is that in humans, the daily utilization of methyl groups in the GAMT reaction approximately equals the daily intake of "labile" methyl groups (Met + choline) on a normal, equilibrated diet (671). Even if de novo Met biosynthesis also is taken into account, Cr biosynthesis still accounts for $\sim 70\%$ of the total utilization of labile methyl groups in the body. Upon lowering of the Met and choline levels in the diet, the deficit in labile methyl groups is compensated for by increased de novo Met biosynthesis, indicating that the delivery of labile methyl groups, in the form of *S*-adenosyl-L-methionine, should normally not become limiting for Cr biosynthesis. It may do so, however, in folic acid and/or vitamin B_{12} deficiency (231, 945) as well as in other physiological and pathological conditions that are characterized by an impairment of *S*-adenosyl-L-methionine synthesis (e.g., Refs. 118, 122, 188, 243).

B. Tissue Concentrations and Subcellular Distribution of Cr and PCr

The highest levels of Cr and PCr are found in skeletal muscle, heart, spermatozoa, and photoreceptor cells of the retina. Intermediate levels are found in brain, brown adipose tissue, intestine, seminal vesicles, seminal vesicle fluid, endothelial cells, and macrophages, and only low levels are found in lung, spleen, kidney, liver, white adipose tissue, blood cells, and serum (61, 127, 175, 525, 547, 568, 570, 693, 759, 1080, 1082, 1083, 1108, 1136). A fairly good correlation seems to exist between the Cr transporter mRNA level and total CK activity which, in turn, also correlates with the tissue concentration of total Cr

(Cr + PCr; Fig. 5). There may be only two exceptions. 1) Kidney displays a much higher Cr transporter content than expected from its CK activity (Fig. 5A, ■), which might be due to an involvement of the Cr transporter in the resorption of Cr from the primary urine. 2) Liver has a considerably lower CK activity than expected from its Cr content (Fig. 5B, ▲), which may be an expression of a strict separation between Cr-synthesizing and CK-expressing tissues in the body. Such a separation may be a crucial prerequisite for independent regulation of Cr biosynthesis on one hand and CK function/energy metabolism on the other hand.

Resting type 2a and 2b skeletal muscle fibers of rodents contain ~32 mM PCr and 7 mM Cr, whereas type 1 fibers comprise ~16 mM PCr and 7 mM Cr (525). The difference in PCr concentration between type 1 and type 2 muscle fibers is less pronounced in humans (337, 844, 1042); nevertheless, the concentration of total Cr seems to parallel the muscle glycolytic capacity in both rodents and humans. In serum and erythrocytes, as opposite extremes, [Cr] amounts to only 25–100 μ M and 270–400 μ M, respectively (175, 776, 1137), implying that Cr has to be accumulated by most Cr-containing tissues against a large concentration gradient from the blood. This accumulation

via the Cr transporter is driven by the electrochemical potential differences of extracellular versus intracellular $[\text{Na}^+]$ and $[\text{Cl}^-]$.

Because both seminal vesicles and seminal vesicle fluid of the rat and mouse contain considerable quantities of Cr and PCr, it was hypothesized “that both compounds are actively secreted by the seminal vesicle epithelium” (542). This hypothesis later turned out to be incorrect, in as far as seminal vesicles were shown to lack AGAT and GAMT but to contain moderate amounts of Cr transporter mRNA (543). Therefore, seminal vesicles most likely accumulate Cr from the blood.

For PCr and Cr, a single cytosolic pool is assumed by most researchers, especially by those who postulate near-equilibrium conditions for the CK reaction throughout the cell. However, tracer studies with ^{14}C Cr suggested distinct cytosolic pools of Cr in rat heart (850) and fast-twitch (white) muscle of the rainbow trout (369). In addition, quantitative X-ray microanalysis revealed that phosphorus compounds (presumably represented mostly by PCr and ATP) are highly compartmentalized in sarcomeric muscle, with a preferential occupancy of the I band as well as the H zone (549). Surprisingly, some researchers detected Cr and PCr in the matrix of heart mitochondria and provided evidence that PCr uptake into the mitochondrial matrix is mediated by the adenine nucleotide translocase (see Refs. 391, 796, 921). In the light of 1) the lower phosphorylation potential within the mitochondrial matrix compared with the cytosol and 2) the lack of Cr-utilizing processes in the mitochondrial matrix, it is questionable whether and to what extent Cr accumulation in the matrix is physiologically relevant or is due to postmortem or other artifacts. Clearly, further studies are needed to get a deeper insight into the subcellular compartmentation of Cr and PCr.

C. Degradation of Cr and PCr in Vertebrates

The degradation of Cr and PCr in vertebrates is, for the most part, a spontaneous, nonenzymatic process, as indicated in the top part of Figure 2. In vitro, the equilibrium of the reversible and nonenzymatic cyclization of Cr to creatinine ($\text{Cr} \leftrightarrow \text{Crn}$) is both pH dependent and temperature dependent. Cr is favored at high pH and low temperature, whereas Crn (M_r 113.1) is favored at elevated temperatures and in acidic solutions (see Ref. 551). In both directions, the reaction is monomolecular. Starting with pure Cr solutions, 1.0–1.3% of the Cr per day is converted into Crn at pH 7.0–7.2 and 38°C. In vitro studies on the stability of PCr revealed that this high-energy phosphate compound is acid labile, yielding P_i and either Cr or Crn upon hydrolysis. Both the rate of PCr hydrolysis and the ratio of Cr to Crn formed depend on temperature and pH and can additionally be influenced in a concen-

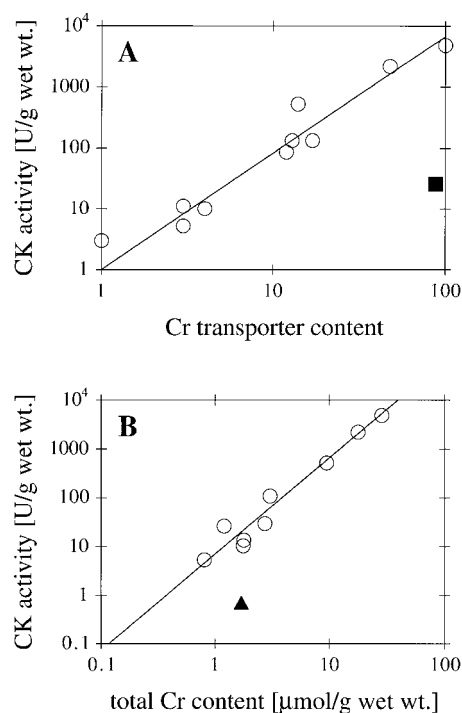


FIG. 5. Correlations between Cr transporter level, CK activity, and total Cr content in different mammalian (rat, human, cat, dog, rabbit, mouse, and guinea pig) tissues. The respective tissues are, from left to right, as follows: A: pancreas, spleen, ovary, lung, small intestine, prostate, brain, colon, heart, kidney (■), and skeletal muscle. B: spleen, kidney, liver (▲), smooth muscle (carotid artery), macrophages, brown adipose tissue, uterus, brain, heart, and skeletal muscle. (Data were taken from Refs. 56, 61, 129, 137, 172, 227, 529, 569, 570, 691, 1020, 1030.)

tration-dependent manner by molybdate (for reviews, see Refs. 226, 669).

In contrast to these *in vitro* studies, experiments with ^{15}N -labeled compounds clearly showed that the conversion of Cr into Crn *in vivo* is an irreversible process (72). Upon feeding of rats with ^{15}N Cr, the isotopically labeled Cr distributed homogeneously over the total Cr pool in the body as well as over the urinary Crn. Even after 5 days, the specific labeling of the urinary Crn and the body Cr were still the same, suggesting that Cr is the only precursor of Crn. Upon feeding with ^{15}N Crn, however, most of the label was directly excreted into the urine, and no significant exchange of the label with the body Cr was observed. In accordance with *in vitro* studies, an almost constant fraction of the body Cr (1.1%/day) and PCr (2.6%/day) is converted nonenzymatically into Crn *in vivo*, giving an overall conversion rate for the total Cr pool (Cr + PCr) of $\sim 1.7\%$ /day (for a review, see Ref. 1077). Consequently, in a 70-kg man containing ~ 120 g of total Cr, roughly 2 g/day are converted into Crn and have to be replaced by Cr from the diet or from *de novo* biosynthesis (Fig. 4) (1050, 1077, 1085). With the assumption of an average content in muscle of 30 mM of total Cr (see above) and a quantitative uptake of the compound by the digestive tract, this loss could be compensated by ingestion of 500 g of raw meat per day. Because Crn is a very poor substrate of the Cr transporter (318, 319, 691), because no other specific saturable uptake mechanism exists for Crn (515), and because Crn, most likely due to its nonionic nature, is membrane permeable, Crn constantly diffuses out of the tissues into the blood and is excreted by the kidneys into the urine (Fig. 4) (759). Because the rate of nonenzymatic formation of Crn from Cr is nearly constant, and because $>90\%$ of the total bodily Cr is to be found in muscle tissue, 24-h urinary Crn excretion is frequently used as a rough measure of total muscle mass (768, 1067). However, this approach suffers various limitations.

Twenty to twenty-five percent of the *in vivo* conversion of PCr into Crn may proceed via phosphorylcreatine (PCrn) as an intermediate (414). Accordingly, [PCrn] in rabbit white skeletal muscle was found to be 0.4% of [PCr], and commercial preparations of PCr (at least several years ago) contained 0.3–0.7% of PCrn.

Crn in mammals, and especially in humans, is still widely believed to be an inert substance that is excreted as such into the urine. Several lines of evidence, however, contradict this view. Using radiolabeled Crn, Boroujerdi and Mattocks (83) showed that in rabbits, some Crn is converted into Cr, Arg, guanidinobutyrate, or guanidinopropionate. Additional routes of Crn degradation become favored in states of renal insufficiency and seem to be relevant for human pathology. They are therefore discussed in detail in section IXH.

V. REGULATION OF CREATINE METABOLISM IN VERTEBRATES

In keeping with the rather complex organization of Cr biosynthesis and degradation in vertebrates, a variety of potential regulatory mechanisms have to be considered, for instance, allosteric regulation, covalent modification, or changes in expression levels of the enzymes involved in Cr metabolism. In addition, changes in the transport capacity and/or permeability of biological membranes for the intermediary metabolites, i.e., Cr, Crn, and guanidinoacetate, are also expected to have an impact on Cr metabolism as a whole (for an extensive review, see Ref. 1077).

A. Regulation of L-Arginine:glycine Amidinotransferase Expression and Activity

The formation of guanidinoacetate is normally the rate-limiting step of Cr biosynthesis (see Ref. 1077). Consequently, the AGAT reaction is the most likely control step in the pathway, a hypothesis that is supported by a great deal of experimental work. Most important in this respect is the feedback repression of AGAT by Cr, the end-product of the pathway, which most probably serves to conserve the dietary essential amino acids Arg and Met. Circumstantial evidence indicates that in folic acid deficiency, where Cr biosynthesis is curtailed and the serum concentration of Cr is likely to be decreased, AGAT expression is upregulated (187). In contrast, an increase in the serum concentration of Cr, due either to an endogenous source or to dietary Cr supplementation, results in concomitant decreases in the mRNA content, the enzyme level, and the enzymatic activity of AGAT, thus suggesting regulation of AGAT expression at a pretranslational level (322, 1053; for a review, see Ref. 1077). Feedback repression of AGAT by Cr is most pronounced in kidney and pancreas, the main tissues of guanidinoacetate formation, but is also observed in the decidua of pregnant rats (see Ref. 1077). Immunological studies suggest the presence of multiple forms (or isoenzymes) of AGAT in rat kidney, of which only some are repressible by Cr, whereas others are not (314). Because the half-life of AGAT in rat kidney is 2–3 days (624), the changes in the AGAT levels described here are rather slow processes, thus only allowing for long-term adaptations.

Cyclocreatine, *N*-acetimidoylsarcosine, and *N*-ethylguanidinoacetate display repressor activity like Cr, whereas Crn, PCr, *N*-propylguanidinoacetate, *N*-methyl-3-guanidinopropionate, *N*-acetimidoylglycine, and a variety of other compounds are ineffective (809, 1077). L-Arg and guanidinoacetate have only “apparent” repressor activity. They exert no effect on AGAT expression by themselves but are readily converted to Cr, which then acts as the true repressor.

In addition to Cr, the expression of AGAT may be modulated by dietary and hormonal factors (for reviews, see Refs. 634, 1053, 1054, 1077). Thyroidectomy or hypophysectomy of rats decreases AGAT activity in the kidney. The original AGAT activity can be restored by injection of thyroxine or growth hormone, respectively. In contrast, injections of growth hormone into thyroidectomized rats and of thyroxine into hypophysectomized rats are without effect, indicating that both hormones are necessary for maintaining proper levels of AGAT in rat kidney. Because enzyme activity, protein, and mRNA contents are always affected to the same extent, regulation of AGAT expression by thyroid hormones and growth hormone occurs at a pretranslational level, very similar to the feedback repression by Cr (322, 625, 1053). Growth hormone and Cr have an antagonistic action on AGAT expression, as evidenced by identical mRNA levels and enzymatic activities of kidney AGAT in hypophysectomized rats simultaneously fed Cr and injected with growth hormone compared with hypophysectomized rats receiving neither of these compounds (322, 1053).

AGAT levels in liver, pancreas, and kidney are also decreased in conditions of dietary deficiency and disease (fasting, protein-free diets, vitamin E deficiency, or streptozotocin-induced diabetes) (273, 1057). These findings seem, however, not to rely directly on the dietary or hormonal imbalance that is imposed. For example, insulin administration to streptozotocin-diabetic rats does not restore the original AGAT activity in the kidney (273). On the contrary, fasting and vitamin E deficiency are characterized by an increased blood level of Cr (248, 480; see also Ref. 1077) which, in all likelihood, represents the true signal for the downregulation of AGAT expression.

Finally, AGAT levels in rat kidney, testis, and decidua may also be under the control of sex hormones, with estrogens and diethylstilbestrol decreasing and testosterone increasing AGAT levels (449; see also Ref. 1077). Oral administration of methyltestosterone to healthy humans not only stimulates AGAT expression and, thus, Cr biosynthesis, but also results in a 70% increase in the urinary excretion of guanidinoacetate (367). This finding might be taken to indicate that at increased levels of AGAT activity, GAMT becomes progressively rate limiting for Cr biosynthesis, thereby leading to an accumulation of guanidinoacetate in the blood. In conflict with this interpretation, dietary Cr supplementation, which is known to decrease AGAT levels in kidney and pancreas, also results in increased urinary guanidinoacetate excretion. Furthermore, guanidinoacetate excretion is much higher when Cr and guanidinoacetate are administered simultaneously than when Cr or guanidinoacetate is given alone (368). Therefore, it is more likely that in situations of elevated Cr concentrations in the blood, the increased levels of Cr in the primary filtrate compete with guanidinoacetate for reabsorption by the kidney tubules (see Ref. 1077).

Based on the findings that GAMT expression in the mouse is highest in testis, caput epididymis, ovary, and liver, and that GAMT expression is higher in female than in male liver, it has been hypothesized that GAMT expression might also be under the control of sex hormones (545). However, removal of either adrenals, pituitaries, gonads, or thyroids and parathyroids or administration of large doses of insulin, estradiol, testosterone, cortisol, thyroxine, or growth hormone had, if any, only minor effects on GAMT activity in rat liver (109). There is some indication that GAMT activity in the liver may be influenced by dietary factors (1019).

In contrast to the described repression by Cr of AGAT in kidney and pancreas, Cr does not interfere with the expression of GAMT or arginase in liver. Cr, Crn, and PCr also do not regulate allosterically the enzymatic activities of AGAT or GAMT *in vitro* (1077). In contrast, AGAT is potently inhibited by ornithine, which may be pathologically relevant, for instance, in gyrate atrophy of the choroid and retina (see sect. 1xA) (897, 1077). A striking parallelism between the enzymes involved in vertebrate Cr metabolism (AGAT, GAMT, CK) is that they all are sensitive to modification and inactivation by sulfhydryl reagents (for reviews, see Refs. 270, 474, 1077). On the basis of current knowledge (e.g., Ref. 496), however, there is no reason to believe that modification by sulfhydryl reagents [e.g., oxidized glutathione (GSSG)] represents a unifying mechanism for the *in vivo* regulation of AGAT, GAMT, and CK.

B. Regulation of Transport of Cr, PCr, ADP, and ATP Across Biological Membranes

Transport of intermediary metabolites across biological membranes represents an integral part of Cr metabolism in vertebrates. Arg has to be taken up into mitochondria for guanidinoacetate biosynthesis. Guanidinoacetate is released from pancreas and kidney cells and taken up by the liver. Likewise, Cr is exported from the liver and accumulated in CK-containing tissues. Finally, inside the cells, ATP, ADP, Cr, and PCr have to diffuse or to be transported through intracellular membranes to be able to contribute to high-energy phosphate transport between mitochondria and sites of ATP utilization. Evidently, all these sites of membrane transport are potential targets for the regulation of Cr metabolism.

In chicken kidney and liver, where AGAT is localized in the mitochondrial matrix, penetration of L-Arg through the inner membrane was found to occur only in respiring mitochondria and in the presence of anions such as acetate or phosphate (301). Consequently, the rate of Arg transport across the mitochondrial membranes might influence Cr biosynthesis.

Cr uptake into CK-containing tissues, e.g., skeletal

muscle, heart, brain, or kidney, is effected by a specific, saturable, Na^+ - and Cl^- -dependent Cr transporter (see sect. viiC). Even though the evidence is not as strong as in the case of AGAT, the expression and/or specific activity of the Cr transporter seems to be influenced by dietary and hormonal factors. A 24-h fast slightly increases [Cr] in the plasma but decreases Cr uptake into tibialis anterior and cardiac muscle of the mouse by $\sim 50\%$ (480). In rats, Cr supplementation of the diet decreases Cr transporter expression (317). Similarly, in rat and human myoblasts and myotubes in cell culture, extracellular Cr downregulates Cr transport in a concentration- and time-dependent manner (571). Na^+ -dependent Cr uptake is decreased by extracellular [Cr] $> 1 \mu\text{M}$, with 50% inhibition being observed at 20–30 μM , i.e., in the range of the physiological plasma concentration of Cr. In media containing 5 mM Cr, transport of Cr is decreased by 50% within 3–6 h, and maximal inhibition (70–80%) is observed within 24 h. Upregulation of Cr transport upon withdrawal of extracellular Cr seems to occur more slowly. Excessive concentrations (5 mM) of guanidinoacetate and GPA also reduce Cr transport significantly, whereas D- and L-ornithine, Crn, Gly, and PCr are ineffective. Because the downregulation of the Cr transporter activity by extracellular Cr is slowed by cycloheximide, an inhibitor of protein synthesis, it has been hypothesized that Cr transport, like Na^+ -dependent system A amino acid transport (331), is controlled by regulatory proteins. However, no conclusive evidence for or against this hypothesis is currently available. It also remains to be clarified how extracellular [Cr] is transformed into an intracellular signal. Loike et al. (571) have presented weak evidence suggesting that Cr has to be taken up into the cells to exert its effect on Cr transporter activity. On the other hand, dietary Cr supplementation in humans and animals, despite an at least 3- to 20-fold increase in the serum concentration of Cr, results in only a 10–20% increase in the muscle levels of Cr (see sect. xi). Because, in addition, this latter increase in muscle [Cr] is much lower than the ones observed during physical exercise, it is difficult to envisage that intracellular [Cr] should be a key regulator of Cr uptake.

In a thorough investigation of the Cr transporter activity in cultured mouse G8 myoblasts, Odoom et al. (711) showed that Cr uptake is stimulated by isoproterenol, norepinephrine, the cAMP analog $N^6,2'$ -O-dibutyryl-adenosine 3',5'-cyclic monophosphate, and the β_2 -agonist clenbuterol, but not by the α_1 -adrenergic receptor agonist methoxamine. Likewise, the stimulatory action of norepinephrine is not affected by α -adrenergic receptor antagonists but is inhibited by β -antagonists, with the β_2 -antagonist butoxamine being more effective than the β_1 -antagonist atenolol. Thus the Cr transporter activity may be controlled predominantly by β_2 -adrenergic receptors that have cAMP as their intracellular signal. In fact, analysis of the Cr transporter cDNA sequence revealed con-

sensus phosphorylation sites for cAMP-dependent protein kinase (PKA) and for protein kinase C (PKC) (691, 927). However, in transiently transfected cells expressing the human Cr transporter, phorbol 12-myristate 13-acetate, an activator of PKC, displayed a small inhibitory effect on Cr uptake, whereas forskolin (an activator of adenylyl cyclase), okadaic acid (a phosphatase inhibitor), A23187 (a calcium ionophore), and insulin were ineffective. The last finding, in turn, contrasts with experiments on rat skeletal muscle where insulin significantly increased Cr uptake, whereas alloxan-induced diabetes had no effect on Cr accumulation (see Ref. 349). Insulin and insulin-like growth factor I also stimulated Cr uptake in mouse G8 myoblasts (711), and insulin at physiologically high or supraphysiological concentrations enhanced muscle Cr accumulation in humans (943). Insulin increases Na^+/K^+ -ATPase activity which, indirectly, may stimulate Cr transporter activity (see Ref. 943). In this context, it seems noteworthy that guanidinoacetate, and to a lower extent Arg and Cr, were seen to stimulate insulin secretion in the isolated perfused rat pancreas (15). Despite using G8 myoblasts and myotubes as Odoom et al. (711; see above), and despite other indications that clenbuterol may exert some of its anabolic effects on muscle by stimulating Cr uptake, Thorpe et al. (1003) failed to detect an effect of clenbuterol on Cr transport.

The contents of Cr, PCr, and total Cr are decreased in hyperthyroid rat cardiac muscle by 13, 62, and 42%, respectively, with these changes being paralleled by an increased sensitivity of the heart to ischemic damage (874). Although this finding might be explained by a direct action of thyroid hormones on the Cr transporter, experiments with colloidal lanthanum suggest that it is due instead to an increased (reversible) leakiness of the sarcolemma. Kurahashi and Kuroshima (519) suggested that the 3,3',5-triiodothyronine-induced creatinuria and decrease in muscle Cr contents is due both to decreased uptake and increased release of Cr by the muscles. On the other hand, Cr uptake into mouse G8 myoblasts was shown to be stimulated by 3,3',5-triiodothyronine and by amylin which, in muscle, is known to bind to the calcitonin gene-related peptide receptor (711).

As to be expected from the Na^+ dependence of the Cr transporter (see sect. viiC), Cr uptake is diminished in deenergized cells and is also depressed by the Na^+/K^+ -ATPase inhibitors ouabain and digoxin (58, 293, 515, 570, 711). When, however, L6 rat myoblasts are preincubated with ouabain or digoxin, and Cr uptake subsequently is analyzed in the absence of these inhibitors, it is even higher than in untreated control cells (58). Finally, in erythrocytes from uremic patients, the Na^+ -dependent component of Cr influx is 3.3 times higher than in normal human erythrocytes. This finding may be due, by analogy, to the known occurrence of inhibitors of Na^+/K^+ -ATPase in uremic plasma (950, 984). Obviously, cells may respond

to decreased $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity, which in turn likely decreases Cr transporter activity, by compensatory up-regulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ (382) and/or Cr transporter expression.

After incubation of L6 rat myoblasts for 20 h under control conditions, replacement of the conditioned medium by fresh control medium decreases Cr uptake by 32–45% (58). This may indicate that conditioned medium from L6 myoblasts contains a modulator of Cr transport.

Despite all these investigations on the regulation of Cr uptake, it cannot be decided yet whether regulation of Cr uptake is effected directly by modulating the expression and/or activity of the Cr transporter or indirectly via alterations of the transmembrane electrochemical gradient of Na^+ which depends primarily on the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. Accordingly, it is still unclear whether Cr uptake via the Cr transporter is under kinetic or thermodynamic control. The findings that Cr uptake is inhibited by ouabain and digoxin and that 3,3',5-triiodothyronine, isoproterenol, and amylin not only stimulate Cr uptake but also increase the $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity and, thus, the membrane potential would favor indirect regulation of the Cr transporter by the electrochemical gradient of Na^+ . However, with the assumption of a Na^+ to Cr stoichiometry of the Cr transporter of 1 or 2, the theoretical concentration ratio of intracellular versus extracellular Cr should be between 900 and 3,000 (286, 711). If the chloride dependence of the Cr transporter were also taken into account, this theoretical ratio would be even higher. In sharp contrast to these values, the actual concentration ratio in resting muscle is around 80. Because, in addition, dietary Cr supplementation over several days or weeks considerably increases [Cr] in human and animal serum, but only slightly enhances the Cr levels in muscle (see sect. XI), and because in rats fed GPA and cyclocreatine, these Cr analogs compete efficiently with Cr uptake into muscle and thereby largely deplete the intracellular pools of Cr and PCr, the hypothesis that the Cr transporter is kinetically controlled seems at present more plausible. Clearly, the question of how Cr uptake is regulated in detail is of importance for a deeper understanding of Cr metabolism in health and disease. In particular, it will be crucial to determine the exact Na^+ and Cl^- stoichiometries of the Cr transporter.

Because part of the Cr that is accumulated in CK-containing tissues is converted to PCr, it might be anticipated that Cr uptake and phosphate uptake influence each other. In fact, in mouse myoblasts that are exposed to extracellular Cr, P_i uptake is transiently stimulated (773). This finding is probably not due to concerted regulation of the Cr and P_i transporters but may rely on a local decrease in P_i concentration due to phosphorylation of intracellularly accumulated Cr. In Langendorff-perfused rabbit hearts, the intracellular concentrations of Cr and of Cr plus PCr remain significantly higher when the

perfusion medium is devoid of phosphate than when it contains 1 mM P_i (286). This effect was attributed to decreased Cr efflux during phosphate-free perfusion.

Only few and inconclusive data are available on Cr efflux from cells. Although in L6 rat myoblasts at 37°C, Cr efflux amounted to 2.8–3.6% of intracellular Cr per hour (571), the respective value for G8 mouse myoblasts at 37°C was 5%/day (711). The latter value is comparable to the fractional conversion rate of Cr to Crn and may indicate that the plasma membrane is largely impermeable for Cr once it is intracellularly trapped. Because the liver is the main site of Cr biosynthesis in the body, the plasma membrane of hepatocytes is expected to be more permeable for Cr than that of muscle cells. This finding agrees with the fact that upon administration of Cr, liver, kidney, and viscera constitute a rapidly expandable pool for Cr, whereas muscle and nervous tissues constitute a slowly expandable pool of Cr plus PCr (480; see also Ref. 1077). On the other hand, when transgenic mice expressing CK in liver were fed 10% Cr in the diet for 5 days, Cr efflux from the liver proved to be insignificant (606). Because high dietary intake of Cr makes de novo biosynthesis of Cr superfluous, a putative transport protein responsible for Cr export from the liver may simply have been down-regulated in this experimental set-up. In any case, this finding should not be taken as evidence against a significant contribution of the liver to de novo biosynthesis of Cr in vertebrates. Finally, cultured Sertoli cells from the seminiferous epithelium of rats were shown to secrete Cr into the medium (665). Cr secretion was stimulated by physiological and toxicological modulators of Sertoli cell function like follicle-stimulating hormone, dibutyl cAMP, mono-(2-ethylhexyl)phthalate, or cadmium.

The permeability itself as well as changes in permeability of the outer mitochondrial membrane may be critical for the stimulation of mitochondrial respiration and high-energy phosphate synthesis, as well as for the transport of these high-energy phosphates between sites of ATP production and ATP utilization within the cell (for reviews, see Refs. 94, 280, 838, 1124). Changes in permeability of the outer mitochondrial membrane pore protein (voltage-dependent anion-selective channel; VDAC) may be accomplished 1) by “capacitive coupling” to the membrane potential of the inner membrane, leading to a voltage-dependent “closure” of the pore, or 2) by a VDAC modulator protein which increases the rate of voltage-dependent channel closure by ~10-fold. To what extent these mechanisms operate in vivo and retard the diffusion of ADP, ATP, P_i , Cr, and PCr remains to be established.

To conclude, the most critical determinant for the regulation of Cr metabolism seems to be the serum concentration of Cr. An elevation of serum [Cr] over an extended period of time would point to excess de novo biosynthesis or dietary intake of Cr and, in addition, would indicate that the tissue pools of Cr and PCr are

replenished. The observed or suspected effects of an elevated serum [Cr], namely, to downregulate the expression and/or activity of AGAT and possibly also the Cr transporter, would therefore help to spare precursors of Cr (Arg, Gly, Met) and to maintain normal, steady levels of Cr and PCr in CK-containing tissues. As a consequence, the rate of Cr biosynthesis is highest in young, healthy, fast-growing vertebrates under anabolic conditions on a balanced, Cr-free diet (1077).

VI. PHOSPHOCREATINE AND CREATINE AS PURPORTED ALLOSTERIC EFFECTORS

In some recent articles (e.g., Refs. 92, 641, 1068), the opinion has still been expressed that Cr and PCr may act as allosteric regulators of cellular processes. As a matter of fact, a number of studies, mainly performed in the 1970s, seemed to demonstrate that physiological concentrations of PCr inhibit glycogen phosphorylase α , phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, lactate dehydrogenase, AMP deaminase, and 5'-nucleotidase from a variety of species. Similarly, PCr was claimed to activate fructose-1,6-diphosphatase from rabbit skeletal muscle, while phosphorylarginine was suggested to inhibit phosphofructokinase from oyster adductor muscle (for references see Refs. 207, 247, 580, 666, 834, 951, 1012, 1099).

Subsequent studies, however, proved that inhibition of at least phosphofructokinase, pyruvate kinase, lactate dehydrogenase, AMP deaminase, and 5'-nucleotidase, but probably also of all other enzymes listed above, is not afforded by PCr itself; rather, these effects were due to contaminants present in the commercial preparations of PCr which, at that time, were no more than 62–75% pure (1099). The contaminating inhibitors were identified as inorganic pyrophosphate for AMP deaminase (1099) and oxalate for lactate dehydrogenase and pyruvate kinase (1012).

When added to the bathing medium of differentiating skeletal and heart muscle cells in tissue culture, Cr increased rather specifically the rate of synthesis as well as the specific activity of myosin heavy chain (406, 1153). In slices of the rat neostriatum, Cr inhibited the GABA-synthesizing enzyme glutamate decarboxylase as well as the veratridine-induced release of GABA, but significant effects were only observed at an unphysiologically high Cr concentration of 25 mM (864). In rat basophilic leukemia cells, PCr was seen to stimulate phospholipase C activity (196). Finally, in cell-free extracts of white gastrocnemius, soleus, heart muscle, and liver of the rat, PCr affected the extent of phosphorylation of various proteins, in particular of phosphoglycerate mutase and of a 18-kDa protein (742). Again, these findings are unlikely to

be due to direct allosteric effects of Cr and PCr but are probably mediated indirectly, e.g., via alterations of the energy status of particular microcompartments or whole cells (195, 563, 742, 1153).

In the unicellular alga *Gonyaulax polyedra*, several functions show circadian rhythmicity, for example, cell division, photosynthesis, bioluminescence, motility, and pattern formation. If cultures of *Gonyaulax* are first grown under a 12:12-h light-dark cycle, and if the conditions are then changed to constant dim light, the circadian rhythmicity persists for several weeks. This condition is called free-running circadian rhythmicity, with its period τ depending on the color and intensity of the constant dim light.

Extracts of several eukaryotic organisms, including bovine and rat brain and muscle, shorten the period of the free-running circadian rhythms in *Gonyaulax*. The substance responsible for this effect was identified as Cr. In the micromolar range (2–20 μ M), Cr accelerates the circadian clock by as much as 4 h/day (Fig. 6A) (816). The Cr effect on τ is very pronounced in constant dim blue light, whereas it is virtually absent in constant dim red light (Fig. 6B) (817). This finding, together with other lines of evidence, suggests that Cr interferes with light transduction pathways and in particular with the pathway(s) coupled to blue-sensitive photoreceptors. In addition to its effect on τ , Cr also affects light-induced phase changes of the circadian rhythmicity (817).

A period-shortening substance with properties similar to Cr is present in extracts of *Gonyaulax* itself (816) and has been identified as gonyauline (*S*-methyl-*cis*-2-[methylthio]cyclopropanecarboxylic acid) (815). Its rather close structural similarity to Cr (Fig. 6C), the complete lack of Cr in extracts of *Gonyaulax* (815), as well as the indication that Cr is not active as such but has to be metabolized to exert its effects on the circadian rhythmicity (see Ref. 817) all suggest that, at least in algae, Cr itself is not a physiological component or modulator of the circadian clock.

To conclude, the data suggesting that Cr and PCr act as direct (allosteric) regulators of cellular processes (other than the repression by Cr of AGAT and possibly also of the Cr transporter) must be treated with skepticism, at least until new, convincing data are presented. There may be four notable exceptions that deserve further attention: 1) PCr stimulates glutamate uptake into synaptic vesicles (1127; see also sect. IXG). A series of control experiments showed that the effect of PCr is not mediated indirectly via CK and ATP. Remarkably, PCr-stimulated glutamate uptake was even higher than that stimulated maximally by ATP. 2) PCr at relatively high concentrations of 10–60 mM was found to promote efficient endonucleolytic cleavage of mammalian precursor RNA in vitro (364), which is a prerequisite for subsequent poly(A) addition. Neither

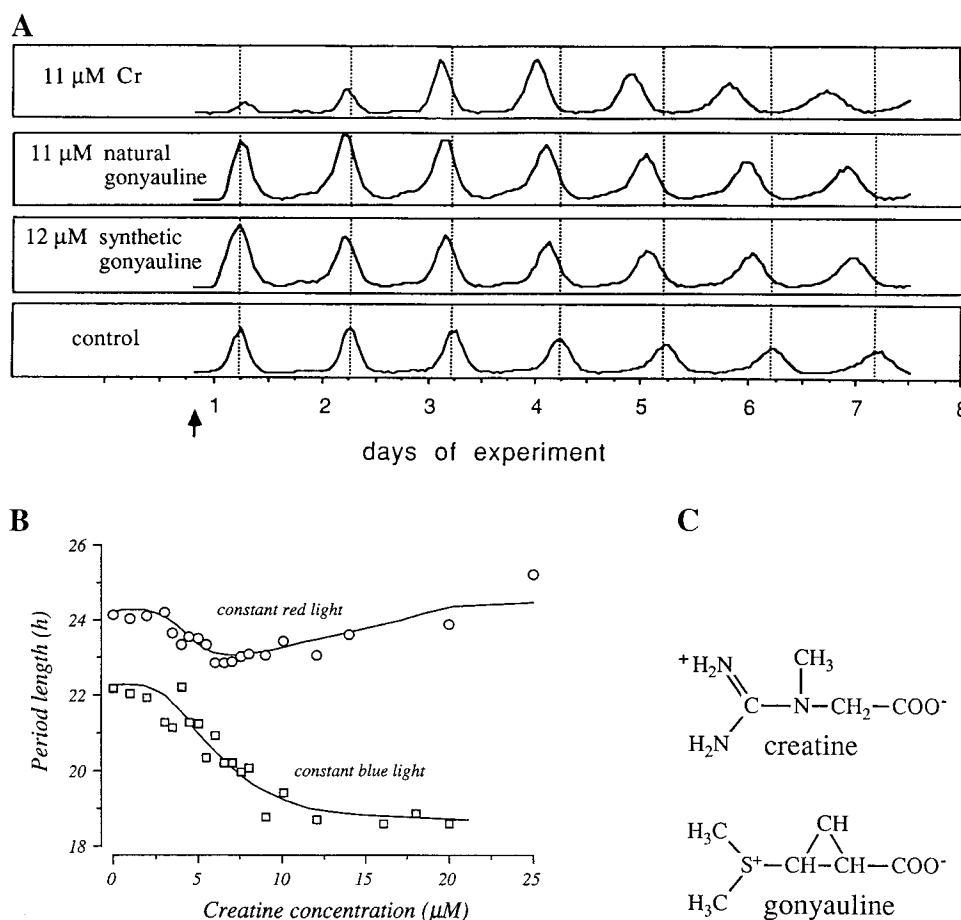


FIG. 6. Effects of creatine and gonyauline on the period of free-running circadian rhythms in the unicellular marine alga *Gonyaulax polyedra*. **A**: effects of authentic natural gonyauline, synthetic gonyauline, and Cr on the bioluminescent glow rhythm of *Gonyaulax*. [Modified from Roenneberg et al. (815).] **B**: relationship between Cr concentration and period length τ , under conditions of constant dim red or constant dim blue light. [Modified from Roenneberg and Taylor (817).] **C**: chemical structures of Cr and gonyauline. For further information see text.

CK nor ATP was required for this effect, and ATP could in fact inhibit 3'-end cleavage. PCr was not hydrolyzed, suggesting that it may act as an allosteric regulator. Phosphorylarginine (PArg) had a similar effect, whereas Cr was ineffective. 3) AMP-activated protein kinase (AMPK) from rabbit skeletal muscle is inhibited by PCr, whereby it is not yet completely clear whether this effect is CK independent or not (774). In turn, AMPK inhibits CK by phosphorylation in vitro and in differentiated muscle cells, and it also activates fatty acid oxidation. These findings suggest that CK, AMPK, and fatty acid oxidation form an intricate regulatory network for meeting energy supply with energy demands. In transgenic mice lacking both M-CK and sarcomeric Mi-CK, due to permanently high levels of PCr even during exercise, AMPK most likely remains inactive and, thus, cannot switch on fatty acid oxidation (774). In fact, these mice are defective in lipid metabolism and show signs of impaired capacity to utilize fatty acids (938). 4) Cr has been identified as an essential cofactor of thiamine-diphosphate (TDP) kinase from pig skeletal muscle, with half-maximal stimulation of enzymatic activity being observed at a [Cr] of 0.2 mM

(881). In contrast, PCr, Crn, Arg, guanidinoacetic acid, and GPA had no effect on TDP kinase activity.

VII. MICROBIAL CREATINE AND CREATININE DEGRADATION PATHWAYS

In contrast to the nonenzymatic conversion of Cr and PCr to Crn in vertebrates, a growing number of microorganisms are being discovered to express specific enzymes for the degradation of Cr and Crn. Several lines of evidence suggest an involvement of microbial Cr and Crn degradation in vertebrate physiology and pathology. Bacteria and fungi capable of degrading Cr and Crn have been identified in chicken and pigeon droppings (278, 772), human urine (499) and feces (204, 992, 1029), as well as the bacterial flora of the human colon (204, 439). The latter bacteria may be particularly relevant to renal disease (see sect. IXH). In uremic patients in whom [Crn] in the serum is highly increased (163), Crn was suggested to diffuse into the intestinal tract where it induces bacterial creatininase, creatinase, and Crn deaminase activity, resulting ultimately in the breakdown of part of the body's Crn pool (439, 438) as well as in partial recycling of Cr (652).

In accordance with experiments on a variety of bacterial strains, 1-methylhydantoin produced by Crn deaminase is not further metabolized by the gut flora (439), but may, instead, be retaken up into the body and degraded there to 5-hydroxy-1-methylhydantoin, methylparabanic acid, *N*⁵-methyloxaluric acid, and oxalic acid plus methylurea (395). Because 1-methylhydantoin and 5-hydroxy-1-methylhydantoin were also detected in rabbit skin after vaccinia virus inoculation, a similar reaction cascade may proceed in inflamed tissue. Further microbial degradation products of Crn (e.g., methylguanidine) may act as uremic toxins (see sect. IXH), carcinogens, or carcinogen precursors (see sect. IXF). Finally, knowledge of the reactions and enzymes involved in Crn degradation may have an impact on routine clinical diagnosis where the Crn-degrading microbial enzymes may be used for specific enzymatic assays of [Crn] and [Cr] in serum and urine (see sect. X).

At least four alternative microbial Crn degradation pathways have to be considered (Fig. 7). 1) In some bacteria (*Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Escherichia*, *Proteus*, and *Pseudomonas* strains) and fungi (*Cryptococcus neoformans* and *C. bacillisporus*), Crn seems to be degraded solely to 1-methylhydantoin and ammonia (see Refs. 278, 484, 660, 772, 884, 895, 992). Crn can therefore be used by these microorganisms as a nitrogen source, but not as a carbon or energy source. In all microorganisms of this group that have been analyzed so far (*Flavobacterium filamentum*,

E. coli, *Proteus mirabilis*, and *Pseudomonas chlororaphis*), a single enzyme displays both cytosine deaminase and Crn deaminase activity (229, 484). The wide distribution of cytosine deaminases in microorganisms and the close structural similarity between cytosine and Crn may thus be the actual reasons why Crn deaminase activity, quasi as a side reaction, is also widely distributed. Although some of the Crn/cytosine deaminases are induced when the bacteria or fungi are grown on media containing Crn or cytosine (484, 772, 992), others are expressed in a constitutive manner or are even repressed by cytosine (484).

2) In several *Pseudomonas*, *Brevibacterium*, *Moraxella*, *Micrococcus*, and *Arthrobacter* strains, as well as in anaerobic *Clostridium* and *Tissierella* strains, 1-methylhydantoin is degraded further to *N*-carbamoylsarcosine and sarcosine. The enzymes involved in this degradation pathway, i.e., Crn deaminase, 1-methylhydantoin amidohydrolase, and *N*-carbamoylsarcosine amidohydrolase, are all highly induced when the bacteria are grown on Crn or 1-methylhydantoin as main source of nitrogen and, in some cases, carbon (see Refs. 170, 335, 357, 484, 714, 883, 884, 892). A comparison of the specific enzymatic activities revealed that the 1-methylhydantoin amidohydrolase reaction is the rate-limiting step of the pathway. Consequently, *N*-carbamoylsarcosine is in most instances either undetectable in these bacteria or is present in much lower concentration than the other intermediates (278, 884). Hydrolysis of 1-methylhydantoin,

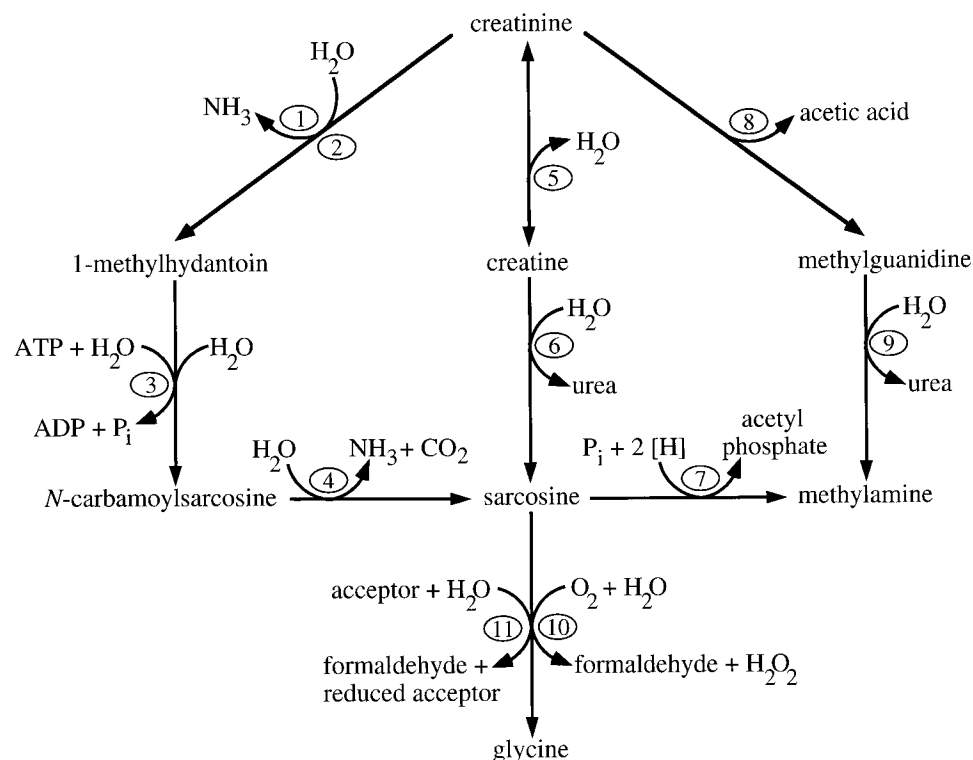


FIG. 7. Schematic representation of the reactions and enzymes involved in microbial Cr and Crn degradation pathways. The respective enzymes are denoted by numbers: 1) creatinine iminohydrolase (creatinine deaminase; EC 3.5.4.21); 2) cytosine aminohydrolase (cytosine deaminase; EC 3.5.4.1); 3) 1-methylhydantoin amidohydrolase [ATP dependent (EC 3.5.2.14) or non-ATP dependent]; 4) *N*-carbamoylsarcosine amidohydrolase (EC 3.5.1.59); 5) creatinine amidohydrolase (creatininase; EC 3.5.2.10); 6) creatine amidohydrolase (creatinase; EC 3.5.3.3); 7) sarcosine reductase (EC 1.4.4.-); 8) not characterized so far; 9) methylguanidine amidohydrolase (EC 3.5.3.16); 10) sarcosine oxidase (EC 1.5.3.1); 11) sarcosine dehydrogenase (EC 1.5.99.1) or dimethylglycine dehydrogenase (EC 1.5.99.2).

as catalyzed by the 1-methylhydantoin amidohydrolases of *Pseudomonas*, *Brevibacterium*, *Moraxella*, *Micrococcus*, and *Arthrobacter* strains, is stoichiometrically coupled with ATP hydrolysis and is stimulated by Mg^{2+} and NH_4^+ or K^+ . In addition, hydantoin is hydrolyzed by these enzymes at a much lower rate than 1-methylhydantoin. In contrast, the 1-methylhydantoin amidohydrolases of anaerobic bacteria (357) are not affected by ATP and Mg^{2+} , and hydantoin is hydrolyzed at a similar rate as 1-methylhydantoin.

3) In various *Alcaligenes*, *Arthrobacter*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, and *Tissierella* strains, still another set of enzymes is induced when they are grown on Cr or Crn as sole source of nitrogen and/or carbon. Creatininase (Crn amidohydrolase) converts Crn to Cr which is then further metabolized by creatinase (Cr amidohydrolase) to urea and sarcosine (see Refs. 115, 257, 335, 487, 708, 884). Even though creatinase has also been detected in human skeletal muscle (655), this finding awaits confirmation and demonstration of its physiological relevance.

Sarcosine formed in *degradation pathways 2* and *3* may be degraded further to Gly by a sarcosine oxidase or sarcosine dehydrogenase (487, 708, 884), or possibly to methylamine by the action of a sarcosine reductase (see Refs. 334, 335, 439). It also seems worth mentioning that glycocyamidine and glycocyamine (guanidinoacetate) can be degraded by microorganisms almost exactly as shown in *pathway 3* for Crn degradation. Glycocyamidinase converts glycocyamidine to glycocyamine, which is then split by glycocyaminase (guanidinoacetate amidohydrolase; EC 3.5.3.2) into Gly and urea (1150, 1151).

4) Finally, *Pseudomonas stutzeri* seems to convert Crn quantitatively to methylguanidine and acetic acid (1049). Methylguanidine was shown to be split in an *Alcaligenes* species by a highly specific methylguanidine amidohydrolase into methylamine and urea (685).

The distinction between four alternative degradation pathways for Crn represents an oversimplification. For example, two of the degradation pathways may occur in the same organism, with the relative expression levels of the individual enzymes depending primarily on the nitrogen source used (884). When the *Pseudomonas* sp. 0114 is grown on Crn as main nitrogen source, Crn is degraded chiefly via Cr. When the same species is grown on 1-methylhydantoin, the 1-methylhydantoin amidohydrolase and *N*-carbamoylsarcosine amidohydrolase activities are induced so that in this case, Crn degradation via 1-methylhydantoin and *N*-carbamoylsarcosine prevails. The different Crn degradation pathways may also overlap. In the *Pseudomonas* sp. H21 grown on 1-methylhydantoin as main nitrogen source, creatinase activity is undetectable. However, Cr can still be degraded, but only indirectly via Crn, 1-methylhydantoin, and *N*-carbamoylsarcosine (884). When the same species is grown on Crn, creatinase is

induced, and Cr can be degraded directly to sarcosine. Finally, the distinction between *pathways 1* and *2* may seem arbitrary, even more so if it is taken into account that *Clostridium putrefaciens* and *C. sordellii* grown on basal medium degrade Cr and Crn solely to 1-methylhydantoin, while the same strains grown on a minced meat medium further degrade 1-methylhydantoin to sarcosine (278).

Clearly, microbial Crn degradation is at present only incompletely understood. To get a deeper insight into this topic, a wide evolutionary screening and detailed characterization of all enzymes involved are essential prerequisites. Relevant questions to be addressed are whether and how the expression of Cr- and Crn-degrading enzymes is regulated, and in which microorganisms Crn and cytosine deamination are catalyzed by a single or by separate enzymes.

VIII. PROTEINS INVOLVED IN CREATINE METABOLISM

A. L-Arginine:glycine Amidinotransferase

Depending on the species, the highest activities of AGAT in vertebrates are found in liver, kidney, pancreas, or decidua (see sect. III). Although the yolk sac of the hen's egg was reported to contain significant amounts of AGAT by Walker (1077), it was suggested not to do so by Ramírez et al. (793). Mostly in kidney and pancreas, but also in rat decidua, the levels of AGAT are influenced by a variety of dietary and hormonal factors. These factors and the underlying mechanisms of AGAT regulation are discussed in detail in section IV. Notably, AGAT expression was shown to be downregulated in Wilms' tumor, a renal malignancy with complex genetic and pathological features (37).

AGAT is confined to the cortex of human and rat kidney (623, 634), which is in line with higher concentrations of GAA in the cortex than in the medulla of rat and rabbit kidney (555). Both immunolocalization and microdissection experiments revealed that AGAT activity and immunoreactivity are restricted to epithelial cells (in a basilar position) of the proximal convoluted tubule of the rat nephron (623, 976, 977). AGAT therefore coincides in location with the site of Arg biosynthesis in the kidney, which was shown to be highest in the proximal convoluted tubule, somewhat lower in the pars recta of the proximal tubule, and almost negligible in all other segments of the nephron (553). In contrast to AGAT, Cr is present in all nephron segments tested, although in varying amounts (974, 977), whereas CK was localized to the distal nephron of the rat kidney in the thick ascending limb of Henle's loop and the distal convoluted tubule (264).

In rat liver, immunostaining with polyclonal antibodies against AGAT is most prominent in cells near the central vein and the portal triad (623). The staining appears to be confined to the cytoplasm of hepatocytes, leaving a negative image of the nucleus. In rat pancreas, despite some earlier, conflicting results suggesting that AGAT is confined to the glucagon-producing α -cells within the islets of Langerhans (623), more recent enzyme activity measurements on isolated islets and acinar tissue as well as immunofluorescence experiments have shown that AGAT is present only in acinar cells (928). For comparison, CK in rat pancreas was suggested to be localized in acinar cells (6) or insulin-producing β -cells (283, 1100).

As far as the subcellular localization is concerned, it is now generally accepted that AGAT is localized in the mitochondria of rat pancreas, rat kidney, and chicken liver (see Refs. 624, 1077). Although in rat kidney AGAT seems to be bound to the outer surface of the inner mitochondrial membrane, it was localized in the mitochondrial matrix of chicken liver. The mitochondrial localization has recently been corroborated by amino acid and cDNA sequencing of rat, pig, and human AGAT, showing that AGAT is synthesized as a precursor protein containing a presequence that is typical for matrix/inner membrane proteins (322, 390). However, an additional cytoplasmic localization of part of the AGAT, due to alternative splicing of human AGAT mRNA, cannot be totally excluded at present (388).

Purification of AGAT from rat and human kidney suggested the presence of two forms each of this enzyme which, in the case of rat, were designated as α - and β -forms (313, 625). In isoelectric focusing experiments, these purified forms were further resolved into multiple bands (313, 314). At least part of this microheterogeneity may be explained by the presence of different AGAT isoenzymes. 1) A monoclonal antibody against rat kidney AGAT, in contrast to polyclonal antibodies, detected the enzyme in kidney, but not in liver and pancreas (623). 2) The α -form of rat kidney AGAT gave a clearly identifiable NH_2 -terminal amino acid sequence, while at least five residues per cycle were recovered for the β -form. 3) *Staphylococcus aureus* V8 digestion yielded different peptide patterns for α - and β -AGAT of the rat (314).

Biophysical characterization of purified native AGAT from hog kidney revealed a M_r of $\sim 100,000$ (134). Native rat α -, rat β -, and human AGAT display similar M_r values (82,600–89,000), are all dimeric molecules (subunit M_r values of 42,000–44,000), and exhibit pI values between 6.1 and 7.6 (313, 314, 625). In vitro translation experiments (624) as well as amino acid and cDNA sequencing (322, 388, 390) demonstrated that AGAT protomers from rat and human kidney are synthesized as 423 amino acid-precursor proteins with a calculated M_r of $\sim 46,500$. The size of the leader sequence, which is cleaved off upon import into the mitochondria, is still under debate and

may range from 35 to 55 amino acids. The amino acid sequences of rat, pig, and human AGAT are highly homologous (94–95% sequence identity). Interestingly, the cDNA sequences of mammalian AGAT also display significant homology (36–37%) to those of L-Arg:inosamine phosphate amidinotransferases from *Streptomyces* bacteria which participate in streptomycin biosynthesis (see Ref. 388).

Extensive kinetic analysis revealed that the AGAT reaction proceeds via a double-displacement (Ping-Pong, bi-bi) mechanism, with a formamidine group covalently attached to a sulfhydryl group of the enzyme as an obligatory intermediate of the reaction (for reviews, see Refs. 389, 634, 1077). The essential sulfhydryl group has recently been assigned by radioactive labeling, X-ray crystallography, and site-directed mutagenesis to Cys-407 of human AGAT (388, 389). The three-dimensional structure of the AGAT protomer displays fivefold pseudosymmetry, resembles a basket with handles, and undergoes a conformational change upon substrate binding (266, 389). It shows no similarity to the three-dimensional structures of CK, creatinase, and N-carbamoylsarcosine amidohydrolase (see below). Aromatic residues play an important role in stabilizing the dimer, but dissociation into monomers has also been observed.

The AGAT reaction is readily reversible, as evidenced by an apparent equilibrium constant (K') of ~ 1 at pH 7.5 and 37°C. The pH optima in the direction of guanidinoacetate formation and Arg formation are 7.2–7.5 and 8.5, respectively. The Michaelis constant (K_m) values for Arg, Gly, guanidinoacetate, and ornithine were determined to be 1.3–2.8, 1.8–3.1, 3.9, and 0.1 mM for hog, rat, and human kidney AGAT (313, 625; see also Refs. 634, 1077). Purified human, rat α -, and rat β -AGAT display maximum velocity (V_{\max}) values at 37°C of 0.5, 0.39, and 0.37 μmol ornithine formed $\cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$, respectively (313, 625), whereas the specific activity at 37°C of purified AGAT from hog kidney was reported to be ~ 1.2 $\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ (134).

AGAT is absolutely specific for the natural L-amino acids. In addition to the physiological substrates (Arg, Gly, ornithine, and guanidinoacetate), canavanine, hydroxyguanidine, 4-guanidinobutyrate, 3-guanidinopropionate (GPA), and homoarginine act as amidine donors, and canaline, hydroxylamine, glycylglycine, 1,4-diaminobutylphosphonate, 4-aminobutyrate (GABA), 3-aminopropionate, and β -alanine as amidine acceptors (769; for reviews, see Refs. 226, 634, 1077). Notably, canavanine, which may be formed and regenerated physiologically in a "guanidine cycle" similar to the urea cycle (692), is at least as effective as Arg in acting as an amidine donor for hog and rat kidney AGAT (see Refs. 975, 976, 1056). All transamidinations catalyzed by AGAT are strongly inhibited by ornithine, even those for which it is the amidine acceptor (see Ref. 1077). In cortex extracts of rat kidney,

the inhibitory constant (K_i) of AGAT for ornithine is $\sim 250 \mu\text{M}$ (897). Interestingly, such inhibition of AGAT by 10- to 20-fold increased concentrations of ornithine most probably is the underlying basis for the decreased levels of tissue Cr in patients with gyrate atrophy of the choroid and retina (see sect. IXA) (897, 899). In isolated tubules of the rat kidney, guanidinoacetate synthesis was shown to be suppressed, besides ornithine, by DL-norvaline and Met (975).

In accordance with the postulate that a sulfhydryl group of AGAT is involved in catalysis, sulfhydryl reagents like *p*-chloromercuribenzoate, DTNB, 2,4-dinitrofluorobenzene (DNFB), and Cu^{2+} inactivate the enzyme (for reviews, see Refs. 634, 1077). Purified human AGAT is also inhibited by Hg^{2+} , Zn^{2+} , and Ni^{2+} (388). Finally, even purified preparations of hog kidney AGAT display hydrolytic activity amounting to $\sim 1\%$ of the transamidinase activity (134). In contrast to the transamidinase activity, the hydrolytic activity of AGAT is not affected by sulfhydryl reagents.

B. S-Adenosyl-L-methionine:N-guanidinoacetate Methyltransferase

In vertebrates, the highest levels of GAMT are found in liver, and it has been estimated that the amount of Cr synthesized in this organ is sufficient to meet the requirements for Cr of the entire animal (1130; for reviews see Refs. 1056, 1077). Intermediate levels of GAMT were detected in mammalian pancreas, testis, and kidney, whereas the specific GAMT activity in spleen, skeletal and cardiac muscle, mouse neuroblastoma cells, and human fetal lung fibroblasts was reported to be rather low (149, 664, 1056, 1077, 1129, 1130, 1135, 1136). It is not yet known to what extent all these tissues contribute to total in vivo Cr biosynthesis. However, because the specific GAMT activity is on the order of $0.2\text{--}0.3 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ in liver, but only <0.005 up to maximally $0.014 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ in heart, skeletal muscle, fibroblasts, and neuroblastoma cells (37°C , pH 7.4–8.0) (149, 713, 1135, 1136), the estimation of Daly (149) that these latter tissues contain 5–20% of the specific GAMT activity of liver seems high. The overestimation may be explained by the facts that 1) in the study of Daly, hepatoma cells instead of authentic liver tissue were chosen as reference and 2) GAMT activity had previously been shown to decrease gradually with the progression of hepatocarcinoma (1019, 1136). Nevertheless, at physiological extracellular concentrations of guanidinoacetate and Cr ($25 \mu\text{M}$ each), cultured mouse neuroblastoma cells synthesized as much Cr as they accumulated from the medium (149). In the liver and pancreas of alloxan-diabetic rats and sheep, respectively, GAMT activity was shown to be decreased by 50–70% (354, 1128).

In thorough studies on the tissue distribution of GAMT expression in the mouse and rat by Northern and Western blotting (543, 545), high levels of GAMT mRNA were observed in the rat in kidney, caput epididymis, testis, brain, and liver; moderate levels in the cauda epididymis; but low or undetectable levels in the lung, pancreas, spleen, vas deferens, prostate, seminal vesicles, coagulating gland, heart, skeletal muscle, and small intestine. In the mouse, highest amounts of GAMT mRNA and protein were detected in testis, caput epididymis, and female liver, followed by ovary and male liver. While barely detectable signals were observed for kidney, skeletal muscle, heart, uterus, and oviduct, no GAMT mRNA or protein at all was found in brain, small intestine, seminal vesicles, lung, vas deferens, cauda epididymis, coagulating gland, or spleen. Most striking is the difference in GAMT expression between female and male liver, which might indicate that liver is the principal site of Cr biosynthesis in the female mouse, whereas testis and caput epididymis take over at least part of this function in the male. It must, however, be kept in mind that this finding might be due instead to the different age of the male and female mice studied. Immunohistochemistry demonstrated that in mouse testis, GAMT is localized primarily in the seminiferous tubules and, in particular, in the Sertoli cells (see also Ref. 664). In the caput epididymis, microvilli of epithelial cells lining the initial segment of the epididymal tubule were most intensely stained. In contrast, spermatocytes, spermatids, cauda epididymis, the stroma of the epididymis, and seminal vesicles displayed no specific signals (545; see also Ref. 693). The fact that seminal vesicles of the mouse and rat nevertheless contain considerable quantities of Cr and PCr seems to be due to Cr uptake from the blood via the Cr transporter (543).

Purified GAMT from rat and pig liver is a monomeric protein with a M_r of 26,000–31,000 (397, 713). This has been corroborated by cDNA and gene sequencing, showing that rat, mouse, and human GAMT are 236-amino acid polypeptides with a calculated M_r of $\sim 26,000$ (409, 427, 712). The human GAMT gene has a size of $\sim 5 \text{ kb}$, contains, like the rat and mouse GAMT genes, six exons, and was mapped to chromosome 19p13.3 that is homologous to a region on mouse chromosome 10 containing the jittery locus (114, 427). Although jittery mice share some of the neurological symptoms of children suffering from GAMT deficiency (see sect. IXG), sequencing of the GAMT gene of jittery mice revealed no mutations in the coding regions (427). Cayman-type cerebellar ataxia, a human genetic disorder exhibiting some overlapping symptoms with GAMT deficiency, has also been mapped to human chromosome 19p13.3 (see Ref. 114).

In contrast to the recombinant protein expressed in *E. coli*, native rat liver GAMT is NH_2 -terminally blocked, with no influence of this modification on the kinetic prop-

erties of the enzyme. The GAMT reaction is essentially irreversible and displays a pH optimum of 7.5 (see Ref. 1077). GAMT binds substrates in an ordered fashion, with binding of AdoMet being an obligatory prerequisite for the subsequent binding of guanidinoacetate (269, 973). For all preparations investigated, the $K_d(\text{AdoMet})$, $K_m(\text{AdoMet})$, and $K_m(\text{guanidinoacetate})$ values were found to be 10–12 μM , 1.2–6.8 μM (49 μM for pig liver GAMT), and 12–98 μM , respectively (149, 269, 270, 397, 712, 713, 917, 970, 973). Purified recombinant rat liver GAMT displays a V_{\max} of 187 nmol product $\cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ at 30°C and pH 8.0 (270, 970). As far as the substrate specificity is concerned, *S*-adenosyl-ethionine can also serve as a substrate, but as a poor one (see Ref. 1077). Adenosylhomocysteine, 3-deaza-adenosylhomocysteine, and sinefungin, a naturally occurring analog of adenosylhomocysteine, inhibit rat and pig liver GAMT competitively with respect to AdoMet, with K_i values between 0.6 and 39 μM (397, 917, 973). In contrast, even 5 mM concentrations of Cr, GPA, guanidosuccinate, Gly, β -alanine, and 4-aminobutyrate display no inhibitory effects (268).

GAMT structure-activity relationships have been investigated extensively by means of site-directed mutagenesis, site-specific labeling reagents, and limited proteolysis. A variety of studies, mostly performed with recombinant rat liver GAMT, focused on the question of how Cys residues are involved in the catalytic mechanism of the enzyme (for a review see Ref. 270). Purified GAMT preparations are stimulated by thiol compounds like 2-mercaptoethanol, Cys, reduced glutathione (GSH), or dithiothreitol. Vice versa, GAMT is inactivated by thiol-specific reagents like DTNB, GSSG, *N*-ethylmaleimide, iodoacetate, or 2-nitro-5-thiocyanobenzoate (496, 713, 970). Although single substrates have no effect on the rate of inactivation, almost complete protection is achieved by sinefungin plus guanidinoacetate.

Four of the five Cys residues of rat liver GAMT, namely, Cys-15, Cys-90, Cys-207, and Cys-219, are amenable to modification by thiol reagents (268, 970; see also Ref. 270). Incubation of the wild-type enzyme with one equivalent of DTNB results in the formation of a disulfide bond between Cys-15 and Cys-90 (268). Site-directed mutagenesis of Cys-90 to Ala has only minor effects on the kinetic properties of the enzyme. However, upon incubation of this mutant enzyme with one equivalent of DTNB, a disulfide bond is now formed between Cys-15 and Cys-219 (970). Both cross-linked species still display 10–16% of the enzymatic activities of the unmodified enzymes (270, 970). In addition, for both wild-type and C90A-GAMT, residual activity is even observed when all Cys residues except Cys-168 are modified by 2-nitro-5-thiocyanobenzoate (970).

GSSG-inactivated GAMT, in which Cys-15 forms a mixed disulfide with GSH, displays a slightly decreased affinity for AdoMet ($K_d = 17 \mu\text{M}$) but does not bind

guanidinoacetate, neither in the absence nor presence of sinefungin (496, 713). Even though the activity of GAMT depends in a hyperbolic manner on $[\text{GSH}]/[\text{GSSG}]$ (496), *in vivo* regulation of GAMT by the redox status of the glutathione system seems unlikely due to the low equilibrium constant of the reaction $\text{E-SH} + \text{GSSG} \leftrightarrow \text{E-SSG} + \text{GSH}$ ($\text{E} = \text{enzyme}$). In conclusion, these results suggest that 1) Cys-15, Cys-90, and Cys-219 are structurally close to each other; 2) Cys-15, Cys-90, Cys-207, and Cys-219 may be important but are not essential for catalysis; and 3) Cys residues are not involved in AdoMet binding. Rather, they may be involved in guanidinoacetate binding or in AdoMet-induced conformational changes which, in turn, may be crucial for the subsequent binding of guanidinoacetate.

Comparison of the amino acid sequences of mammalian AdoMet-dependent, non-nucleic acid methyltransferases revealed the presence of three short homologous regions (residues 63–71, 133–136, and 159–165 in rat GAMT) which, therefore, might be involved in AdoMet binding. Inspection of the three-dimensional structure of catechol *O*-methyltransferase (1064) suggests that some of these residues may, in fact, be in or near the active site. Site-directed mutagenesis of rat GAMT in the region of Lys-160 to Leu-165 had only minor effects on the catalytic properties of the enzyme but strongly influenced tryptic susceptibility of Arg-20 (292). Likewise, rather small effects on the kinetic properties of the enzyme were observed for mutations in nonhomologous regions (E89Q, D92N, W143F, and W143L) (326). On the other hand, the G67A and G69A mutants were completely inactive (326). This does not necessarily mean that these residues are involved in substrate binding, since effects of the mutations on the overall conformation of the enzyme were not excluded.

In the presence of radioactive AdoMet, exposure of GAMT to ultraviolet light results in cross-linking of the enzyme with its ligand. Tyr-136 was identified as the labeled residue, implying that it is involved in AdoMet binding (971). This conclusion is corroborated by the fact that the Y136F and Y136V mutants display slightly or even drastically increased K_m values for both AdoMet and guanidinoacetate (326). The Y133V mutant had a 25-fold increased K_m for guanidinoacetate (326). The two Asp residues that are located next to Tyr-136, Asp-129 and Asp-134, are both preceded by a hydrophobic amino acid and followed successively by a small neutral and a hydrophobic residue. This sequence motif is shared by most AdoMet-dependent methyltransferases (399), thus raising the possibility that either Asp-129 or Asp-134 is critical for catalysis. As a matter of fact, site-directed mutagenesis of Asp-134 to Glu or Asn decreased V_{\max} 3- and 120-fold, respectively, and increased the K_m values for AdoMet and guanidinoacetate 50- to 800-fold (973). In contrast, only minor

changes in kinetic properties were observed for the D129N, D129A, Y133F, and T135A mutant enzymes.

Scrutiny of the reactivity of the amino groups of recombinant rat GAMT toward radioactive acetic anhydride revealed that Lys residues 38, 83, 104, 108, 152, and 180 are moderately reactive, that lysines 113 and 160 are weakly reactive, and that lysines 178 and 234 are nonreactive (972). The amino group of the NH₂-terminal Ser residue, on the other hand, displays high reactivity, indicating that it is exposed to the solvent. Although AdoMet has no influence on the reactivity of the Lys residues, a substantial reduction in radioactive labeling of Lys-38 is brought about when both AdoMet and guanidinoacetate are present. Because, however, even excess concentrations of these substrates do not suppress the reactivity of Lys-38 completely, this residue was suggested not to be involved directly in substrate binding.

Finally, trypsin, chymotrypsin, and elastase all cleave native and recombinant rat liver GAMT preferentially between amino acids 19 and 25 (269, 713). Cleavage is paralleled by inactivation of the enzyme and by a large decrease in the affinities for both AdoMet and guanidinoacetate. The presence of either substrate has no effect on the rate of inactivation by trypsin, but a substantial protection from inactivation is observed when both substrates are present, or when Cys-15 is cross-linked with Cys-90 via a disulfide bond (269). These results suggest that residues 19–25 of GAMT are flexible and exposed to the solvent and that the NH₂-terminal region is not involved directly in substrate binding, but plays a role in catalysis.

C. Cr Transporter

Investigation of Cr transport across the plasma membrane has been hampered for many years by the problems inherent in the study of integral membrane proteins. However, recent cDNA and gene sequencing of the Cr transporters from rabbit (319), rat (295, 619, 840, 860), mouse (697), human (52, 214, 415, 691, 843, 927), and electric ray (*Torpedo*) (318) has given a fresh impetus in this particular field. These DNA sequencing approaches have shown that Cr transporters are composed of 611–636 amino acid residues and have a calculated M_r of ~70,000. The Cr transporters are members of the Na⁺-dependent “neurotransmitter” transporter family. They are most closely related to the GABA/taurine/betaine transporter subfamily (46–53% amino acid sequence identity), while the homology to Gly, Pro, catecholamine, and serotonin transporters is somewhat less pronounced (38–44%). All Cr transporters lack a hydrophobic NH₂-terminal signal sequence and display 12 putative transmembrane domains like other members of the Na⁺-dependent neurotransmitter transporter family. Consequently, the NH₂ and COOH

termini of the polypeptide chain are probably both directed toward the cytosol.

By classical biochemical means, a saturable uptake mechanism for Cr was identified in rat and mouse skeletal muscle (150, 250, 711), human fibroblasts, human uterine and calf aortic smooth muscle cells (150), human red blood cells (965), human monocytes and monocyte-derived macrophages (570), as well as in rat astroglia cells (659). In addition to the saturable component of Cr uptake, kinetic analysis often revealed a second component, displaying a K_m for Cr of ≥ 1.3 mM or being not saturable (see Refs. 150, 570, 965). In the light of a Cr concentration in the serum of 25–50 μ M (175), this latter component seems to be irrelevant for Cr uptake in vivo and may represent passive diffusion of Cr across the plasma membrane.

Investigation of the tissue distribution of the Cr transporter by Northern blotting and in situ hybridization techniques gave somewhat contradictory results. It remains to be established whether the quantitative differences observed are due to methodological artifacts or to pronounced species differences. So far, expression of Cr transporter mRNA seems to be highest in kidney, heart, and skeletal muscle and somewhat lower in brain, small and large intestine, epididymis, testis, vas deferens, seminal vesicles, prostate, and adrenal. Only very low amounts or no Cr transporter mRNA at all are found in ovary, uterus, placenta, liver, lung, spleen, pancreas, and thymus (295, 319, 415, 543, 619, 691, 840, 860, 927). In brain, the regional distribution of Cr transporter transcripts is heterogeneous, but again, the quantitative measures published on this issue differ to some extent (295, 332, 359, 619, 840, 860). Nevertheless, in situ hybridization experiments suggested that Cr transporter expression in rat brain closely correlates with the localization of CK (332). In the digestive tract of the rat, moderate levels of Cr transporter transcripts were observed in the small and large intestine, but only low levels were seen in the stomach (295). In contrast, Cr transporter mRNA was undetectable in human gut. In rat and human kidney, expression of the Cr transporter seems to be slightly higher in the cortex than in the medulla (295, 691). Finally, the level of Cr transporter expression in transformed cells is likely to depend on the tumor type. In human colonic tumor cell lines, only very low levels of Cr transporter mRNA were detected (295). On the other hand, 1 h after intravenous injection of radioactive Cr into tumor-bearing mice, Ehrlich ascites tumor cells and intestine displayed the highest specific radioactivities, suggesting that they have considerable Cr transporter activities (1135).

Experiments in the late 1960s on Cr uptake into rat skeletal muscle yielded an unusually high K_m (Cr) of 0.5 mM (250, 251). More recent studies, with improved techniques, have shown that the cloned and overexpressed Cr transporters from rabbit, rat, human, and *Torpedo* as well

as the saturable component of Cr uptake into the mammalian tissues examined display K_m values for Cr of 15–128 μM (150, 318, 319, 515, 570, 659, 691, 711, 860, 874, 927, 965). The published V_{\max} values are somewhat difficult to compare, since they were given relative to different measures of tissue mass (intracellular water volume or tissue dry weight or mass of protein) (250, 515, 570, 659, 711, 874).

The Cr transporter is Na^+ dependent (150, 319, 570, 619, 659, 691, 711, 840, 927), with a $K_m(\text{Na}^+)$ of 55 mM and a suggested transport stoichiometry of 2 Na^+ for 1 Cr (659). Substitution of Na^+ by Li^+ , guanidinium, or choline strongly depresses Cr transporter activity (319, 659, 927). Expression of the Cr transporter in COS-7 (derived from monkey kidney) or HeLa cells further revealed that Cr uptake is Cl^- dependent (319, 840). Cr transporter activity is slightly reduced when Cl^- is replaced by Br^- , but activity is almost completely abolished when succinate is chosen as anion. Finally, Cr uptake was shown not to depend on subsequent phosphorylation to PCr (570, 850).

Uptake of Cr by the Cr transporter is inhibited most efficiently and in a competitive manner by GPA ($K_i = 8.8$ –120 μM) and 3-guanidinobutyrate. 1-Carboxymethyl-2-imino-hexahydropyrimidine, *N*-methyl-amidino-*N*-methylglycine, 4- and 2-guanidinobutyrate, *N*-ethylguanidinoacetate, guanidinoacetate, Ala, *p*-guanidinobenzoate, and succinamic acid are somewhat less inhibitory. In contrast, Arg, sarcosine, choline, GABA, citrulline, carnitine, D- and L-ornithine, PCr, epoxycreatine, taurine, β -alanine, guanidine, and succinamide have negligible effects on Cr transport (150, 246, 251, 318, 319, 515, 570, 659, 691, 840, 860, 927). Although Crn is a weak inhibitor of the human Cr transporter, it seems to have no effect on the rabbit or rat orthologs. Likewise, 2-amino-3-guanidinobutyrate was found to be a weak inhibitor of the rabbit and human Cr transporters as well as of Cr transport in COS-7 cells (319, 927), whereas in other studies, it had no influence on both the human and *Torpedo* Cr transporters (318, 691).

In addition to simply inhibiting Cr uptake, GPA and other Cr analogs are likely to be transported themselves by the Cr transporter. In rat skeletal muscle, GPA is accumulated by a saturable process displaying kinetic properties almost indistinguishable from Cr transport. GPA uptake is competitively inhibited by Cr and, to a lesser extent, guanidinoacetate (251). Furthermore, in animals fed GPA, cyclocreatine, or homocyclocreatine, the accumulation of these Cr analogs within the tissues is paralleled by a decline in intracellular [Cr] and [PCr] (249, 637, 810). Administration of GPA and of other Cr analogs has therefore been used widely as an experimental means of depleting tissue Cr and PCr in vivo, with the final goal to unravel the physiological functions of the CK system (see sect. VIII). In contrast to Cr and its analogs, no specific uptake via the Cr transporter was observed for taurine, choline, serotonin, dopamine, norepinephrine, Glu, Gly,

Ala, Ser, carnitine, 3-hydroxybutyrate, putrescine, GABA, pyruvate, ornithine, and urea (318, 319, 619, 691).

Some further points seem worth mentioning. DNA sequencing and gene localization revealed two Cr transporter genes on human chromosomes Xq28 (CT1) and 16p11.1–11.2 (CT2) (52, 214, 309, 415, 691, 843) that may have arisen from a transposition of a gene cluster from Xq28 to near the 16p11.1/16p11.2 boundary (see Refs. 214, 415). The Xq28 locus has been linked to the genes for several hereditary neuromuscular disorders, which raises the possibility of causal links between muscle diseases and disturbances of Cr transporter expression and/or activity (see sect. IXA). While CT1 is likely to be expressed in all tissues mentioned above including testis, CT2 seems to be restricted solely to the testis. It has been postulated that the existence of autosomal homologs of X-linked genes is a compensatory response to the inactivation of the X chromosomal genes in spermatozoa before meiosis (621). The finding of CT2 and its expression in testis therefore stress the importance of the CK system for normal sperm function. On the amino acid level, the two Cr transporter isoproteins share 98% identity. Whether CT2 in fact has 50 additional COOH-terminal amino acids as suggested by Iyer et al. (415) or whether the Cr transporter gene on human 16p11.1–11.2 only represents a nonfunctional pseudogene as suggested by Eichler et al. (214) remains to be established.

In perfused liver of transgenic mice expressing rat B-CK in this organ, the intracellular [Cr] and [PCr] are 25 and 8 mM, respectively, at a [Cr] of 2 mM in the perfusion medium (97). This suggests either that a mechanism for the accumulation of Cr also exists in normal liver (415) or that expression of CK and/or accumulation of PCr in transgenic liver are regulatory signals that stimulate expression of the Cr transporter. In human red blood cells, Cr concentration decreases with cell age from 11 to 0.15 mM. Mathematical modeling has shown that this decrease may be due to progressive degradation of the Cr transporter (378). Finally, in tissue cultures derived from embryonic or newborn rats, Cr transporter activity is high in astroglial cells, but almost undetectable in neuron-rich primary cultures. Thus Cr transport might be an astroglial rather than a neuronal function (659). This notion is in agreement with the presence of higher amounts of CK (both in terms of protein concentration and mRNA level) and PCr in glial cells compared with neurons (see Refs. 353, 372). On the other hand, in situ hybridization experiments demonstrated expression of Cr transporter mRNA in glial, neuronal, as well as nonneuronal cells of the rat brain (332), and primary rat astroglial cells in culture are able to synthesize guanidinoacetate and Cr from radioactively labeled Gly (197), suggesting that astrocytes may actually provide Cr to other cell types, e.g., neurons.

D. CK

Physiological and biochemical aspects of the CK isoenzyme system have been briefly introduced at the beginning of this article and were the subject of recent reviews (837, 838, 1084, 1124). Instead of repeating the relevant arguments, some major achievements made in the last few years are summarized here.

Transgenic animals lacking single isoenzymes of CK or combinations thereof have recently been analyzed carefully (for reviews, see Refs. 502, 838; see also Refs. 73, 527, 818, 848, 942, 1026, 1047, 1091). Mutant mice lacking the muscle form of cytosolic CK, M-CK, are viable and fertile and display neither overt abnormalities nor alterations in absolute muscle force (1043). However, the gastrocnemius-plantaris-soleus muscle complex of these M-CK knockout mice lacks the ability to perform burst activity, i.e., at 1- and 5-Hz stimulation, the isometric twitch force is considerably lower for the first 70 and 25 s, respectively, than in controls. After these periods, the ratios invert and mutants develop even greater force than controls.

Graded mutants displaying a two-, three-, or sixfold depression of MM-CK activity relative to controls showed intermediary properties in as far as force generation is concerned (1045). Interestingly, no flux through the CK reaction could be detected by the ^{31}P -NMR inversion transfer technique in muscles having 0, 16, and 34% of wild-type MM-CK activity, whereas significant fluxes of similar size were measured in muscles having 50 and 100% of wild-type activity. These findings clearly indicate that a considerable portion of the CK flux in muscle must be NMR invisible, and therefore question the usefulness of NMR experiments for measuring total CK flux.

Single mutants lacking the sarcomeric mitochondrial CK (Mi_b -CK) gene showed no abnormalities in skeletal muscle morphology and short-term performance (939). Oxidative phosphorylation capacity also was normal in both skeletal muscle and heart, except for an impaired stimulation of mitochondrial respiration by Cr (73, 939). These findings might raise questions about the physiological functions of Mi-CK. It has to be kept in mind, however, that the proportion of Mi-CK to total CK activity in skeletal muscle is generally rather low. Because this proportion is considerably higher in rodent heart, it will be more interesting to study the effects of the Mi_b -CK knockout mutation on heart function.

Double mutants deficient in both M-CK and Mi_b -CK, despite being apparently normal, displayed even more pronounced disturbances in gastrocnemius and diaphragm muscle performance than M-CK-deficient single mutants (527, 938, 1091). Tetanic force, power, work, and the rates of tension development and relaxation were considerably decreased. The enhanced endurance performance seen in M-CK-deficient gastrocnemius muscle was

not observed in double mutants. Cultured myotubes of CK-deficient double mutants showed abnormal Ca^{2+} handling in response to depolarization by ACh or KCl, as evidenced by a 30% decrease in the $[\text{Ca}^{2+}]$ amplitude, a 60% increase in the plateau level of Ca^{2+} after stimulation, and decreased rates of Ca^{2+} release and sequestration. Furthermore, double mutants displayed ultrastructural abnormalities, most notably conspicuous tubular aggregates of SR membranes in fast skeletal muscle fibers. Such tubular aggregates are also seen in various myopathies with electrolyte disturbances and may represent a metabolic adaptation to abnormal Ca^{2+} handling.

Isolated perfused hearts from wild-type, M-CK-deficient, and both M- and sarcomeric Mi-CK-deficient mice displayed comparable isovolumic left ventricular contractile performance at baseline and responded to increased heart rate and perfusate $[\text{Ca}^{2+}]$ with similar increases in rate-pressure product (848). During baseline perfusion, [ADP] was significantly higher in M- plus Mi-CK-deficient hearts (214 μM) than in either wild-type or M-CK-deficient hearts (150 μM). In contrast, the ΔG for ATP hydrolysis was not significantly different between the three groups. Increasing heart rate and perfusate $[\text{Ca}^{2+}]$ increased [ADP] in M- plus Mi-CK-deficient hearts to 407 μM and decreased $|\Delta G_{\text{ATP}}|$ by 3.6 kJ/mol. On the other hand, in either wild-type or M-CK-deficient hearts, it had no significant effect on [ADP] and decreased $|\Delta G_{\text{ATP}}|$ by only 1.7 kJ/mol. Consequently, increases in cardiac work in M- plus sarcomeric Mi-CK-deficient mice become more "energetically costly" in terms of high-energy phosphate use, accumulation of ADP, and decreases in the free energy of ATP hydrolysis (848).

Because the importance of the CK/PCr/Cr system for high-energy phosphate transport had been demonstrated most elegantly in sea urchin spermatozoa (1011), it was straightforward to test the effects of a disruption of the proposed PCr shuttle in mouse spermatozoa which contain ubiquitous mitochondrial CK (Mi_a -CK) and the brain isoenzyme of cytosolic CK (B-CK) (942). Surprisingly, transgenic mice deficient in ubiquitous Mi-CK displayed normal viability and fertility as well as normal motility patterns of isolated spermatozoa. In summary, the mild phenotype of some of the CK knockout mutants was rather unexpected. Upon closer examination, however, the CK-knockout mice stress the importance of the CK system for high-energy phosphate metabolism and transport, proper Ca^{2+} handling and, thus, muscle performance.

In contrast to these CK-knockout mice, gain of function effects were studied in transgenic mice expressing B-CK or ubiquitous Mi-CK in liver, a tissue that is normally devoid of CK (32, 96, 347, 454, 502, 642, 643, 717, 846). These livers, in the presence of Cr and PCr, revealed a protection of [ATP] and decreased adenine nucleotide degradation during a fructose load (96), delayed ATP

depletion and onset of cellular damage in low-oxygen stress situations (642), accelerated regeneration of liver mass following major hepatectomy (32, 846), and increased endotoxin tolerance which was reflected in depressed necrosis and apoptosis in liver tissue and in higher survival rates of transgenic mice relative to controls after intraperitoneal injection of either lipopolysaccharide (LPS) alone or LPS combined with D-galactosamine (347, 454). Furthermore, ubiquitous Mi-CK together with Cr or cCr was suggested to inhibit mitochondrial permeability transition induced in liver mitochondria from transgenic mice by Ca^{2+} plus atractyloside, a finding that may have significant implications for ischemia and reperfusion injury (717). Even though the detailed mechanisms of action of CK in these experimental settings are not yet clear, and although appropriate controls have not always been included, the results underscore the critical importance of the CK/PCr/Cr system for proper tissue function.

With regard to mitochondrial permeability transition, complexes between hexokinase, porin of the mitochondrial outer membrane, and adenine nucleotide translocase (ANT) of the mitochondrial inner membrane, when isolated from rat brain and reconstituted into liposomes or black lipid membranes, displayed permeability and conductance properties similar to those of the mitochondrial permeability transition pore which may play a critical role in cellular apoptosis (see Ref. 64). On the other hand, complexes between Mi-CK, porin, and ANT showed properties of the mitochondrial permeability transition pore only after dissociation of Mi-CK octamers into dimers, suggesting that the Mi-CK octamer is able to suppress the structural transition of the ANT into a channel that leads to permeability transition. It will be a challenging task for the future to establish how Mi-CK is involved in mitochondrial permeability transition and apoptosis in vivo, and how potential modulators of the Mi-CK dimer-to-octamer ratio (e.g., CK substrates, cCr, or reactive oxygen species; Refs. 717, 935) may influence these processes.

In studies on the intact, isolated rat diaphragm, Zeleznikar, Goldberg, Dzeja, and co-workers, by using endogenously generated $[\gamma\text{-}^{18}\text{O}]\text{ATP}$, measured net rates of ADP and PCr production catalyzed by adenylate kinase and CK, respectively, as opposed to total phosphate exchange between ATP, ADP, and PCr which can be determined by ^{31}P -NMR saturation or inversion transfer experiments (for a review, see Ref. 206). With the former technique, they obtained evidence for a peculiar organization of high-energy phosphate metabolism in muscle, with CK activity being associated primarily with oxidative phosphorylation and adenylate kinase activity with glycolysis. When oxidative phosphorylation was inhibited with cyanide or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide (FCCP) or by incubating the tissue in N_2 -equili-

brated medium, or when CK activity was gradually inhibited with 2,4-dinitrofluorobenzene, net synthesis of $[\text{O}^{18}]\text{PCr}$ progressively decreased. The changes in net CK rates were compensated by corresponding increases in net adenylate kinase (AdK)-catalyzed phosphorylation of AMP, thus demonstrating a shift in phosphotransferase catalysis from the CK to the AdK system. These results have the following implications: 1) there is a rather complex structural organization of high-energy phosphate metabolism in muscle, allowing for a preferential functional coupling between AdK and glycolysis on one hand and between CK and oxidative phosphorylation on the other hand. 2) When the capacity of the CK/PCr/Cr system is compromised, the AdK system is able, at least in part, to take over its function, as evidenced by the compensatory increase in net AdK rate. 3) The conception that the CK and AdK reactions are near equilibrium in muscle may be invalid. 4) The results question the usefulness of ^{31}P -NMR data for evaluating functional properties of the CK system. 5) These experiments provide a plausible explanation for the rather mild phenotype of some CK knockout mice. Only by disrupting both the AdK and PCr shuttles may it be possible to estimate their real relevance for intracellular high-energy phosphate transport.

Roughly 70 years after the discovery of PCr (213, 244) and after several unsuccessful attempts, the first three-dimensional structure of a CK isoenzyme, namely, of octameric Mi-CK from chicken heart, has recently been reported (267, 445). X-ray crystallography confirmed previous conclusions drawn from electron micrographs of single Mi-CK molecules, showing that they are cubelike structures with fourfold symmetry, a side length of ~ 90 Å, and a Mi-CK protomer in every edge of the cube. The protomers surround a central channel with a diameter of 20 Å that runs through the entire molecule. This channel may be a structural prerequisite for the proposed function of Mi-CK to allow for efficient metabolic channelling of high-energy phosphates out of the mitochondria (see Ref. 1124). In the meantime, two new three-dimensional structures for dimeric rabbit MM-CK (795) and for a transition state-analog complex of monomeric ArgK from the horseshoe crab *Limulus polyphemus* (1162) have been determined. The individual CK and ArgK protomers share the same subunit topology and consist of two domains each, a small α -helical domain (residues 1–112; numbering according to chicken Mi-CK) and a large domain containing an eight-stranded antiparallel β -sheet flanked by seven α -helices (residues 113–380). The active site is located between the two domains and is covered by residues that are conserved within the CK (guanidino kinase) family. These three-dimensional structures should help to further establish the catalytic mechanism as well as structure-function relationships of guanidino kinases.

For a long time, CK isoenzymes were known to contain a single highly reactive sulfhydryl group per protomer

(Cys-278 in Mi-CK, Cys-283 in cytosolic CK). There has been a long-lasting debate on whether this sulfhydryl group is essential for catalysis or not. Conclusive evidence was not available until recently when site-directed mutagenesis of Mi-CK clearly demonstrated that Cys-278, even though it has a critical impact on specific CK activity, is nonessential (275). X-ray crystallography has now located Cys-278 of chicken Mi-CK near the γ -phosphate group of bound ATP (267); in horseshoe crab ArgK, the equivalent residue, Cys-271, interacts with the nonreactive guanidinyll nitrogen of the substrate, Arg (1162).

Nothing was known so far on whether CK activity can be reversibly regulated posttranslationally. In case of excess CK activity and the reaction being near equilibrium, reversible regulation would seem useless. If, however, the CK reaction were rate limiting for high-energy phosphate transport under certain conditions in vivo, regulation of CK activity might have an impact on energy metabolism. Recently, MM-CK was shown in vitro and in differentiated muscle cells to be phosphorylated and thereby inhibited by AMP-activated protein kinase, which may be part of an intricate regulatory network of energy metabolism in muscle (774; see also sect. v). Furthermore, nitric oxide (NO) was shown to reversibly inhibit CK and to decrease the contractile reserve of the rat heart, most probably by modifying the reactive sulfhydryl group mentioned above (see Refs. 29, 444). Similarly, reversible S-thiolation of the reactive sulfhydryl group may be a way for both inhibiting CK activity and for protecting CK against irreversible damage during periods of oxidative stress in the heart (133, 351). As a matter of fact, CK isoenzymes were identified as (prime) targets of irreversible modification and inactivation by reactive oxygen species (see sect. IXC).

All these achievements represent major steps forward in understanding CK structure and function and are expected to be strong stimuli for further progress in this field.

E. Guanidinoacetate Kinase, Arginine Kinase, and Other Guanidino Kinases

In vertebrates, considerable amounts of Cr, PCr, and CK activity are found in almost all tissues with high and fluctuating energy demands (1081). Most tissues of invertebrates, however, lack the CK system. In these tissues, other guanidines (Fig. 8) together with the corresponding phosphagens and guanidino kinases may play a role very similar to Cr, PCr, and CK in vertebrates (for a review, see Ref. 668). PArg is the only phosphagen in arthropods, and it is also found in almost all echinoderms investigated so far, sometimes in combination with PCr. The most pronounced phosphagen diversity is observed in the annelid phylum where all guanidines shown in Figure 8 and the

corresponding guanidino kinases have been identified and where up to three different phosphagens and guanidino kinases may be present in the same organism (668, 996). The fact that echiuroid worms dispose of L-lombricine and L-thalassemine (with the serine moiety of these molecules being in the L-configuration), whereas only D-lombricine has been detected in annelids, further adds to the complexity of phosphagen metabolism in invertebrates.

Only limited information is available on the biosynthesis and degradation pathways for invertebrate guanidines (for a review, see Ref. 995). Contrary to expectation, taurocyamine (2-guanidinoethanesulfonic acid) does not seem to be synthesized in annelids by transamidation from taurine (2-aminoethanesulfonic acid). Instead, hypotaurocyamine (2-guanidinoethanesulfonic acid), formed by transamidation from hypotaurine (2-aminoethanesulfonic acid) and Arg, serves as an intermediate that is then converted to taurocyamine in an enzymatic or non-enzymatic oxidation reaction. The backbone structure of lombricine and thalassemine is provided by (D- or L-) serine and ethanolamine which are incorporated into (D- or L-) serine ethanolamine phosphodiester. Subsequent transamidation, with Arg as amidine donor, yields (D- or L-) lombricine which, in the echiuroid worm *Thalassema neptuni*, is further methylated to (L-) thalassemine (996). Degradation of lombricine in the oligochaete *Lumbricus terrestris* is most likely initiated by a phosphodiesterase that cleaves the molecule into serine and guanidinoethyl phosphate (see Ref. 995). Finally, methylation of guanidinoethyl phosphate is the last step in the biosynthesis of opheline.

Although interest in evolutionary aspects of the structure and function of guanidino kinases and phosphagens diminished in the mid 1970s, the field has recently been revived by cDNA or amino acid sequencing of arginine kinase from the fruit fly *Drosophila melanogaster*, the honey bee *Apis mellifera*, the grasshopper *Schistocerca americana*, the lobster *Homarus vulgaris*, the horseshoe crab *Limulus polyphemus*, the chiton *Liolophura japonica*, the turbanshell *Battilus cornutus*, the sea anemone *Anthopleura japonicus*, the abalone *Nordotis madaka*, and the shrimp *Penaeus japonicus*; of glyco-cyamine (guanidinoacetate) kinase from the polychaete *Neanthes diversicolor*; of lombricine kinase from the earthworm *Eisenia foetida*; as well as of guanidino kinases with unknown substrate specificity from the parasitic trematode *Schistosoma mansoni* and the nematode *Caenorhabditis elegans* (see DDBJ/EMBL/GenBank databanks). All invertebrate guanidino kinases display pronounced biochemical and biophysical similarity as well as considerable sequence homology to the CK isoenzymes (668, 672, 961). cDNA sequencing as well as biochemical and biophysical characterization of additional members of this enzyme family are expected to further our understanding of guanidino kinase evolution and to provide

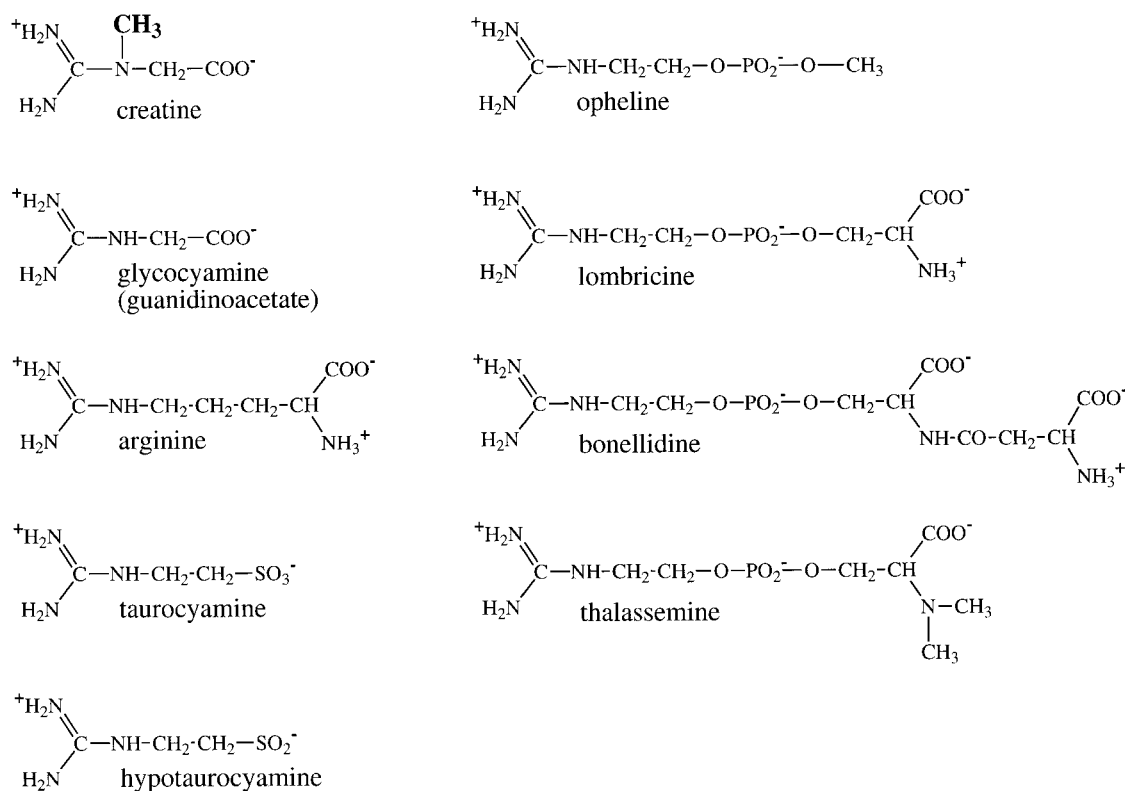


FIG. 8. The natural “phosphagen precursors” are as follows: creatine (Cr), guanidinoacetate = glycocyamine (Gc), arginine (Arg), taurocyamine (Tc), hypotaurocyamine, opheline, lombricine (L), bonellidine, and thalassemine. Note that they all share a guanidino group (drawn on the left of the chemical formulas). Cr is unique in having a disubstituted guanidino group (the additional methyl group is shown in bold), with this feature explaining some of the distinctive chemical properties of PCr. In the corresponding phosphagens, a phosphate group is covalently attached to the guanidino moiety of the molecule.

plausible answers to several intriguing questions (see also sect. XIII A). Is there a common ancestor of all guanidino kinases, and what was its substrate specificity? Why is PCr the sole phosphagen in vertebrates, whereas a series of different phosphagens are present in invertebrates? Why do many invertebrates express CK exclusively in spermatozoa, but other guanidino kinases in their bodily tissues? Does the CK/PCr/Cr system represent a “functional improvement” over the invertebrate guanidino kinase systems? And do the invertebrate guanidino kinases play a protective role in hypoxia?

F. Creatinine Amidohydrolase (Creatininase) and Creatine Amidohydrolase (Creatinase)

Both creatininase (EC 3.5.2.10) and creatinase (EC 3.5.3.3) are inducible enzymes, being expressed in bacteria only when Crn or Cr is provided as main source of carbon or nitrogen (7, 27, 459, 813, 884, 1022). Creatininase activity has been detected in *Alcaligenes*, *Pseudomonas*, *Arthrobacter*, and *Flavobacterium* species (see Refs. 7, 407, 459, 1022), and creatinase activity has been

detected in *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, and *Pseudomonas* species (for references, see Refs. 115, 960). Whereas creatininase and creatinase are found intracellularly in *Pseudomonas* (806, 1022), the same enzymes from *Alcaligenes* seem to be located extracellularly (407).

Creatininase has been purified partially or to homogeneity from *Arthrobacter* species (460, 708), *Pseudomonas* species (191, 806), and *Alcaligenes* (407). The creatininases from *Arthrobacter* and *Pseudomonas* are most likely octameric molecules with a subunit M_r of $\sim 30,000$, whereas *Alcaligenes* creatininase is a dimer composed of two identical 80-kDa subunits. Gene sequencing revealed that *Pseudomonas* and *Arthrobacter* creatininase are 258- to 259-amino acid proteins sharing 36% sequence identity (708, 1131).

Creatininases are stable over quite a broad pH and temperature range and display pH optima between 7 and 9 in both directions of the reaction (7, 191, 407, 460, 806, 1022). As evidenced by an equilibrium constant, $K = [\text{Cr}]/[\text{Crn}]$, of ~ 1.2 at 30°C and pH 6–9, the creatininase reaction is readily reversible. The K_m values for Cr and Crn are

80–162 and 26–66 mM, respectively. The V_{\max} values of *Pseudomonas* creatininase were determined to be 390–1,400 and 1,510 $\mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$, respectively, in the direction of Cr and Crn formation (at pH 7 or 8 and 30°C). *Alcaligenes* creatininase also catalyzes the cyclization of *N*-ethylguanidinoacetate, *N*-propylguanidinoacetate, and guanidinoacetate, with V_{\max}/K_m values (in % relative to Cr) of 228, 103, and 0.09, respectively (809). In the opposite direction of the reaction, *N*-ethylglycocyamidine serves as a substrate with a V_{\max}/K_m value almost identical to Crn, whereas glycocyamidine may be a poor substrate.

Atomic absorption spectrophotometry revealed that *Pseudomonas* creatininase contains one zinc atom per subunit (806), and it is highly probable that the creatininases of *Alcaligenes* and *Arthrobacter* are metal-containing enzymes as well (407, 460). Accordingly, creatininases are inactivated by EDTA, with the metal-free enzyme being completely inactive. In addition, the native (metal-containing) creatininases are inactivated to different degrees by Co^{2+} , Zn^{2+} , Fe^{2+} , Fe^{3+} , Cu^{2+} , and Hg^{2+} . Reactivation of metal-free *Pseudomonas* creatininase, on the other hand, is reached, in order of decreasing effectiveness, with Mn^{2+} , Co^{2+} , Mg^{2+} , Fe^{2+} , Ni^{2+} , and Zn^{2+} . Sulfhydryl reagents decrease the enzymatic activity by no more than 40%, while *N*-bromosuccinimide, *o*-phenanthroline, ethoxyformic anhydride, and photooxidation cause strong or even complete inactivation.

Creatinase has been purified partially from *Arthrobacter ureafaciens* (460) and to apparent homogeneity from *Pseudomonas putida* (1152; see also Refs. 370, 869, 871), *Bacillus* (960), *Arthrobacter* sp. TE1826 (708), and *Alcaligenes* (608). The M_r of native *Arthrobacter ureafaciens* creatinase was estimated to be 100,000, while the subunit M_r of creatinase from *Arthrobacter* sp. TE1826 determined by SDS-PAGE is 48,000. *Pseudomonas* and *Bacillus* creatinase are composed of two identical 43- to 47-kDa subunits each, while *Alcaligenes* creatinase is a monomer with a M_r of 51,000. The creatinases of *Alcaligenes* and *Pseudomonas* both display a *pI* of 4.7–4.8, a pH optimum of 7.5–8.0 (in the direction of Cr breakdown), and are inactivated by sulfhydryl reagents like *p*-chloromercuribenzoate. Complete inactivation of *Pseudomonas* creatinase is achieved upon modification of a single sulfhydryl group per protomer. *Alcaligenes* creatinase has a 65 times higher specific activity than its *Pseudomonas* counterpart, whereas the K_m values for Cr are in a similar range (17.2 vs. 1.3–25 mM). Cr is the preferred substrate of *Pseudomonas* creatinase, but *N*-acetimidoylsarcosine is also hydrolyzed readily (809). In contrast, guanidinoacetate, GPA, *N*-ethylguanidinoacetate, *N*-propylguanidinoacetate, *N*-methyl-3-guanidinopropionate, and cyclocreatine do not or only poorly serve as substrates. *Pseudomonas* creatinase also catalyzes the degradation of pseudothiohydantoic acid to urea and thio-

glycolic acid, with this reaction being inhibited by carbamoyl sarcosine, succinic acid, sarcosine, succinamic acid, Cr, and some other compounds (132).

DNA sequencing revealed that the amino acid sequences of creatinase from *Pseudomonas putida* (370), *Bacillus*, *Flavobacterium* (960), *Arthrobacter* (708), and *Alcaligenes faecalis* (Geneseq accession no. W11861) share a high degree of homology and all have a length of 403–411 residues. Crystal structure determination of creatinase from *Pseudomonas putida* further corroborated the biochemically determined size of the molecule and showed that the two identical subunits are linked in the dimer by more than 20 hydrogen bonds and four salt bridges (132, 370). Each subunit is composed of two domains: a smaller NH_2 -terminal domain (residues 1–160) and a larger COOH -terminal domain (161–402). Residues 155–160 form a hinge region connecting the two domains and possibly allow for movements of the two domains relative to each other. Cocrystallization of creatinase with Cr, carbamoylsarcosine, succinamic acid, or sarcosine revealed that the active site is buried in the interior of the large domain and is partly covered by the small domain of the neighboring subunit. The entrance to the cavity is blocked by two Arg residues. Pocket opening and closure is most likely brought about by a rotation and/or translation of the two domains relative to each other.

When bound to the enzyme, the guanidinium and carbamoyl groups of Cr and carbamoylsarcosine, respectively, are not planar but display a distorted geometry that is characterized by disrupted electron delocalization. This distortion therefore facilitates nucleophilic attack by a water molecule (Fig. 9). His-B232 is close to the guanidinium group of Cr and probably plays a central role in catalysis by serving as a proton donor and acceptor.

All residues of *Pseudomonas* creatinase interacting with the substrate Cr, the product sarcosine, and the competitive inhibitor carbamoylsarcosine are fully conserved in the *Bacillus*, *Flavobacterium*, *Arthrobacter*, and *Alcaligenes* creatinase sequences. Alkylation of Cys-298 causes inactivation of the enzyme. Because this residue is far from the active site, it is tempting to speculate that inhibition is due to an impairment of domain (or subunit) motion, with the enzyme being locked in either an open or closed conformation (132).

Comparison of the amino acid sequences and three-dimensional structures revealed that the COOH -terminal domain of *Pseudomonas putida* creatinase is related to methionine aminopeptidase (AMPM; EC 3.4.11.18) and proline-specific aminopeptidase (AMPP; EC 3.4.11.9) from *E. coli* (see Refs. 681, 1104). Within an ~260-residue chain segment, 218 C^α atoms of the creatinase and AMPM structures superimpose within 2.5 Å, but only 41 of these overlapping positions (19%) feature identical amino acids. AMPM (AMPP) is activated by Co^{2+} (Mn^{2+}) which is coordinated by Asp-97, Asp-108, His-171, Glu-204, and

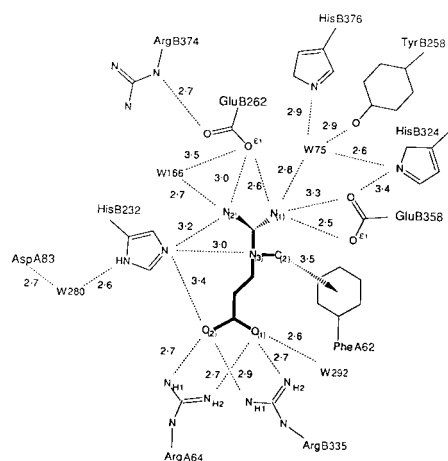


FIG. 9. Schematic representation of the active site of creatinase from *Pseudomonas putida*. The active site of subunit B of the dimeric molecule is shown in the Cr complex at pH 7.4. Amino acids of subunits A and B are marked with the respective letter. The letter W stands for water molecules. Hydrogen bonds are represented by broken lines. A van der Waals contact between C₍₂₎ of Cr and PheA62 is also shown. [From Coll et al. (132), with permission from Academic Press.]

Glu-235 (Asp-260, Asp-271, His-354, Glu-383, and Glu-406). The structurally equivalent residues of creatinase, Asn-249, Ala-260, His-324, Glu-358, and His-376, are substantially different. Accordingly, creatinase is not a metal-dependent enzyme. Searches of protein data banks using sequence and structure-based profiles revealed other enzymes, including aminopeptidase P (EC 3.4.11.9), prolidase (proline dipeptidase, EC 3.4.13.9), eIF-2-associated p67 factors, and agropine synthase, that likely share the same "pita-bread" fold common to creatinase and AMPM.

In very preliminary studies, Miyoshi et al. (654, 655) reported on the detection and partial characterization of creatinase from human skeletal muscle. The enzyme displayed a native M_r of ~50,000 and a pH optimum of 6.2. Whereas creatinase from nonmyopathic patients revealed normal Michaelis-Menten behavior with a $K_m(\text{Cr})$ of 80 μM , the enzymatic activity of creatinase from patients with Duchenne muscular dystrophy depended in a sigmoidal manner on Cr concentration, with half-maximal velocity at 360 μM . In the light of the widespread belief that nonenzymatic conversion to Crn represents the only pathway for Cr degradation in vertebrates, these findings clearly await confirmation.

G. Creatinine Iminohydrolase (Creatinine Deaminase) and Cytosine Aminohydrolase (Cytosine Deaminase)

In microorganisms, cytosine deaminase (EC 3.5.4.1) and Crn deaminase (EC 3.5.4.21) activities overlap considerably. Both reactions are catalyzed by one and the same enzyme in *Pseudomonas putida*, *Pseudomonas*

chlororaphis, *Escherichia coli*, *Proteus mirabilis* (484, 883), *Flavobacterium filamentosum* (229), and baker's yeast (469). Crn inhibits competitively cytosine deaminase activity, and vice versa, implying that both activities result from catalysis at the same active site (229). Interestingly, the ratio of the enzymatic activities with the two substrates, Crn and cytosine, depends on the metal content of the *Flavobacterium* enzyme and can thus be varied (see below). In contrast to the enzymes mentioned so far, the Crn deaminases of *Corynebacterium lilium* (1033), an anaerobic *Clostridium* sp. (357), and of *Tissierella creatinini* (278, 298; see Ref. 335) displayed no cytosine deaminase activity, while the cytosine deaminases of *Pseudomonas ovalis* (484), *Alcaligenes denitrificans*, and of *Arthrobacter* species were unable to utilize Crn as a substrate (485).

Enzymes displaying Crn deaminase and/or cytosine deaminase activity have been purified from a variety of bacteria and fungi (e.g., Refs. 229, 278, 298, 357, 408, 468, 469, 781, 883, 968, 1033). Most of the bacterial enzymes are oligomeric proteins composed of 4–16 identical subunits with a M_r of 35,000–72,000 each. More specifically, the M_r values of the native Crn deaminases from *Flavobacterium filamentosum*, *Pseudomonas putida*, and *Tissierella creatinini* were shown by different techniques to be 245,000–288,000, while SDS-PAGE revealed subunit M_r values of 44,300–53,000. Therefore, these Crn deaminases are most likely hexameric molecules. On the other hand, the fungal enzymes from *Aspergillus fumigatus* and baker's yeast as well as the cytosine deaminases from *Alcaligenes denitrificans* and from an *Arthrobacter* species are likely to be active (also) as monomers with M_r values of 32,000–41,000, while Crn deaminase from *Corynebacterium lilium* seems to be a monomeric protein with a M_r of ~200,000. The cloned Crn deaminase from a *Bacillus* sp. (Geneseq accession no. R79013) codes for a 394-amino acid protein that shares considerable sequence homology with a cytosine deaminase from *E. coli* (see below) (36), but distinctly lower homologies with the cytosine deaminases from *S. cerevisiae* and *Candida albicans* (DDBJ/EMBL/GenBank accession nos. U55193 and U55194, respectively).

The Crn deaminases investigated so far are relatively thermostable, pH resistant, and have pH optima between 7 and 10 (229, 278, 298, 408, 468, 469, 772, 883, 968, 1033). Cr, Arg, urea, Gln, guanidine, cytidine, CMP, and other nucleotides do not serve as substrates. On the other hand, 5-fluorocytosine, 5-methylcytosine, and 3-methylcytosine are deaminated by the enzymes from *Flavobacterium filamentosum* and baker's yeast which display both Crn deaminase and cytosine deaminase activity. The K_m values for Crn of the Crn deaminases investigated so far range between 0.15 and 18 mM, whereas those for cytosine, 5-fluorocytosine, and 5-methylcytosine are between 0.17 and 5 mM.

Purified Crn deaminase from *Flavobacterium fila-*

mentosum was shown by atomic absorption analysis to contain variable amounts of metal ions (229). Zinc (0.87–2.5 g atom/mol enzyme) and iron (0.1–1.2 g atom/mol enzyme) are consistently present in all preparations, whereas nickel is found in some but not in others. Treatment of the enzyme with 1,10-phenanthroline decreases the specific Crn deaminase and cytosine deaminase activities by 98%. Reactivation of the metal-free apoenzyme is achieved by incubation with FeCl_2 , ZnCl_2 , CoCl_2 , CdCl_2 , or NiCl_2 . Several other salts (BaCl_2 , CaCl_2 , CuCl_2 , MgCl_2 , SnCl_2 , SrCl_2 , FeCl_3 , PbAc_2 , and HgCl_2) are ineffective at reactivating the apoenzyme. Remarkably, the ratio of the specific Crn deaminase and cytosine deaminase activities strongly depends on the nature and the proportion of the metal ions present. For example, the activity ratio is 1:3.75 in the presence of FeCl_2 , 1:0.9 in the presence of ZnCl_2 , and 1:0.06 in the presence of NiCl_2 . For different preparations of the purified holoenzyme, this ratio ranged from 1:0.45 to 1:1.10, suggesting that alterations in the metal content may even be a physiologically relevant mechanism for the regulation of this enzyme. Although the effect of metal ions on the specific activity has been investigated thoroughly, no information is yet available about their influence on the K_m values for Crn and cytosine.

In contrast to the *Flavobacterium* enzyme, Crn deaminase from *Clostridium paraputrificum* [the identity of this strain must be considered with caution, since in a later investigation (660), two strains of *Clostridium paraputrificum* were found to lack Crn deaminase activity] is inhibited by divalent cations and stimulated by trivalent cations (Fe^{3+} , Al^{3+}) as well as by di- and polyvalent anions (e.g., P_i , PP_i) (968). The enzymatic activity of the Crn deaminases from *Corynebacterium lilium* and *Cryptococcus* is not affected by metal ions, EDTA, 1,10-phenanthroline, and 2,2'-bipyridine, suggesting that these two proteins are not metal dependent (772, 1033).

Crn deaminase activity is strongly inhibited by sulfhydryl reagents like *p*-chloromercuribenzoate, Hg^{2+} , Ag^+ , and Cu^{2+} , with these inhibitions being reversed by Cys or reduced glutathione (468, 469, 883, 968, 1033). The lack of an effect of Crn on the inactivation by *p*-chloromercuribenzoate implies that the reactive sulfhydryl groups are not involved directly in substrate binding and catalysis.

Somewhat contradictory results have been obtained for *E. coli* cytosine deaminase which, based on gel filtration, SDS-PAGE, and DNA sequencing results, seems to be a tetramer or hexamer composed of 427-amino acid subunits with a calculated M_r of 47,561 each (36, 151, 468, 484, 781). Although the purified enzyme was suggested by some authors to have Crn deaminase activity (469), it was shown by others not to do so (D. Porter, unpublished results). Two alternative interpretations have to be considered. 1) *E. coli* contains two different cytosine deaminases, with only one of them displaying Crn deaminase activity. 2) More likely, *E. coli* cytosine deaminase is a

metal-dependent enzyme like the one from *Flavobacterium filamentosum*, with the cytosine deaminase-to-Crn deaminase activity ratio depending on the metal content of the enzyme. In fact, *E. coli* cytosine deaminase was shown to contain a catalytically essential divalent metal ion and to be inactivated by >95% upon treatment with 1,10-phenanthroline (781). Reactivation of the metal-free apoenzyme was achieved by incubation with Fe^{2+} , Mn^{2+} , Co^{2+} , and Zn^{2+} .

To more clearly define the evolutionary and functional relationships between the various microbial cytosine deaminases and Crn deaminases, more detailed studies will be required in the future on the substrate specificity, the metal ion dependence, as well as on the DNA and amino acid sequences of these enzymes.

H. 1-Methylhydantoin Amidohydrolase and *N*-carbamoylsarcosine Amidohydrolase

1-Methylhydantoin amidohydrolase (EC 3.5.2.14) and *N*-carbamoylsarcosine amidohydrolase (EC 3.5.1.59) activities have been detected in bacteria from the genera *Brevibacterium*, *Moraxella*, *Micrococcus*, *Arthrobacter*, *Pseudomonas*, and *Clostridium* (see Refs. 170, 357, 714, 883, 884, 892). Both enzymes are expressed only when Crn or 1-methylhydantoin are provided as sole sources of nitrogen and/or carbon.

1-Methylhydantoin amidohydrolase has been purified from *Brevibacterium*, *Moraxella*, *Micrococcus*, and *Arthrobacter* species (892) as well as from *Pseudomonas putida* 77 (482, 714, 883). The native *Pseudomonas* enzyme displays a M_r of 300,000, a *pI* of 4.15, and seems to be a heterotetramer composed of two α -subunits (M_r 80,000) and two β -subunits (M_r 70,000). The cloned 1-methylhydantoin amidohydrolase from an *Arthrobacter* sp. codes for 1,288 amino acid residues with a calculated M_r of ~140,000 (870) and displays significant homology to other hydantoinases. Hydrolysis of 1-methylhydantoin to *N*-carbamoylsarcosine, as catalyzed by these 1-methylhydantoin amidohydrolases, is stoichiometrically coupled with ATP hydrolysis, with K_m values for 1-methylhydantoin and ATP of 20–32 and 7.5–800 μM , respectively, and with a pH optimum of 7.5–9.0. Hydantoin, L-5-methylhydantoin (but not D-5-methylhydantoin), glutarimide, and succimide are also hydrolyzed, although with distinctly higher K_m and lower V_{max} values than 1-methylhydantoin. In addition, glutarimide and succimide hydrolysis rates are ~50% lower than the respective ATP hydrolysis rates, thus showing that the stoichiometric coupling between the two enzymatic activities is lost. Dihydrouracil, dihydrothymine, uracil, thymine, and a variety of other compounds even stimulate ATP hydrolysis without being hydrolyzed themselves.

In addition to ATP, a series of other nucleoside

triphosphates and even ADP are hydrolyzed by the enzyme. In the presence of 1-methylhydantoin or dihydrouracil, ATP and dATP display the lowest K_m and highest V_{max} values. In the absence of an amide substrate, however, ATP and dATP are not hydrolyzed at all, whereas considerable hydrolytic activity was observed with the other nucleoside triphosphates tested. These findings point to a pronounced change in the specificity for nucleoside triphosphates upon binding of an amide substrate like 1-methylhydantoin or dihydrouracil.

The enzymatic activity of the 1-methylhydantoin amidohydrolases critically depends on both divalent and monovalent cations. EDTA almost completely abolishes enzymatic activity. The potencies for reactivation of the metal-free enzyme decrease in the order $NH_4^+ > Rb^+ > K^+ > Cs^+$ and $Mg^{2+} > Mn^{2+} > Co^{2+}$. 1-Methylhydantoin amidohydrolases are also inhibited by sulfhydryl and carbonyl group reagents, several metal ions, and some other compounds.

In its catalytic properties, 1-methylhydantoin amidohydrolase closely resembles two other amide-hydrolyzing enzymes, 5-oxoprolinase (EC 3.5.2.9) and urea amidolyase (EC 3.5.1.45) (see Refs. 482, 714). All three enzymes depend on ATP, Mg^{2+} or Mn^{2+} , and a monovalent cation (NH_4^+ or K^+) for catalysis. In analogy to 1-methylhydantoin amidohydrolase, ATP hydrolysis by 5-oxoprolinase is stimulated by L-2-imidazolidone-4-carboxylate and dihydroorotate, which are not hydrolyzed themselves by the enzyme. Therefore, it is tempting to speculate that the three enzymes are evolutionarily close.

In the case of anaerobic bacteria, evidence has been provided for ATP-independent 1-methylhydantoin amidohydrolases displaying K_m values for 1-methylhydantoin of 4.0–18.7 mM (278, 357). The enzyme from *Tissierella creatinini* (see Ref. 335) had a pH optimum of 8.9. Its enzymatic activity critically depended on dithioerythritol and was inhibited rather than stimulated by NH_4^+ and Mg^{2+} . In addition, a 20% higher rate of hydrolysis was observed with hydantoin compared with 1-methylhydantoin. If confirmed, these findings raise the question whether ATP-dependent and ATP-independent 1-methylhydantoin amidohydrolases are characteristic of aerobic and anaerobic bacteria, respectively, or whether they represent adaptations to other physiological constraints.

N-carbamoylsarcosine amidohydrolase has been purified from *Arthrobacter*, *Micrococcus*, and *Moraxella* species (170) as well as from *Pseudomonas putida* 77 (486, 883). Moreover, the kinetic properties of the enzyme have been characterized preliminarily in a cell-free extract of *Tissierella creatinini* (278; see Ref. 335). For the *Pseudomonas* enzyme, gel permeation chromatography and ultracentrifugation experiments yielded a M_r of 75,000–102,000, but an unusually high $s_{20,w}$ of 13.9 S.

Because the subunit M_r was determined to be 27,000 by SDS-PAGE, the native molecules might be trimers, tetramers, or even larger aggregates. The *Arthrobacter* enzyme was shown by biochemical analysis, DNA sequencing, and crystal structure determination to be a tetrameric molecule composed of identical 264-amino acid subunits (819, 1156). Two subunits each form compact dimers which, in turn, are held together in the tetramer by just a few contacts. The four active sites are located at the intersubunit interfaces of the compact dimers. Experiments with reversible and irreversible inhibitors strongly suggest that catalysis involves nucleophilic attack of *N*-carbamoylsarcosine by the reactive thiol group of Cys-177, thereby forming a covalent enzyme-thiohemiacetal intermediate. Arg-202 is also likely to play a critical role, since it does not only interact with the ligands but also blocks the entry to the catalytic cleft. Arg-202 must move to allow exchange of ligands.

Attempts to demonstrate the reversibility of the reaction failed so far. The enzyme displays remarkable substrate specificity: *N*-methyl-*N*-carbamoyl-DL-alanine, *N*-carbamoylglycine, *N*-carbamoyl-DL-alanine, and a variety of other *N*-carbamoyl compounds were reported to be hydrolyzed either slowly (<13% of the activity with *N*-carbamoylsarcosine) (486) or not at all (1156). Likewise, these substances are, if at all, only weak inhibitors of *N*-carbamoylsarcosine hydrolysis. Crn, Cr, sarcosine, 1-methylhydantoin, and hydantoin also do not serve as substrates. *N*-carbamoylsarcosine amidohydrolase displays stereospecificity, with the D- but not the L-isomers of the *N*-carbamoyl compounds being hydrolyzed.

The K_m for *N*-carbamoylsarcosine as well as the pH optimum of the enzyme seem to depend on buffer composition, with respective values of 0.125–7.1 mM and 7.0–8.5. The V_{max} of the purified enzyme at pH 7.5–8.0 and 25–37°C is on the order of 2 μ mol *N*-carbamoylsarcosine hydrolyzed \cdot min $^{-1}$ \cdot (mg protein) $^{-1}$. *N*-carbamoylsarcosine amidohydrolase is potentially inhibited by thimerosal (= merthiolate), *p*-chloromercuribenzoate, DTNB, Ag^+ , Hg^{2+} , Cu^{2+} , Zn^{2+} , and sodium arsenite, thus supporting the reaction mechanism proposed above. Other metal ions as well as 1,10-phenanthroline and 2,2'-bipyridine had no significant effects on enzyme activity.

I. Sarcosine Oxidase, Sarcosine Dehydrogenase, and Dimethylglycine Dehydrogenase

In various microorganisms, sarcosine is metabolized further to glycine (8; for a review, see Ref. 958). In most bacteria belonging to the genera *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Corynebacterium*, and *Pseudomonas* as well as in fungi from the genera *Cylindrocarpon* and *Streptomyces*, this degradation is achieved by a sarcosine oxidase (EC 1.5.3.1; see Refs. 27, 500, 707, 803, 883,

958, 959). On the contrary, sarcosine degradation is catalyzed in some *Pseudomonas* strains by a sarcosine dehydrogenase (EC 1.5.99.1; see Refs. 729, 884, 1022). Sarcosine oxidase and sarcosine dehydrogenase activities are induced in these microorganisms only when grown on a medium containing Crn, Cr, 1-methylhydantoin, sarcosine, dimethylglycine, choline, or betaine as main source of carbon and/or nitrogen.

The sarcosine dehydrogenase of *Pseudomonas putida* is a homotetrameric flavoprotein composed of $\sim 45,000$ M_r subunits (729). The purified enzyme displays a pI of 5.9, a pH optimum of 8.0–9.0, and a K_m for sarcosine of 29 mM. At least two classes of microbial sarcosine oxidases have to be discriminated, all of which are flavoproteins with a K_m for sarcosine of 0.9–12.2 mM (125, 633, 706; for reviews see Refs. 883, 958). *Arthrobacter*, *Bacillus*, *Cylindrocarpon*, and *Streptomyces* species express monomeric sarcosine oxidases with ~ 390 amino acid residues and a M_r of 42,000–45,000. On the other hand, heterotetrameric sarcosine oxidases with a M_r of 160,000–185,000 have been identified in *Corynebacterium*, *Pseudomonas*, and *Arthrobacter denitrificans*. The corynebacterial enzyme is composed of an α -subunit (M_r 103,000), β -subunit (44,000), γ -subunit (21,000), and δ -subunit (11,000), with the covalent flavin being attached to the β -subunit (124). In addition to the covalent flavin, a noncovalent flavin and an NAD^+ molecule have been identified as coenzymes of corynebacterial sarcosine oxidase (for references, see Ref. 124). While the flavins are involved in catalysis, NAD^+ is not reduced by sarcosine and seems not to be in redox equilibrium with the flavins. Finally, the sarcosine oxidase of *Alcaligenes denitrificans* was suggested to be a 190,000- M_r heterodimer or heterotrimer composed of an α -subunit (M_r 100,000) and one or two β -subunits (M_r 55,000) (483). However, it cannot be ruled out at present that this protein also is a heterotetrameric enzyme, with the γ - and δ -subunits having escaped detection so far.

All bacterial sarcosine oxidases catalyze the oxidative demethylation of sarcosine to yield glycine, H_2O_2 , and formaldehyde. The heterotetrameric sarcosine oxidases, in contrast to the monomeric ones, also use tetrahydrofolates as substrates, giving rise to 5,10-methylenetetrahydrofolates instead of formaldehyde as reaction products (1070). In this regard, heterotetrameric sarcosine oxidases resemble mammalian sarcosine and dimethylglycine dehydrogenases (EC 1.5.99.2) that catalyze the last two steps in the consecutive degradation of choline to betaine, dimethylglycine, sarcosine (=methylglycine), and glycine.

Dimethylglycine dehydrogenase and sarcosine dehydrogenase have been purified from rat liver and are monomeric flavoproteins with M_r values of $\sim 90,000$ and 105,000, respectively (530, 780, 1111). The former displays

both dimethylglycine and sarcosine dehydrogenase activity in a ratio of 4:1, whereas the latter is specific for sarcosine. Cr was shown not to be decomposed by both of these dehydrogenases (1112). In contrast to the mitochondrial localization of both mammalian dehydrogenases, a peroxisomal sarcosine oxidase with 390 amino acids and a calculated M_r of $\sim 44,000$ has recently been purified and/or cloned from rabbit kidney and liver (803) as well as from mouse liver (356). Sarcosine, L-pipecolic acid, and L-proline are oxidized with similar catalytic efficiencies, whereas dimethylglycine, D-pipecolic acid, D-proline, and other amino acids are rather poor substrates of this enzyme.

Interestingly, sequence comparisons revealed pronounced homology between the β -subunit of heterotetrameric corynebacterial sarcosine oxidase, monomeric bacterial sarcosine oxidases, rabbit peroxisomal sarcosine oxidase, pipecolic acid oxidase from mammalian liver, amino acid deaminase from *Proteus mirabilis*, a (duplicated) protein with unknown function from *Caenorhabditis elegans*, and the NH_2 -terminal half of mammalian dimethylglycine dehydrogenase (125, 356, 803). Likewise, the α -subunit of heterotetrameric corynebacterial sarcosine oxidase displays sequence homology to the A subunits of octopine and nopaline oxidases from *Agrobacterium tumefaciens*, to T proteins from *E. coli* and several eukaryotes (components of the multienzyme glycine cleavage system), and to the COOH-terminal half of dimethylglycine dehydrogenase. These results therefore point to common evolutionary ancestors for all of these (flavo)proteins.

J. Methylguanidine Amidinohydrolase

Under aerobic conditions, methylguanidine was shown to be used by an *Alcaligenes* species as sole source of carbon and nitrogen (685). Purification yielded a homogeneous methylguanidine amidinohydrolase (EC 3.5.3.16) preparation with a specific activity of $102 \mu\text{mol} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$ (30°C, pH 10.0). The purified enzyme displayed marked substrate specificity. Ethylguanidine, *n*-propylguanidine, *n*-butylguanidine, agmatine, and guanidine were hydrolyzed with 75- to 500-fold lower rates than methylguanidine, whereas no enzymatic activity was observed with guanidinoacetic acid, 3-guanidinopropionic acid, 4-guanidinobutyric acid, or guanidinosuccinic acid. Unfortunately, Cr and methylguanidine have never been tried as substrates of methylguanidine amidinohydrolase and creatinase, respectively, so that at present, nothing is known about the evolutionary and functional relationships between these two enzymes.

IX. USE OF CREATINE ANALOGS AND INVERTEBRATE PHOSPHAGENS AS TOOLS FOR THE STUDY OF THE PHYSIOLOGICAL FUNCTIONS OF THE CREATINE KINASE SYSTEM

A. PCr in Comparison With Invertebrate Phosphagens and Synthetic Analogs: Thermodynamic and Kinetic Considerations

In the first account on PCr, Eggleton and Eggleton (213) mentioned that "the supposed inorganic phosphate of muscle is in certain conditions mainly organic phosphate of a very labile nature [= PCr], which is so unstable in acid solution." This statement explains 1) why this substance escaped detection before and 2) why it was termed "phosphagen". Whereas PCr was found predominantly in vertebrates, a variety of other phosphorylated guanidino compounds were subsequently identified in invertebrates and were shown to play a physiological role similar or identical to PCr in vertebrates (Fig. 8) (for reviews, see Refs. 221, 226, 668, 669, 995, 1092, 1093). Consequently, the term *phosphagen* nowadays is used generally for all phosphorylated guanidino compounds that may serve to regenerate ATP. PCr is unique in this family, in so far as it is the only natural phosphagen with a methyl group attached to the guanidino moiety of the molecule (bold in Fig. 8). This feature may explain some of the distinctive chemical properties of PCr.

All of the phosphagens are rather stable in alkaline solution but are susceptible to acid hydrolysis, with the acid lability decreasing in the order PCr > PArg > PTc > PGc (see Refs. 226, 669). Although the rate of PCr hydrolysis increases with increasing acidity, the rate of hydrolysis of PArg, PGc, and PTc displays a maximum at pH 1.0–3.5. Acid molybdate accelerates the hydrolysis of PCr but retards that of PArg, PGc, and PTc. Depending on temperature, molybdate concentration, and pH, acid hydrolysis of PCr results in the formation of Cr plus P_i and/or Crn plus P_i. In contrast, no cyclic products are formed upon acid hydrolysis of other phosphagens.

The *N*-methyl group of PCr eliminates almost all resonance states in the guanidino group (221, 226). In consequence, PCr is thermodynamically less stable ($\Delta G^{\circ'}$ for PCr hydrolysis = −44.6 kJ/mol at 35°C, pH 7.25 and 4 mM Mg²⁺) than PArg, PGc, PTc, and PL ($\Delta G^{\circ'}$ = −39.4, −41.4, −41.5, and −41.7 kJ/mol, respectively) (221). The differences in thermodynamic stability between the various phosphagens are also reflected in the different electronic environments of the phosphorus nuclei as visualized by ³¹P-NMR. While the phosphorus nucleus of PCr displays a chemical shift of −2.57 ppm relative to an external standard of *o*-phosphoric acid, the respective values for PArg, PGc, PTc, and PL are −3.00, −3.03, −3.03, and −3.01 ppm, respectively (221).

The $\Delta G^{\circ'}$ for PCr hydrolysis is also more negative than that for ATP hydrolysis (−45.1 vs. −35.7 kJ/mol in the absence of Mg²⁺, −45.0 vs. −31.8 kJ/mol at 1 mM Mg²⁺, pH 7.0, 38°C, *I* = 0.25 M) (538; see also Refs. 451, 474). This implies, under the assumption of near-equilibrium conditions for the CK reaction in the cytosol, that the free energy change (= affinity) of ATP hydrolysis {defined as $A = -dG/d\xi = -\Delta G^{\circ'} + RT \ln([ATP]/[ADP][P_i])$, where $d\xi$ stands for the advancement of the reaction} and thus the phosphorylation potential in a cell can be buffered efficiently at much higher values by PCr and Cr than by ATP and ADP alone. Buffering of the phosphorylation potential, in turn, seems to be crucial for some cellular processes, especially for the Ca²⁺-ATPase of the SR which depends on a free energy change for ATP hydrolysis of at least 52 kJ/mol to allow proper muscle relaxation (310, 451). The notion that CK may help in maintaining high phosphorylation potentials in the intimate vicinity of crucial ATPases is indeed supported by the close functional coupling and the colocalization observed for CK and Ca²⁺-ATPase of the SR (see Ref. 646).

Based on the $\Delta G^{\circ'}$ values for ATP and PCr hydrolysis, the equilibrium constant for the reaction ATP + Cr \leftrightarrow ADP + PCr, $K' = ([\Sigma ATP][\Sigma Cr])/([\Sigma ADP][\Sigma PCr])$, where Σ represents the sum of all ionized and Mg²⁺-complexed forms in solution and where pH is taken to be constant at 7.0, was calculated to be 37.8 in the absence of Mg²⁺ and 166 at a free Mg²⁺ concentration of 1 mM (38°C, *I* = 0.25 M) (538). Because K' critically depends on pH, temperature, [Mg²⁺], and probably also on the concentrations of other monovalent and divalent cations (see Refs. 287, 538), reported values for K' (e.g., Refs. 421, 518, 574) should be taken as rough estimates and should not be compared directly without considering differences in experimental conditions.

It may be asked at this stage why evolution has "chosen" PCr for the vertebrates and a variety of more stable phosphagens for the invertebrates. Over many years, the hypothesis that PArg is simply the evolutionary precursor of PCr has attracted much attention (see, for example, Refs. 1092, 1093). Evidently, this hypothesis implies that PCr in some respect represents a functional improvement over PArg. Even though the common ancestral gene of all guanidino kinases may in fact have been an arginine kinase (ArgK), only unsatisfactory arguments are available for explaining the current distribution of the various phosphagens in the animal kingdom.

PCr is the only phosphagen in vertebrates, but it is also found in spermatozoa of a wide variety of invertebrates (for reviews, see Refs. 221, 811, 1092). In these invertebrate species, PCr in spermatozoa may coexist with one or even two other phosphagens in other tissues (pluriphosphagen phenomenon) (221, 668, 811, 1092). In sea urchins of the class Echinoidea, for example, PCr is the sole phosphagen in spermatozoa, whereas the differ-

ent lantern muscles contain both PArg/ArgK and PCr/CK in varying proportions (220, 1092). CK and ArgK in these lantern muscles were suggested to be associated more with tonic and phasic contractions, respectively (1092). Clearly, if PCr simply represented a functional improvement over PArg, it would be inconceivable why PArg in these invertebrates was replaced by PCr only in some tissues, but not in others. The following excursus shall provide an alternative explanation (see also Ref. 221).

To test the effects of Cr analogs on energy metabolism during ischemia, Walker and co-workers (reviewed in Ref. 1076) fed chickens either with a control diet or with the same diet supplemented with cyclocreatine (cCr; 1-carboxymethyl-2-iminoimidazolidine; see Fig. 10), homocyclocreatine (hcCr; 1-carboxyethyl-2-iminoimidazolidine), or Cr. cCr and hcCr compete with Cr for uptake into the tissues, are accumulated instead of Cr inside the cells, and are finally phosphorylated by intracellular CK. PhcCr is ~200,000-fold less potent than PCr and 1,500-fold less potent than PcCr in acting as a substrate for CK, and both PhcCr and PcCr display a $\Delta G^{\circ'}$ of hydrolysis ~7.3–8.4 kJ/mol less negative than that of PCr (37°C; see Table 1) (810, 1076).

Hearts of chickens fed cCr for at least 6 days accumulate $15 \mu\text{mol} \cdot (\text{g wet wt})^{-1}$ of cCr plus PcCr. This accumulation is paralleled by a triplication of the glycogen levels and by a decrease in the Cr + PCr levels from

the normal $6 \mu\text{mol/g}$ to $1.8 \mu\text{mol/g}$ (1028). During total ischemia in vitro, these hearts utilize PcCr for the regeneration of ATP, display greatly prolonged glycolysis, and exhibit a two- to fivefold delay in ATP depletion relative to controls. Accumulation by chicken hearts of a comparable amount of PhcCr is also paralleled by a triplication of the glycogen levels, but by a decrease in Cr + PCr levels to only $4.2 \mu\text{mol/g}$. During ischemia, these hearts exhibit prolonged glycolysis but do not utilize PhcCr and manifest no delay in ATP depletion relative to controls. Therefore, the cCr effects on energy metabolism during ischemia are most probably not due to the increased glycogen levels. Similar to hcCr, no delay in ATP depletion was observed during ischemia in hearts of Cr-fed chickens having accumulated $14.8 \mu\text{mol/g}$ of Cr plus PCr.

In further investigations, long-term feeding of cCr was found 1) to increase the total high-energy phosphate levels in mouse brain and skeletal muscle (24, 1119); 2) to delay ATP depletion during ischemia in mouse brain, mouse skeletal muscle, rat heart, rat skin, and chicken breast muscle (24, 147, 419, 808, 1027, 1119); 3) to delay onset of rigor contraction in ischemic rat heart and mouse skeletal muscle (24, 419, 808); and 4) to enhance survival of experimental skin flaps in the rat (147). On the basis of these results, it may be hypothesized that at the lower phosphorylation potentials and at the more acidic pH values characteristic of later stages of ischemia, substantial levels of a kinetically competent and thermodynamically rather stable phosphagen are advantageous to assist glycolysis in buffering decreases and oscillations in the ATP/ADP and ATP/(ADP · P_i) ratios (1028). In contrast, PCr and PhcCr are not suited for this purpose for thermodynamic reasons and because of kinetic limitations, respectively. Very similar to the synthetic phosphagen PcCr, the phosphagens characteristic of invertebrates (i.e., PArg, PGc, PTC, PL) display $\Delta G^{\circ'}$ values of hydrolysis that are 2.9–5.2 kJ/mol less negative than that of PCr (221). Provided that the corresponding guanidino kinases are also present, these phosphagens may thus be advantageous in invertebrate phyla under conditions of hypoxia or intracellular acidosis (221, 1124).

In vertebrates, which almost perfectly maintain intracellular homeostasis and where pronounced acidification is only observed in extreme situations, PCr may in fact represent a functional improvement over PArg, since it can efficiently buffer the cytosolic phosphorylation potential at a higher value. In many invertebrates, on the other hand, changes in the extracellular environment may cause pronounced fluctuations in intracellular composition (e.g., pH, temperature, oxygen and substrate concentrations). Intracellular acidosis, for example, is a common occurrence, especially in mollusks (see Ref. 221). Under these conditions, PCr may be quite susceptible to hydrolysis, resulting in net energy dissipation. PArg and the other invertebrate phosphagens, on the other hand, are

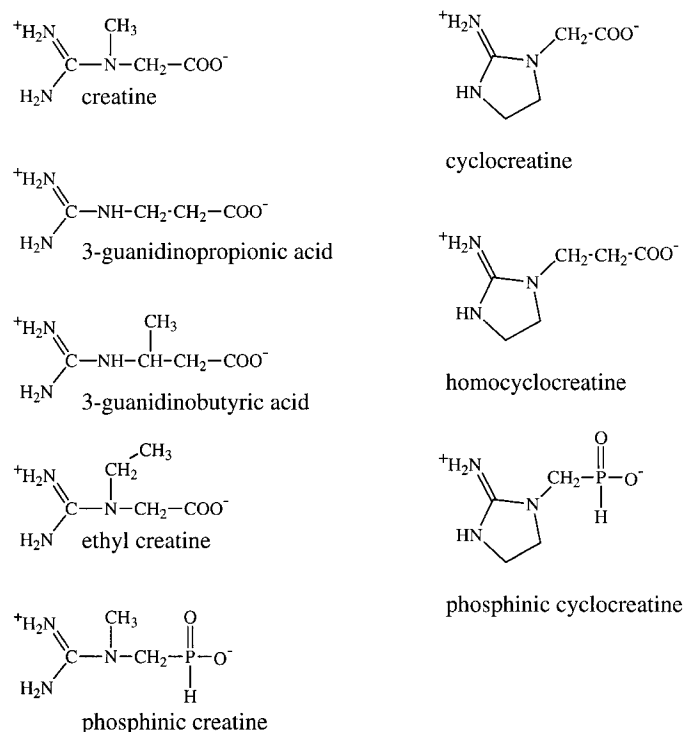


FIG. 10. Chemical formulas of synthetic creatine analogs. Phosphinic creatine, *N*-methyl-*N*-amidino-aminomethylphosphinic acid; phosphinic cyclocreatine, 2-iminoimidazolidine-1-methylphosphinic acid; ethyl creatine, *N*-ethyl-*N*-amidinoglycine.

TABLE 1. Capacity of Cr analogs to act as substrates of CK and to compete with Cr for uptake into rat skeletal muscle and heart

Cr Analog (X)	V_{\max}/K_m as Substrate of CK, in % relative to Cr	V_{\max}/K_m of the Phosphorylated Form (PX) as Substrate of CK, in % relative to PCr	Inhibition of Cr Uptake Into Skeletal Muscle, in % relative to Cr	Inhibition of Cr Uptake Into Heart, in % relative to Cr	Equilibrium Constant K of the Reaction $\text{PCr} + \text{X} \leftrightarrow \text{Cr} + \text{PX}$
Creatine	100	100	100	100	(1)
Creatinine					0.039
Guanidinoacetate	0.30–0.69		47	11	
3-Guanidinopropionate	0.091	0.043–0.067	80	98	3.06
DL-2-Guanidinopropionate	0.021		26	35	
DL-3-Guanidinobutyrate	ND	ND	84	89	
DL-2-Guanidinobutyrate			50	65	
N-ethyl-N-amidinoglycine	1.9–5.1	0.5–3.3	35	22	
N-propyl-N-amidinoglycine	0.094				
N-methylamidino-N-methylglycine	ND	ND	70	68	
N-methyl-N-amidino- β -alanine	0.007–0.066	~0.2	16	8.4	
(R,S)- or (R)-N-methyl-N-amidinoalanine	1.0–1.7				
1-Carboxymethyl-2-imino-hexahydropyrimidine	ND	ND	69	75	
(R)-N-amidinoazetidine-2-carboxylic acid	6.4				
(S)-N-amidinoazetidine-2-carboxylic acid	ND				
(R)-N-amidinoproline	0.18				
(S)-N-amidinoproline	ND				
1-Carboxymethyl-2-iminoimidazolidine (cCr)	16–38	0.40–0.67			26–34.3
1-Carboxyethyl-2-iminoimidazolidine (hcCr)	~0.01	~0.0005			17
N-methyl-N-amidino-aminomethylphosphinic acid	2.7–12.6	2.1			
2-Iminoimidazolidine-1-methylphosphinic acid	2.0	0.020			

To determine the potencies of various analogs to competitively inhibit creatine (Cr) uptake into rat skeletal muscle and heart, rats were given 50 μmol of the respective test compound and 0.21 μmol of [^{14}C]Cr intraperitoneally. One hour later, the rats were killed, and radioactivity was measured in tibialis anterior muscle and heart. [Data from Fitch and Chevli (246).] The inhibition of [^{14}C]Cr uptake exerted by 50 μmol of “cold” Cr was taken as 100%. ND, not detectable; CK, creatine kinase; K_m , Michaelis constant; V_{\max} , maximum velocity. Other data listed in the table are from Refs. 23, 60, 120, 246, 414, 474, 809, 810, 1109.

less susceptible to acid hydrolysis than PCr and still efficiently buffer [ATP]/[ADP] at the lower phosphorylation potentials characteristic of hypoxia. In invertebrates, the CK/PCr/Cr system may thus be expressed specifically in those tissues that critically depend on a high phosphorylation potential, with spermatozoa obviously fulfilling this criterion. Clearly, these arguments may only explain why thermodynamically more stable phosphagens are advantageous for invertebrates. They provide no answer, however, for the question why highly complex phosphagens like PL and phosphoryl-thalassemine are formed in certain echinoid and annelid worms when, on a thermodynamic and kinetic basis, the much simpler phosphagens PGc and PTc might do the same job. This aspect of comparative physiology awaits investigation and elucidation.

In addition to cCr and hcCr mentioned above, a variety of other synthetic Cr analogs have been synthesized (some of them listed in Table 1; for references, see Refs. 59, 644, 645) and have been used to investigate the physiological role of the CK/PCr/Cr system, to study the regulation of Cr biosynthesis, and to evaluate the relationships between abnormalities of Cr metabolism and muscle disease (see sect. IXA). For instance, Cr analogs have been used very elegantly to prove that Cr is the physio-

logical feedback repressor of liver and kidney AGAT (for a review see Ref. 1077). For allowing clear-cut conclusions, an “ideal” Cr analog for in vivo investigations should either 1) completely inhibit Cr biosynthesis, 2) completely prevent Cr uptake by muscle and nerve cells in vivo, 3) completely and specifically inhibit CK activity in vivo, or 4) completely replace Cr and PCr, with the synthetic phosphagen possessing markedly different thermodynamic and kinetic properties relative to PCr (1077). None of the currently used Cr analogs fulfills any of these criteria, and undesired side effects therefore have to be taken into account (see sect. VIII B).

The extent to which a synthetic phosphagen is accumulated within a given tissue and the degree to which PCr and Cr are replaced depend on a variety of factors (810): the maximal concentration of the Cr analog that can be tolerated in the diet, the average concentration of this analog in the serum maintained by the rates of uptake (intestine) and excretion (kidney), the V_{\max}/K_m values for uptake and release of the Cr analog into and from a given tissue, the fraction of the Cr analog that is phosphorylated by CK (to yield the synthetic phosphagen), the V_{\max}/K_m values for the synthetic phosphagen and for the Cr analog as substrates of the CK isoenzymes, the rate of metabolic

side reactions (biodegradation), and the extent of feedback repression of AGAT exerted by the Cr analog.

To get a feeling for the potencies of the Cr analogs that have been used so far, the available knowledge will shortly be summarized (Table 1). The Cr analogs and corresponding synthetic phosphagens cover a wide range of potencies to act as substrates for CK. cCr and PcCr are rather good substrates, both in vitro and in vivo (810). On the other hand, GPA and PGPA, with 1,000- to 1,500-fold decreased V_{\max}/K_m values, are expected to be poor CK substrates. Nevertheless, upon changes in workload in the perfused myocardium of control and GPA-treated rats, [PCr] and [PGPA], respectively, displayed very similar changes, both as far as the absolute concentration changes and the kinetics of the alterations are concerned (135). This finding points to an "overcapacity" of the CK system and suggests that under normal in vivo conditions as well as under most experimental regimens, PGPA is a satisfactory substitute of PCr. Therefore, conclusions as to the function of PCr in organs of animals fed these analogs may be misleading. hcCr and especially PhcCr are extremely poor substrates of CK. As a matter of fact, PhcCr is very slowly consumed under in vivo conditions (810). 3-Guanidinobutyrate is not phosphorylated at all in vivo (1163). Even though, on first sight, hcCr and 3-guanidinobutyrate seem to be perfectly suited for investigations of the physiological functions of the CK/PCr/Cr system, they both suffer the limitations to be accumulated rather inefficiently by the tissues and to replace PCr and Cr only incompletely (810, 1163). These may be the reasons for GPA being the most widely used Cr analog in physiological experiments.

When porcine carotid arteries, containing almost no Mi-CK, were superfused with either GPA, methyl-GPA (mGPA), guanidinoacetic acid (GAA), or cCr, all of these Cr analogs entered the tissue and became phosphorylated (74). When, subsequently, oxidative metabolism was inhibited by cyanide, PCr, PGPA, PGAA, and PmGPA were consumed over a similar time course, despite different kinetic properties of these analogs in vitro. PcCr, on the other hand, was utilized at a significantly lower rate. Nevertheless, the results show that these Cr analogs may all serve as substrates for cytosolic CK. In contrast, only Cr and cCr stimulated respiration in isolated rat heart mitochondria, whereas GAA, mGPA, and GPA were ineffective (74, 130, 1163; see also Ref. 1044). These findings may indicate that Mi-CK displays narrower substrate specificity than the cytosolic CK isoenzymes or, more unlikely, that the outer mitochondrial membrane is impermeable to GAA, mGPA, and GPA.

Compared with CK, the Cr transporter seems to be considerably less specific toward Cr analogs (Table 1). This conclusion has recently been corroborated by experiments on the cloned Cr transporters from *Torpedo*, rabbit, and human (see also sect. VII C) (318, 319, 691, 840,

927). In cells transiently transfected with Cr transporter cDNA, nonlabeled Cr and GPA inhibit the uptake of radioactive Cr very efficiently and to approximately the same extent. 4-Guanidinobutyrate, guanidinoacetate, and 2-amino-3-guanidinopropionate are somewhat less inhibitory, while Crn has only little effect. In experiments on the repression of AGAT activity in embryonic and developing chicken liver, Cr, cCr, *N*-ethylguanidinoacetate, and *N*-acetimidoylsarcosine proved to be powerful repressors, whereas *N*-acetimidoylglycine, 1-carboxymethyl-2-imino-hexahydropyrimidine, *N*-propylguanidinoacetate, and *N*-methyl-3-guanidinopropionate caused either no or just a small reduction in AGAT activity (809, 1078).

To conclude, an ideal Cr analog unfortunately does not exist. Therefore, experiments with Cr analogs have to be analyzed and interpreted with caution. Definite conclusions should only be based on a detailed comparison of several analogs with different kinetic and thermodynamic properties and/or on a variety of experimental approaches: ultrastructural, physiological, and biochemical.

B. Cr Analog Administration as a Means of Studying CK Function: Facts and Potential Pitfalls

Cr analogs have been used frequently for studying the relevance of the CK system for cellular energetics in various tissues. Most of these studies have been performed on skeletal muscle where Cr analog (in particular, GPA) administration caused a series of functional changes (for a review see Ref. 1125). For example, GPA or 3-guanidinobutyrate (GBA) administration decreases developed muscle tension as well as the rates of tension development and relaxation (556, 636, 637, 656, 890, 1044, 1073, 1074), suppresses posttetanic twitch potentiation and the staircase phenomenon (see Refs. 636, 637), improves endurance performance (556, 637, 983, 1044, 1073, 1074), and causes ultrastructural abnormalities (535) (see also below). Even though these results have been taken to indicate that the CK system is important for proper muscle function, some authors arrived at opposite conclusions by finding either no or only minor changes in muscle mechanics upon GPA feeding (889). Either way, results and conclusions on CK function based on Cr analog-feeding experiments must be interpreted with caution, since these compounds may have a series of confounding side effects.

GPA is a physiological constituent of mammalian blood plasma, erythrocytes, brain, liver, kidney, aorta, and urine, and its concentration is considerably increased in uremic patients (see, e.g., Refs. 163, 181, 321, 554, 555, 597, 628, 998, 1132). GPA may be formed by AGAT through transamidination between Arg and β -alanine (1089) (see also sects. VII A and IX H). It may thus be

envisaged that administration of massive amounts of GPA interferes with the physiological metabolic pathways in which Arg, β -alanine, and GPA are implicated.

GPA- and 3-GBA-treated animals commonly display a significantly lower body weight and growth retardation relative to normal controls (e.g., Refs. 3, 5, 246, 371, 556, 584, 629, 656, 724, 727, 741, 754, 983, 1072–1074). The toxicity seems to be most pronounced in frogs, where a mortality of 50% was observed (180), and in young animals. Young chickens fed 1% GPA stopped eating and often died within 1 wk (809, 810). Similarly, cCr was found to be toxic to weanling mice and rats and to developing chicken embryos in the early stages of their development (30, 311, 1078). On the other hand, GPA has been tolerated by adult mice at a level of 1% of the diet for several weeks up to 19 mo (584).

Cr analogs may also be toxic to red blood cells. GPA and GBA cause hemolysis when incubated with human blood samples at concentrations of 100 μ M to 3 mM (285). In red blood cells, GPA may decrease the activity of the hexose monophosphate shunt (453) and inhibits glucose-6-phosphate dehydrogenase activity (877). In addition, a negative correlation was observed between the level of reduced glutathione in red blood cells and the plasma concentration of GPA.

GPA is a potent inhibitor of GAT3 and GAT4 GABA transporters (564, 565). At a concentration of 100 μ M, transport is inhibited by 82–91%. The K_i of GPA for GABA uptake into mouse brain microsomes is 19 μ M. GPA also is a potent taurine analog that inhibits sodium-dependent transport of taurine into rat brain synaptosomes with a K_i of 46 μ M (384). At a considerably higher concentration (10 mM), GPA and GBA inhibit the phytohemagglutinin-induced stimulation of proliferation of normal human lymphocytes (878) which may cause immunological disturbances. In rat pituitary tumor cells, GPA greatly increases prolactin secretion stimulated by thyrotropin-releasing hormone (TRH) (787). This effect of GPA was suggested to be due to activation of TRH-dependent and phospholipase C-mediated hydrolysis of phosphoinositides. Finally, administration of GPA to rats in vivo and to cultured chicken chondrocytes in vitro revealed that this agent inhibits normal development of cartilage (274).

In addition to these potential side effects, feeding of Cr analogs over extended periods of time—at least a few weeks are needed to cause a considerable decrease in the tissue levels of Cr and PCr—was shown to induce metabolic adaptation. These adaptive alterations include a decrease in the diameter of fast-twitch skeletal muscle fibers, giving rise to a decreased size of fast-twitch muscles, whereas the size of slow-twitch muscles is unaffected (528, 556, 887, 1044); an increase in the content and activity of mitochondrial proteins (cytochrome *c*, cytochrome oxidase, citrate synthase, succinate dehydrogenase, 3-hydroxyacyl-CoA dehydrogenase, Mi-CK, adenine

nucleotide translocase) as well as in the rate of oxidative phosphorylation, preferentially in fast-twitch skeletal muscles (e.g., Refs. 5, 262, 263, 528, 556, 656, 718, 719, 725, 802, 889, 983, 1044, 1073); an increase in GLUT-4 glucose transporter activity (802); an increase (or decrease?) in hexokinase activity (183, 184, 262, 718, 802); a decrease in glycolytic enzymes and lactate content as well as an increase in glycogen content (184, 724, 802, 887, 983, 1044); a decrease in AMP deaminase protein content and activity which, however, was unrelated to actual IMP production (800, 827); a shift in myosin isoenzyme expression from fast-type myosins to slow-type myosins, coupled with a decreased energy cost of maintaining tension (= higher contractile economy) (3–5, 556, 656, 657, 801); a decrease in CK activity (183, 656, 718, 719, 887); an increase in β -adrenoceptor density in soleus and extensor digitorum longus muscle (726); a decrease in parvalbumin content (657); as well as an up to 50% decrease in ATP content in fast-twitch muscles (e.g., Refs. 656, 724, 801, 889, 983). In addition, GPA feeding increases resting oxygen consumption of rats but has no effect on maximum oxygen consumption or on the oxygen transport capacity of blood (983).

Notably, many of the effects of GPA feeding, both as far as muscle performance and biochemical changes are concerned, are very similar to those observed in CK-deficient mice (see, e.g., Refs. 527, 941, 1026) and to those elicited by endurance training, chronic electrical stimulation, cross-reinnervation with nerves that normally innervate slow muscle, or hypoxia (4, 82, 657, 726, 762, 802, 861, 887). Some further points seem worth mentioning. The effects of GPA feeding depend on the duration of GPA administration (528) as well as on the age of the animals at which GPA feeding is initiated (3, 5). Significant changes in myosin isoenzyme expression were observed when GPA feeding was started in weaning animals, whereas virtually no changes were induced in adult rats. Furthermore, the effects of GPA feeding are tissue specific. They differ not only between fast- and slow-twitch skeletal muscles but also between skeletal muscles, heart, and brain. GPA feeding of rats decreased total CK activity in heart and soleus muscle by ~40%, whereas CK activity was increased in brain by a factor of 1.4–2 (371, 374, 718). The specific activities of adenylate kinase and succinate dehydrogenase were increased 100–165% in brain and soleus muscle of GPA-fed animals, whereas they were unchanged or even slightly decreased in the heart. In general, the heart seems to be much less affected by GPA feeding than fast- and slow-twitch skeletal muscles (887). The considerable increase of adenylate kinase activity in both soleus muscle and brain of GPA-fed rats may indicate that the adenylate kinase system takes over part of the function of the CK system, a conclusion that is in line with experimental findings of Dzeja et al. (206) (see sect. VII D).

GPA feeding of rats caused an increase in the mass of brown adipose tissue (BAT) as well as in BAT DNA, glycogen, and total protein content, suggesting a tendency to hyperplasia of the BAT (727, 1072, 1133). On the other hand, the contents of mitochondrial protein and uncoupling protein showed a tendency to be decreased, which correlated with decreases in thermogenic activity and both colonic and skin temperature. It was suggested that the impairment of BAT thermogenic activity reflects a sparing of ATP in compensation for a reduction in high-energy phosphate levels, with resultant hypothermia.

The results published on the effects of GPA on mitochondrial composition and function require further comment. As mentioned above, the contents and activities of several mitochondrial proteins were considerably increased in skeletal muscle by GPA feeding. In isolated mitochondria, however, the specific activities of pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, NAD^+ -isocitrate dehydrogenase, hydroxyacyl-CoA dehydrogenase, and citrate synthase were virtually unchanged (887), suggesting a general increase in mitochondrial mass. On top of that, there seems to be an additional specific increase in the protein contents of Mi-CK and adenine nucleotide translocase (718–720) that is thought to be a metabolic adaptation to compensate for the decreased tissue levels of Cr and PCr. The increased amounts of Mi-CK tend to aggregate and form large paracrystalline intramitochondrial inclusions (see Refs. 718–720, 1125). Mi-CK-rich intramitochondrial inclusions were seen in skeletal muscle, to a lesser extent in heart, and not at all in brain, liver, and kidney of GPA-treated rats (718, 719). Because similar intramitochondrial inclusions were also seen in adult rat cardiomyocytes cultured in medium devoid of Cr (228), their formation is unlikely to be due to a toxic side effect of GPA, but rather to Cr deficiency. In line with the expectation that the aggregated Mi-CK may be nonfunctional, Cr-stimulated mitochondrial respiration was severely impaired in skinned cardiac and soleus muscle fibers as well as in isolated heart mitochondria of GPA-treated rats (130, 718–720).

Although not strictly related to the findings discussed so far, one additional effect of GPA administration is worth mentioning. In KKA^y mice, an animal model of non-insulin-dependent diabetes, GPA and GAA decreased the plasma glucose level, whereas 4-GBA, Arg, β -alanine, Cr, or guanidino-undecanoic acid were ineffective (629). GPA was more potent than even metformin, a clinically effective antidiabetes agent. Glucose incorporation into glycogen was increased by GPA in hindlimb muscle, but not in cardiac muscle. GPA's antihyperglycemic effect was corroborated in two other models of non-insulin-dependent diabetes, namely, in *ob/ob* mice and in insulin-resistant rhesus monkeys, but was absent in normoglycemic animals, insulinopenic Chinese hamsters, and streptozotocin-diabetic rats. Together, these studies sug-

gest that the antihyperglycemic action of GPA requires the presence of at least some circulating insulin as well as hyperglycemia, and that improved insulin sensitivity is the mode of action for GPA. Not fully in line with these results, GPA-fed normal mice and rats displayed a decreased plasma concentration of insulin, a tendency to decreased blood glucose levels, and significantly increased glycogen contents in both tibialis anterior muscle and liver (724). In addition, glucose tolerance was considerably enhanced, i.e., after intraperitoneal infusion of glucose, its blood concentration increased less in GPA-fed animals than in controls. In a phase I study in terminal cancer patients as well as upon intravenous infusion in rabbits (O'Keefe et al. and Schimmel et al., unpublished data), cCr decreased blood glucose levels significantly. This effect was reversible upon addition of a dextrose solution. It therefore seems attractive to speculate that Cr analogs may become a new class of antidiabetes compounds. Interestingly, in diabetic *db/db* mice, administration of Cr reduced the concentrations of both *N*-carboxymethyllysine and hydroxyproline and, thereby, most probably inhibited the accumulation of collagen type IV in the kidney (578). Thus Cr supplementation may reduce diabetic long-term complications in this mouse model of type II diabetes. Hypoglycemic effects of guanidino compounds, including Cr (see Refs. 208, 820), as well as hypotheses on how high-energy phosphate and glucose metabolism may influence each other in both pancreatic β -cells and peripheral tissues (281), have also been reported in other studies.

To conclude, all these results demonstrate that the effects of Cr analogs, and of GPA in particular, are not strictly muscle specific and that clear-cut discrimination between (beneficial or toxic) side effects and effects related entirely to energy depletion may often be difficult, if not impossible. Nevertheless, the functional and biochemical alterations elicited by this Cr analog are strikingly similar to those observed in transgenic CK knock-out mice, thus suggesting that the changes are due to a considerable extent to the functional deficit in the CK/PCr/Cr system.

X. CREATINE METABOLISM AND (HUMAN) PATHOLOGY

A. Cr Metabolism and Muscle Disease

In mammals, the highest concentrations of Cr and PCr and the highest specific CK activities are found in skeletal muscle. Consequently, it is thought that the CK/PCr/Cr system plays an important role in the energy metabolism of this tissue. In accordance with this concept, a multitude of experimental findings suggest a close relationship between disturbances of Cr metabolism and various muscle diseases.

On one hand, manipulations of the CK/PCr/Cr system were shown to induce myopathic changes. 1) Skeletal muscle of transgenic mice lacking MM-CK and/or sarcomeric Mi-CK displayed structural and functional alterations such as impaired burst activity, decreased rate constants for changes in muscle tension, and abnormal Ca^{2+} handling (see sect. VII D). The facts that these mice survive and reproduce, and that the phenotype is milder than previously suspected, may indicate that other systems (e.g., adenylate kinase) take over in part the function of CK (see sect. VII D). 2) With the caveat that the reagent may not be sufficiently specific, injection of the CK inhibitor 2,4-dinitrofluorobenzene (DNFB) into the aorta of rats caused a metabolic myopathy characterized by spontaneous muscle contractures in the hindlimbs and by selective destruction of type I fibers in both soleus and gastrocnemius muscles (233). 3) When fed to experimental animals, the Cr analog GPA competes with Cr for uptake into muscle and therefore results in considerable depletion of the muscle stores of Cr and PCr. In line with the fact that GPA and its phosphorylated counterpart PGPA represent poor CK substrates, a variety of pathological changes have been observed in skeletal muscles of these animals (see sect. VIII B) (741, 1125).

On the other hand, many (neuro)muscular diseases with different underlying defects are accompanied by a variety of disturbances in Cr metabolism. Examples are Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD), facioscapulohumeral dystrophy, limb-girdle muscular dystrophy, myotonic dystrophy, spinal muscle atrophy, amyotrophic lateral sclerosis, myasthenia gravis, poliomyelitis anterior, myositis, or diabetic myopathy, to name just a few (for references, see Refs. 639, 826, 955, 1002, 1123). Common findings are increased Cr concentrations in serum and urine; stimulation of creatinuria by oral supplementation with Gly or Cr; decreased urinary Crn excretion; depressed muscle levels of Cr, PCr, P_i , glycogen, and ATP; increased serum CK activities; as well as an increased MB/MM-CK ratio in skeletal muscle, with the latter suggesting induction of B-CK expression in regenerating muscle fibers. In addition, a 67–86% decrease in Mi-CK activity or mRNA levels was reported for chickens with hereditary muscular dystrophy and rats with diabetic myopathy (585, 955). Depending on the particular muscle disease, these disturbances are more or less pronounced. Unfortunately, no sufficiently detailed studies have been published in recent years, whereas the older investigations were performed mostly with rather nonspecific analytical methods. Therefore, the above-mentioned findings await corroboration and expansion, which will hopefully allow us to unravel potential causal links between individual muscle diseases and disturbances of Cr metabolism.

In DMD, increased plasma membrane fragility and subsequent leakage of cytosolic components due to dys-

trophin deficiency are generally accepted to be the primary defects. The muscle concentrations of Cr, PCr, and ATP, the ATP/ADP, PCr/Cr, and PCr/ATP ratios, as well as the phosphorylation potential are significantly decreased, whereas the calculated ADP concentration and intracellular pH are increased (88, 143, 211, 472). Conversely, serum [Cr] is increased, resulting in creatinuria, in considerably reduced tolerance toward orally administered Cr, and, very likely due to competition of Cr and GAA for reabsorption in the kidney, in elevated urinary excretion of GAA. The total bodily Cr pool is reduced because of both muscle wasting and a reduced Cr concentration in the remaining muscle mass, with the consequence that Crn production and urinary Crn excretion are largely decreased. By use of radioactively labeled Cr, Cr turnover was shown to be increased in DMD patients relative to controls, with half times for the decrease in isotope content of 18.9 ± 5.1 and 39.8 ± 2.6 days, respectively (245). This latter finding may be due either to impaired Cr uptake into muscle (57) or to an impaired ability of muscle to retain Cr.

Most probably due to leakage of the plasma membrane and to continued necrosis of immature muscle fibers, both the total CK activity and the proportion of MB-CK in serum are dramatically increased (79, 190, 222, 755). Finally, disturbances of ion gradients across the plasma membrane were observed in skeletal muscle from DMD patients. The muscle concentration of Na^+ as well as the free intracellular $[\text{Ca}^{2+}]$ are increased, whereas the muscle levels of K^+ and P_i are decreased. In serum, on the other hand, $[\text{K}^+]$, $[\text{Ca}^{2+}]$, and $[\text{P}_i]$ are increased, whereas $[\text{Na}^+]$ and $[\text{Cl}^-]$ are decreased (see Refs. 143, 199, 755).

Disturbances very similar to those seen in DMD were observed in *mdx* mice that display the same primary defect as DMD patients, namely, dystrophin deficiency, and in other dystrophic animal strains (see Refs. 143, 199, 201–203, 790, 799). Additionally, in skeletal muscles of *mdx* mice, the resting membrane potential was shown to be “decreased” from -70 to -59 mV (see Ref. 199). Remarkably, all pathological changes, i.e., muscle fiber necrosis as well as the disturbances in membrane permeability, in Cr and high-energy phosphate metabolism, and in serum CK activities, were not evident in *mdx* mice at birth, but only developed after 2–6 wk of life (202, 799, 982). Consequently, dystrophin deficiency alone does not seem to be sufficient to induce muscle damage, thus calling for other factors that may act in conjunction with dystrophin deficiency to bring about plasma membrane damage and muscle cell necrosis.

Two hypotheses may be put forward to explain how disturbances in Cr metabolism may contribute to the progression of DMD and of other muscle diseases (see also Ref. 1123). 1) Loike et al. (571) have shown that increasing concentrations of extracellular Cr downregulate Cr transport activity in rat and human myoblasts and

myotubes. Similarly, Cr supplementation of the diet downregulates Cr transporter expression in rat skeletal muscle (317). In muscle diseases that are characterized by decreased tissue levels of Cr and PCr, the muscle should respond to this deficit by an increased Cr uptake across the plasma membrane. However, because of the chronically increased serum concentration of Cr that is observed in many muscle diseases, the Cr transport activity may even be depressed, thereby resulting in a further depletion of the muscle stores of Cr and PCr. This progressive Cr depletion would likely compromise the energy metabolism of muscle and would make the muscle cells more vulnerable to (membrane) damage upon further use. 2) Let us assume that the changes in membrane permeability and the concomitant disturbances of ion gradients across the plasma membrane represent early events in pathological muscle fiber degeneration. Because the Cr transporter is driven by the electrochemical gradients of Na^+ and Cl^- across the plasma membrane (see sect. ivB), the consequences would be a diminished rate of Cr uptake into muscle and partial depletion of the intracellular high-energy phosphate stores which, in turn, may further deteriorate ion homeostasis. If either of these purported vicious circles 1 or 2 were in fact operative, oral Cr supplementation may represent a promising strategy to alleviate the clinical symptoms and/or to slow or even halt disease progression. If only *hypothesis 2* is correct, continuous supplementation with Cr is indicated. If, however, *hypothesis 1* is valid, intermittent short-term supplementation with high doses of Cr is expected to provide superior results. In support of these hypotheses, preincubation of primary *mdx* muscle cell cultures for 6–12 days with 20 mM Cr prohibited the increase in intracellular Ca^{2+} concentration induced by either high extracellular $[\text{Ca}^{2+}]$ or hyposmotic stress (790). Furthermore, Cr enhanced *mdx* myotube formation and survival.

Patients with chronic renal failure commonly present with muscle weakness and display disturbances in muscular Cr metabolism (see Refs. 93, 716). Histochemical

studies revealed type II muscle fiber atrophy. In skeletal muscle of uremic patients, [ATP], [PCr], and [ATP]/[P_i] are significantly decreased both before and after hemodialysis, whereas [Cr] and [P_i] may either be unchanged or increased. Disturbances in ion homeostasis similar to those observed in DMD were also reported for uremic myopathy (99) and may be due, in part, to depressed $\text{Na}^+-\text{K}^+-\text{ATPase}$ activity (648, 950). Nevertheless, the benefit of oral Cr supplementation for uremic subjects has to be questioned, since the plasma level of Cr most likely is normal, and since an increase in the total body Cr pool would be paralleled by a further increase in the plasma concentration of Crn which, in turn, is a precursor of the potent nephrotoxin methylguanidine (see sect. ixH).

In gyrate atrophy of the choroid and retina, the disturbances of Cr metabolism seem to be brought about by a different series of events (Fig. 11). Gyrate atrophy is an autosomal recessive tapetoretinal dystrophy. The clinical phenotype is mainly limited to the eye, beginning at 5–9 yr of age with night blindness, myopia, and progressive constriction of the visual fields. By age 20–40 yr, the patients are practically blind. In addition to the retinal degeneration, type II muscle fiber atrophy, an increase in the proportion of type I muscle fibers with age, as well as the formation of tubular aggregates in affected type II fibers were observed in vastus lateralis muscle of gyrate atrophy patients (900). The underlying primary defect is a deficiency in mitochondrial matrix L-ornithine:2-oxo-acid aminotransferase (OAT; EC 2.6.1.13), the major enzyme catabolizing ornithine (see Refs. 95, 398, 792). Because of this deficiency, ornithine accumulates in the body, with the plasma concentration being raised 10- to 20-fold (450–1,200 μM vs. ~ 40 –60 μM in controls) (897, 899). Ornithine, in turn, inhibits AGAT ($K_i = 253 \mu\text{M}$) (897), the rate-limiting enzyme for Cr biosynthesis, and therefore slows production of both GAA and Cr (899). Accordingly, [GAA] is decreased in plasma and urine by a factor of 5 and 20, respectively. Similarly, [Cr] is reduced in plasma,

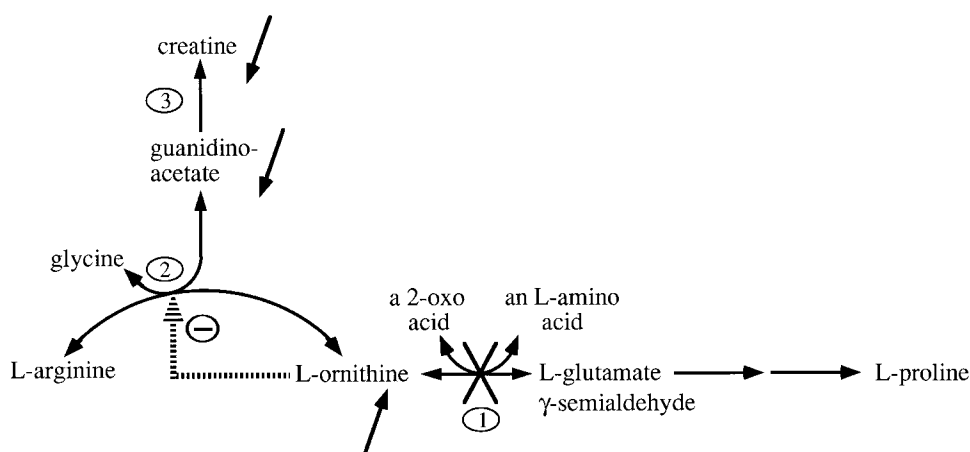


FIG. 11. Disturbances of Cr metabolism in gyrate atrophy of the choroid and retina. Due to a block of L-ornithine:2-oxo-acid aminotransferase, ornithine accumulates, competitively inhibits AGAT, and thereby depresses the rate of GAA and Cr biosynthesis. 1) L-ornithine:2-oxo-acid aminotransferase; 2) AGAT; 3) GAMT.

urine, cerebrospinal fluid, erythrocytes, and vastus lateralis muscle by a factor of 2–6 (901).

The effects of oral Cr supplementation (0.75–1.5 g/day) have been tested in 13 patients with gyrate atrophy for periods of 12 mo (898) and 5 yr (1052). Cr supplementation caused the disappearance of tubular aggregates in type II muscle fibers as well as an increase in the diameter of type II muscle fibers from 34 to 49 μm . In contrast, there was no significant increase in the diameter of type I fibers. In the few patients that discontinued Cr supplementation, the pathological muscle changes promptly reappeared. Somewhat less promising results were obtained with regard to eye pathology. Although during the first 12 mo of therapy no further constriction of the visual fields became apparent, the 5-yr follow-up study demonstrated continued deterioration of visual function in all of the patients. The velocity of the progression varied considerably between individuals and was, in general, rapid in young patients and slow at more advanced stages. It remains to be established whether the apparent discrepancy between the effects of Cr supplementation on muscle and eye pathology are due to limited permeability of the blood-eye barrier for Cr.

The finding of hyperornithinemia that are not accompanied by gyrate atrophy casts doubt on a potential causal link between disturbances in Cr metabolism on one hand and muscle and eye pathology on the other hand in gyrate atrophy of the choroid and retina (see Refs. 189, 350, 898). Unfortunately, it has not been established so far whether Cr biosynthesis is depressed in all of these hyperornithinemia. For example, it might be anticipated that hyperornithinemia is caused by a defect of ornithine transport across the mitochondrial membranes (234). In this case, the intramitochondrial concentration of ornithine and therefore also the rates of GAA and Cr formation may be normal. As an alternative, it has been proposed that the clinical symptoms of gyrate atrophy are caused by proline deficiency rather than Cr deficiency. Only by further investigation will it be possible to discriminate between these and further possibilities.

Mitochondrial (encephalo-) myopathies—e.g., chronic progressive external ophthalmoplegia (CPEO); mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS); and Kearns-Sayre syndrome—deserve special attention. They commonly display a phenotype of so-called ragged-red fibers that are characterized by an accumulation of abnormal and enlarged mitochondria as well as by the occurrence of highly ordered crystal-like inclusions in the intermembrane space of these mitochondria (see Refs. 463, 751, 760, 936, 1046, 1079). Remarkably, investigation by enzyme cytochemistry, immunoelectron microscopy, and optical diffraction of electron micrographs demonstrated that Mi-CK represents the major constituent of these intramitochondrial inclusions (906, 936; see also Refs. 281,

1124, 1125). There is evidence that in muscles displaying ragged-red fibers and/or Mi-CK-containing intramitochondrial inclusions, the specific Mi-CK activity relative to both protein content and citrate synthase activity is increased (89, 906). Further hints as to the pathogenesis of the inclusions come from a comparison with two additional sets of experiments. Cr depletion through feeding of rats with GPA caused the appearance of mitochondrial intermembrane inclusions immunoreactive for sarcomeric Mi-CK in skeletal muscle and heart (719, 720). Similarly, in cultured adult rat cardiomyocytes, large, cylindrical mitochondria displaying crystal-like inclusions that are highly enriched in Mi-CK appear when the cells are cultured in a Cr-free medium, or when the intracellular Cr stores are depleted through incubation with GPA (228). The large mitochondria and the Mi-CK crystals rapidly disappear when the cardiomyocytes are resupplied with external Cr. Therefore, it seems plausible to postulate that in both the rat cardiomyocyte model and in human mitochondrial myopathies, an initial depletion of intracellular Cr pools causes compensatory upregulation of Mi-CK expression. Although, at first, overexpression of Mi-CK may be a physiological adaptation process, it becomes pathological when, at a given limit, Mi-CK starts to aggregate and forms the highly ordered intramitochondrial inclusions. Inherent in this hypothesis are the postulates that in the respective myopathies, the muscle concentrations of Cr, PCr, and total Cr are decreased; that Cr supplementation reverses crystal formation (see Ref. 502); and that Cr supplementation may alleviate some of the clinical symptoms. In fact, in a 25-yr-old male MELAS patient, Cr supplementation resulted in improved muscle strength and endurance, reduced headache, better appetite, and an improved general well-being (323). Similarly, a randomized, controlled trial of Cr supplementation in patients with mitochondrial myopathies (mostly MELAS) revealed increased strength in high-intensity anaerobic and aerobic type activities, but no apparent effects on lower intensity aerobic activities (986). It will be interesting to investigate whether intramitochondrial inclusions seen in other myopathies are also enriched in Mi-CK, e.g., in ischemic myopathy (464), HIV-associated or zidovudine-induced myopathy (557, 667), congenital myopathy (783), oculopharyngeal muscular dystrophy (1116), inclusion body myositis (31, 732), hyperthyroid myopathy (567), mitochondrial myopathy of transgenic mice lacking the heart/muscle isoform of the adenine nucleotide translocator (300), in the diaphragm of patients with chronic obstructive pulmonary disease (566), in cultured human muscle fibers overexpressing the β -amyloid precursor protein (31), or in myocytes of isolated rat hearts exposed to oxygen radicals (352). Remarkably, ragged-red fibers of patients with mitochondrial encephalomyopathies were recently shown to overexpress the neuronal and endothelial isoenzymes of NO synthase (NOS) in the subsarcolem-

mal region (728). Therefore, it might be interesting to test the hypothesis that ragged-red fibers are exposed to oxidative stress, that Mi-CK (which is sensitive to oxidative inactivation; see sect. viiD) is inactivated by NO or peroxynitrite, and that the modified Mi-CK displays an increased tendency to form crystalline aggregates in subsarcolemmal mitochondria. This interpretation would be in line with a similar conclusion by O'Gorman et al. (720).

In patients with muscle hypotonotrophy of the thigh due to knee osteoarticular lesions, intravenous injection of 1 g PCr daily during the rehabilitation phase significantly accelerated recovery of muscle strength and power peak torque (847). After 30 days of treatment, the difference between PCr-treated and nontreated patients was 13% in muscle flexion and 18% in extension. Intramuscular injection of PCr in the rat before 4 h of ischemia followed by 30 min of reperfusion prevented the increase in membrane ion conductance and the loss of excitability of the muscle fibers upon reperfusion (1016).

Finally, recent gene localization studies revealed interesting relationships. The gene for the Cr transporter is localized on human chromosome Xq28, a locus to which several (neuro)muscular disorders have been mapped, for example, Emery-Dreifuss muscular dystrophy, Barth syndrome, or myotubular myopathy (see Refs. 309, 317, 691). Similarly, the gene for M-CK on human chromosome 19q13.2–19q13.3 is one of the most tightly linked markers of myotonic dystrophy (101, 506). The genes for ubiquitous Mi-CK and AGAT on human chromosome 15q15.3 and for sarcomeric Mi-CK on human chromosome 5q13.3 are in close proximity to the genes for limb-girdle muscular dystrophy type 2A (LGMD2A) and for proximal spinal muscular atrophy, respectively (260, 805, 940). So far, however, evidence is lacking that mutations in the Cr transporter, CK, or AGAT genes may be the cause of the respective muscle diseases (see, e.g., Ref. 42).

To conclude, a wealth of experimental evidence suggests that muscle diseases and disturbances of Cr metabolism are related. However, little is known so far about the causal links, either direct or indirect, between the disturbances of Cr metabolism on one hand and the primary defects or the clinical expression of the disease on the other hand. Future studies should not only provide the missing links but may also hint at alternative therapeutic approaches for muscle diseases. Possibly, oral Cr supplementation may turn out to be a simple and practicable way for alleviating at least some of the clinical symptoms in a broad range of muscle diseases. Just very recently, Tarnopolsky and Martin (985) provided experimental support for this hypothesis, in that Cr supplementation in fact increased handgrip, dorsiflexion, and knee extensor strength in more than 80 patients with neuromuscular disease (mitochondrial cytopathies, neuropathic disorders, dystrophies/congenital myopathies, inflammatory

myopathies, and miscellaneous conditions), with no obvious differences between subgroups.

B. CK, Phosphorylcreatine, and Cardiac Disease

The question whether the capacity of the CK/PCr/Cr system critically determines cardiac function is still a matter of debate. Some authors believe that the CK system simply serves a backup role by buffering [ATP] and [ADP] intracellularly, with no major impact of changes in PCr and Cr contents or CK activity on cardiac performance. Others, however, have accumulated evidence in favor of a close correlation between the functional capacity of the CK/PCr/Cr system and cardiac mechanical performance. If these latter correlations in fact turn out to be valid, disturbances in Cr metabolism may be one of the underlying causes of cardiac disease.

There are numerous arguments supporting a correlation between cardiac performance and CK function. 1) In rat heart, flux through the CK reaction was shown by ³¹P-NMR saturation transfer measurements to increase in parallel with the workload imposed, thus suggesting a close coupling between the rate of ATP synthesis and/or utilization on one hand and flux through the CK reaction on the other hand (see Refs. 401, 402, 516, 518, 611, 620, 1155). 2) Exposure of isolated perfused rat hearts to iodoacetamide causes rather selective inhibition of CK and, concomitantly, contractile dysfunction. After iodoacetamide exposure, [ATP] and [PCr], end-diastolic pressure, left ventricular developed pressure, rates of tension development and relaxation, coronary flow rate, and heart rate were maintained in the control range at low levels of developed pressure. In contrast, large changes in these parameters relative to controls were observed at increased workloads (259, 329, 402, 611, 1005; see also Ref. 1006). Matsumoto et al. (611) observed a linear correlation between CK flux and rate-pressure product in these iodoacetamide-treated rat hearts. 3) Similarly, perfusion of rabbit hearts with the CK inhibitor DNFB strongly depressed left ventricular output at a time when [ATP] and [PCr] were decreased by only 16 and 20%, respectively (282). Furthermore, in frog hearts subjected to metabolic inhibition by cyanide, the decrease in developed tension did correlate neither with electrical activity (Ca²⁺ metabolism, action potential amplitude or duration) nor with metabolic acidosis but with [PCr] (1061). 4) Very recently, Gross et al. (315) presented an interesting hypothesis in that NO may exert its physiological effects on cardiac contractile performance by reversibly inhibiting myocardial CK activity. In control rat hearts, a high Ca²⁺ challenge (3.5 mM) transiently increased the rate-pressure product by 74% and decreased [PCr], while [ATP] was maintained. In hearts perfused with the NO donor *S*-nitrosoacetylcysteine, on the other hand, the

Ca^{2+} -induced increase in rate-pressure product was strongly depressed, [ATP] declined significantly while [PCr] was maintained, and CK activity was reduced to 39% of control. Therefore, NO can regulate contractile reserve, possibly by reversible nitrosothiol modification of CK at the reactive Cys residue identified in previous investigations (see Ref. 275).

5) In isolated perfused hearts of transgenic mice lacking both M-CK and sarcomeric Mi-CK, with a 96% decrease in total CK activity, [ADP] was significantly higher during baseline perfusion than in controls (848). Increasing heart rate and perfusate $[\text{Ca}^{2+}]$ caused no differences in cardiac contractile response or myocardial oxygen consumption but increased [ADP] and decreased the free energy of ATP hydrolysis significantly more in CK-deficient than in control hearts. Consequently, cardiac work is more "energetically costly" in hearts with low CK activity (see also sect. VII D).

6) As described in detail in section VIII B, GPA or 3-GBA feeding is a means of depleting the Cr and PCr stores of a tissue. Again, no differences were found in the basic characteristics of contraction and relaxation among the hearts of control, GPA-treated, and 3-GBA-treated rats at low workloads. At high workloads, however, a series of functional deficits became apparent. Hearts from GPA- or 3-GBA-fed animals displayed a decrease in maximal work capacity (456, 457, 517, 754, 1163), a considerable decrease in the length of time during which 75% of the maximal cardiac output could be maintained (91), a decrease in right ventricular (RV) and left ventricular (LV) systolic pressure and, hence, mean aortic pressure (4, 456–458, 754, 1163), an increase in RV and LV (end) diastolic pressure and stiffness (91, 456–458, 754), as well as delayed kinetics of pressure development and relaxation (4, 456, 501, 754). Increased LV stiffness and incomplete myocardial relaxation may impair LV filling and may thus be the underlying cause of the diminished cardiac output at high workloads. Furthermore, Mi-CK activity and Cr-stimulated mitochondrial respiration were depressed in GPA-treated hearts (130, 458, 718–720, 754). Electron microscopic examination revealed structural abnormalities of both myofilaments and mitochondria (91, 718–720). Only in some investigations, GPA feeding induced cardiac hypertrophy (130, 135, 631). The fact that functional deficits of GPA-treated hearts were observed in some studies but not in others (888) may be due to different extents of Cr depletion and to different maximal workloads imposed. As a matter of fact, cardiac work and the rates of pressure development and relaxation were shown in rat heart to decrease only when total Cr was reduced to $\leq 20\%$ of control (456, 517).

7) All these experimental findings have recently gained theoretical support, in as far as mathematical modeling revealed that in the systolic phase of the rat and mouse cardiac cycle, all CK isoenzymes may be displaced

from chemical equilibrium (11, 831). This, again, signifies that CK does not merely serve a backup role for buffering [ATP] and [ADP] in the rat heart but that it may critically determine high-energy phosphate transport within the cells.

Although all approaches discussed so far have provided a consistent and convincing picture, it shall not be ignored that some authors have arrived at contradictory results (e.g., Refs. 470, 614, 782, 1048) and that several approaches (e.g., inhibition of CK by iodoacetamide or DNFB; GPA administration) may not be sufficiently specific to have the CK/PCr/Cr system as their only target. Even more importantly, the results obtained on the rat heart cannot necessarily be extrapolated to the human heart since the cardiac CK/PCr/Cr system and energy metabolism in general differ considerably between the two species (see Refs. 402, 824). The rat heart displays lower total CK activity, a lower proportion of MM-CK, and higher proportions of Mi-CK and MB-CK than the human heart (60, 30, and 10% vs. 90%, 10%, and trace amounts).

In case the correlation between the capacity of the CK/PCr/Cr system and cardiac performance is accepted, it might also be anticipated that cardiac disease is intimately linked with disturbances in CK function and/or Cr metabolism, with these disturbances representing either an expression or, conversely, the underlying cause of the pathological condition. In fact, alterations of the CK/PCr/Cr system present themselves as one of the key characteristics of cardiac disease and have been observed in various animal models, e.g., the spontaneously hypertensive rat (68, 400, 403, 404, 882); the hypertensive and hypotensive rat of the Lyon strain (575); the hyperthyroid rat (55, 400, 403, 576, 875); in rat diabetic cardiomyopathy (see Refs. 38, 458, 611, 658, 777, 854, 955, 988, 1060); in pressure-overload hypertrophy induced in the rat by aortic banding, pulmonary banding, or clipping of the renal artery (35, 256, 400, 401, 752, 914, 1007); in rat cardiomyopathies induced by the drugs adriamycin (= doxorubicin), norepinephrine, or isoprenaline (38, 457, 458, 703, 812); in hereditary dilated or hypertrophic cardiomyopathy of the Syrian hamster (38, 107, 458, 476, 599, 689, 832, 1008, 1058, 1060); in right ventricular hypertrophy and failure in the cat due to constriction of the main pulmonary artery (775); in cardiac hypertrophy due to pressure or volume overload in the dog (33, 40, 400, 401, 404, 737, 865, 1159); in a guinea pig model of autoimmune cardiomyopathy (863); in turkey poults with furazolidone-induced cardiomyopathy (117, 558); as well as in hypertrophied baboon heart (for reviews see Refs. 55, 401, 739, 1058). Disturbances in the CK/PCr/Cr system were also found in patients with LV hypertrophy due to aortic stenosis, in patients with coronary artery disease with and without hypertrophy, and in patients with aortic valve disease, mitral regurgitation, and dilated cardiomyopathy (139, 403, 425, 476, 690, 778, 832, 967; for reviews and

references see Refs. 140, 401, 700). The most consistent findings in these pathological conditions are decreases in the tissue concentrations of Cr and PCr, in total CK activity and in the chemical flux through the CK reaction, in the specific activities and relative proportions of Mi-CK and MM-CK, and in Cr-stimulated mitochondrial respiration. With the exception of the diabetic rat heart (see below), the relative proportions of MB- and BB-CK are increased. It has been suggested that the relative increase in MB- and BB-CK, which have higher affinity for Cr and PCr than MM-CK, represents a metabolic adaptation to the decreased tissue concentrations of these metabolites (405). No increase in MB-CK has been observed in cardiac hypertrophy induced by physiological stimuli where [Cr] and [PCr] most likely are normal, i.e., in mild hypertrophy caused by swimming in the rat heart or in moderate hypertrophy in hearts of greyhounds (see Ref. 401).

For illustration, some examples shall be discussed in more detail. In the heart of rats suffering from streptozotocin-induced diabetic cardiomyopathy, total CK activity, flux through the CK reaction, mRNA levels of B-CK and M-CK, specific enzymatic activities of all CK isoenzymes, as well as the relative proportion of MB-CK are all significantly decreased (38, 611, 777, 854, 955). Although in control hearts CK flux increased in parallel with the workload imposed, it did not do so in diabetic hearts (611). In addition, maximal and Cr-stimulated mitochondrial respiration were considerably depressed, suggesting a functional deficit at the level of both mitochondrial oxidative phosphorylation and Mi-CK (458, 851, 1060). Chronic insulin treatment of streptozotocin-diabetic rats normalized all deviating parameters. In line with experiments on GPA- and iodoacetamide-treated animals, contractile dysfunction became apparent in diabetic rat hearts only at high work loads (611).

Spontaneously hypertensive rats show progressive cardiac hypertrophy and undergo transition from stable compensated hypertrophy to failure between 12 and 18 mo of age. At 12 mo, despite pronounced hypertrophy, LV tissue displayed normal total CK activity, normal proportions of MM- and Mi-CK, and normal CK flux at a given level of cardiac performance (68, 400, 403, 404). On the other hand, the proportions of MB- and BB-CK were increased, and the total Cr content decreased at all ages studied (6, 12, and 18 mo). With the development of pump failure between 12 and 18 mo of age, total CK activity decreased by 30–45%, the relative proportion of Mi-CK by ~50% (with apparently no change in mitochondrial volume), and the flux through the CK reaction by at least 24%. Antihypertensive treatment with hydralazine or guanethidine reversed both the biochemical and functional changes. Whether the coincidence between the time course of changes in the CK/PCr/Cr system and the mechanical deterioration of the hearts is accidental or not remains to be clarified.

The time course of development of cardiac failure and of alterations in the CK/PCr/Cr system has also been studied in Syrian hamsters with hereditary hypertrophic cardiomyopathy (strain T02) (1008). Compared with controls, total CK activity and total Cr content were first decreased at 17 wk of age, with these changes persisting to the last time point analyzed, i.e., 43 wk of age. Baseline isovolumic contractile performance, measured as rate-pressure product (RPP), was first decreased at 30 wk. Contractile reserve, on the other hand, which was assessed as the increase in RPP elicited by high calcium stimulation, was already reduced at 17 wk of age. Interestingly, for both normal and myopathic hamsters, the very same linear relationship was observed between the energy reserve via the CK reaction, estimated as the product of total CK activity and total Cr content, and the contractile reserve of the heart (Fig. 12). Based on these experiments, the authors concluded that “depletion of energy reserve occurs early in heart failure and is likely to be one of the many predisposing factors to further functional deterioration” (1008).

In this same strain of cardiomyopathic Syrian hamsters, the effect of the angiotensin converting enzyme (ACE) inhibitor enalapril on cardiac bioenergetics was tested (689). Results from clinical trials had shown that therapy of congestive heart failure with ACE inhibitors ameliorates patients' symptoms, slows the progression to heart failure, and reduces mortality. Enalapril treatment of cardiomyopathic hamsters did not prevent cardiac hypertrophy but improved isovolumic contractile performance and survival rate. The 67% decrease in CK flux

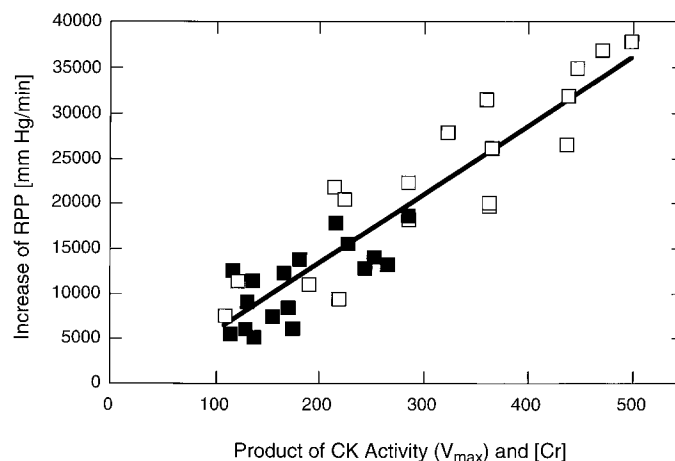


FIG. 12. Relationship between the functional capacity of the CK/PCr/Cr system and contractile reserve of the heart. The increase in rate pressure product (RPP) observed in isolated isovolumic hearts upon increasing total calcium concentration in the perfusate from 1.75 to 4 mM was taken as a measure of contractile reserve. The product of total CK activity and total Cr content was taken as a measure of energy reserve via the CK reaction. V_{\max} , maximum velocity; \square , hearts from normal hamsters; \blacksquare , hearts from cardiomyopathic Syrian hamsters, strain T02. [Data redrawn from Tian et al. (1008).]

observed in untreated cardiomyopathic hamster hearts was almost completely reversed. Surprisingly, however, enalapril treatment normalized neither [PCr], [Cr], total CK activity, nor CK isoenzyme distribution (see also Ref. 865), thus calling for an alternative explanation on how the increase in CK flux was brought about.

Changes in the CK/PCr/Cr system similar to those in hereditary dilated cardiomyopathy in the Syrian hamster (458, 476, 599, 1058) were also observed in dilated cardiomyopathy in humans: total CK, MM-CK, and Mi-CK activities, Cr-stimulated mitochondrial respiration, and total Cr content were all considerably decreased, whereas MB-CK activity was significantly increased (403, 476, 690, 778, 832, 967). Mi-CK activity was also decreased relative to citrate synthase activity, which excludes a general loss of mitochondria. Energy reserve via the CK reaction, defined as above, was reduced by 83% in the failing human myocardium (690). A decrease in mitochondrial adenine nucleotide translocase activity—despite increased ANT mRNA and protein levels—that is due most likely to autoimmune reactions against the ANT protein may contribute to the disturbances in cardiac energy metabolism in dilated cardiomyopathy (193, 967).

Surprisingly, total CK, MM-, and Mi-CK activities as well as total Cr content were also decreased considerably in organ donors maintained in an intensive care unit before heart harvesting, i.e., in hearts presumed to be normal (690). It has been hypothesized that these latter changes are due to increased production of stress hormones associated with intensive life support treatment, an idea that is supported by the fact that an increased adrenergic drive can modulate the contents of Cr and CK in the heart. The more important lesson to be learned from these findings is that energy metabolism of the donor heart may already be compromised at the time of organ transplantation, which may have a serious impact on the outcome of the intervention. Perhaps, simple Cr or PCr infusion in the organ donor, either alone or in conjunction with parasympathetic cholinergic agents like carbachol (1075) or β -adrenergic receptor antagonists like propranolol, atenolol, and bisoprolol (117, 533), may preserve the energetic reserve in the donor heart and thereby improve the survival rate of the transplanted organ (see also sect. IXC).

As early as in the 1930s, ingestion of the Cr precursor Gly was proposed as a therapy for cardiac disease and was reported to have favorable effects (see Ref. 55). It is only in recent years that the idea of treating heart failure with Cr supplementation has been revived (138, 140, 241, 297). In the study of Constantin-Teodosiu et al. (138), sustained hypertension was induced in the rat by prolonged oral administration of N^G -nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS. Concomitant ingestion of Cr prevented the decreases in [ATP], [PCr], [Cr], and [total Cr] as well as the increase in lactate concentration in ventricular tissue asso-

ciated with L-NAME treatment, but failed to ameliorate the mechanical disturbances of the heart at intermediate workloads. In chronic heart failure in humans, Cr supplementation improved LV ejection fraction neither at rest nor during exercise (297). However, it increased the concentrations of Cr, PCr, and total Cr in quadriceps femoris muscle by 12–24%, which was accompanied by a significant increase in different measures of skeletal muscle performance (see also Ref. 22).

Evidently, there is no *strict* mutual dependence between disturbances in Cr metabolism and defects in cardiac contractile performance. For example, defects in excitation-contraction coupling may reduce contractile performance in the absence of any changes in the CK/PCr/Cr system. Furthermore, disturbances in the CK/PCr/Cr system do not have to be expressed at all stages of the disease, and both functional and biochemical deficits may not be observed at low or intermediate workloads. Therefore, it comes as no surprise that in some studies on cardiac disease, no defects in the CK system were detected (e.g., Refs. 54, 753, 856, 1122).

Despite these latter caveats, a wealth of evidence suggests a close correlation between the functional capacity of the CK/PCr/Cr system and cardiac contractile performance. Even though most arguments in support of such a correlation come from studies on small laboratory animals like the rat and hamster, having a cardiac CK/PCr/Cr system admittedly quite distinct from that of humans, similar conclusions can also be drawn from investigations on human cardiac disease. Although initial experiments on Cr supplementation in cardiac disease failed to demonstrate a beneficial effect on the mechanical function of the heart (138, 297), even though supplementation of the diet of normal rats with 1–7% Cr for 40 days neither increased total Cr content, flux through the CK reaction, or [PCr]/[ATP], nor had an impact on the mechanical function of the heart (380), and in spite of conflicting arguments (241), interventions toward improving the energy reserve via the CK reaction as a means for treating cardiac disease should be given further thought.

C. Low-Oxygen Stress, CK Function, and the Potential of Cyclocreatine for Organ Transplantation

Although a strict separation from what has been discussed in section IXB is impossible, this section focuses on the impact of hypoxia, anoxia, ischemia, reoxygenation and reperfusion on the functional capacity of the CK/PCr/Cr system, as well as on the possibilities for preventing damage induced by low-oxygen stress and subsequent reperfusion.

1. Introduction

Functional recovery after a period of hypoxia, anoxia, or ischemia depends on environmental factors (e.g., temperature) as well as on the severity and duration of low-oxygen stress. Myocardial reperfusion after brief periods of ischemia is associated with a long-lasting depression of contractile force despite no evidence for cellular necrosis or ultrastructural damage. Several factors have been proposed to contribute to this phenomenon commonly referred to as "myocardial stunning" (see Refs. 200, 253, 478, 509, 830); nevertheless, the underlying mechanism is still unknown. One of the potentially contributing factors is the considerable loss of adenine nucleotides during ischemia. The progressive fall in [ATP] is accompanied by a corresponding increase in [ADP] that is degraded consecutively to AMP, adenosine, inosine, and hypoxanthine by way of adenylate kinase, 5'-nucleotidase, adenosine deaminase, and purine nucleoside phosphorylase (1037; for a review, see Ref. 430). The latter purine nucleosides and bases are membrane permeable and are therefore washed out of the tissue upon resumption of cardiac perfusion. De novo resynthesis of adenine nucleotides in the postischemic or postanoxic myocardium is known to be a slow process (16, 852, 853, 963). Thus the reduced adenine nucleotide pool size may be anticipated to limit cardiac contractility. However, a series of arguments are in contradiction to this hypothesis (see Refs. 430, 478, 509, 788, 835). The depressed contractile function of the stunned myocardium might also be due to a decrease in the capacity of mitochondrial oxidative phosphorylation and high-energy phosphate production (see Refs. 873, 1118, 1126). As evidenced most convincingly by the PCr overshoot phenomenon (see below), this hypothesis again seems unlikely (253, 393, 830, 835).

Prolonged regional or global ischemia of the heart results in irreversible loss of myocardial contractile activity. Again, the factors determining irreversible functional and biochemical deterioration of the heart are largely unknown despite a variety of hypotheses raised. Notably, even after prolonged ischemia and reperfusion, skinned ventricular strips from rat hearts displayed normal resting tension, maximal tension, Ca^{2+} sensitivity, and response to quick length changes in the presence of both PCr and MgATP (1062), thus excluding a functional defect at the level of the myofilaments. What, hence, are the disturbances of the CK/PCr/Cr system induced by low-oxygen stress and subsequent reperfusion, and how do they relate to the contractile dysfunction observed?

2. The response of the CK/PCr/Cr system to low-oxygen stress and reperfusion

Because the heart relies almost exclusively on mitochondrial oxidative phosphorylation for high-energy phosphate production, a decrease in oxygen delivery be-

low a critical limit—due to pathological block of adequate blood supply, asphyxia, poisoning, experimental, or surgical intervention—will challenge cardiac energy metabolism. In line with the notion that the CK reaction is near equilibrium in the heart under low workload conditions, [PCr] declines in the initial stages of low-oxygen stress with just a minor decrease in [ATP]. Only at later stages when the PCr stores are largely depleted, [ATP] also decreases considerably (e.g., Refs. 14, 90, 185, 254, 277, 316, 429, 452, 695, 702, 796, 1036, 1063, 1101). Contractile performance decreases precipitously and ceases when 75% of PCr, but only ~20% of ATP is depleted (316). Even though a direct causal relationship is unlikely, the time courses of the decrease in [PCr] on one hand and of the changes in cardiac contractility are strikingly similar (14, 695, 1101).

The effect of hypoxia on cardiac CK function was investigated by ^{31}P -NMR spectroscopy of rats ventilated with either 21, 10, or 8% O_2 (67). Under hypoxic conditions, [PCr] and [ATP] were decreased by 11–24% and ~12%, respectively. Chemical flux through the CK reaction, however, was decreased by 40% at 10% O_2 and by 75% at 8% O_2 , relative to normoxic conditions. For comparison, ventilation with 10 and 8% O_2 reduced the rate-pressure product by 34 and 49%, respectively. No changes in total CK activity, proportion of Mi-CK, or total Cr content were observed in tissue extracts of these hypoxic rat hearts. It remains to be elucidated why CK flux was considerably decreased despite no or only minor changes in total CK activity and substrate concentrations, and whether the decreased CK flux is, at least in part, responsible for the reduced cardiac contractile performance under hypoxic conditions. In a similar set of experiments on the isolated perfused rat heart, mild hypoxia caused a 52% decrease in forward CK flux, a 55% reduction in rate-pressure product, and a 38% decrease in left ventricular pressure (299). In both normo- and hypothermic rat hearts as well as under hypoxic conditions, there seemed to be an almost linear relationship between forward CK flux and rate-pressure product. In chronic anemic hypoxia induced in rats by dietary iron deficiency, Mi-CK activity and the capacity of Cr-stimulated mitochondrial respiration as well as MB- and BB-CK activity were significantly increased, whereas total CK activity remained unchanged (242). On the other hand, MM-CK activity and the concentrations of ATP, PCr, and total Cr were considerably depressed.

Ischemia itself had either no effect on total CK activity (48, 420, 461, 462) or decreased the level of CK protein or activity only after several hours (879, 894). An exception may be the pig heart where loss of CK immunoreactivity was observed within 15 min of acute right ventricular ischemia (933). On the other hand, even after short periods of ischemia, reperfusion resulted in a considerable decrease in total CK activity, CK flux measured by

^{31}P -NMR saturation transfer, MM- and Mi-CK activity, mitochondrial adenylate kinase activity, total Cr content, as well as in Cr- and AMP-stimulated mitochondrial respiration (48, 69, 420, 461, 462, 509, 622, 699, 771, 835, 879, 1010). In reperfused isolated rat hearts following varying periods of ischemia, the decrease in Cr-stimulated mitochondrial respiration was even identified as the first detectable functional deterioration (822). The functional capacity of Mi-CK may be reduced in two different ways in reperfused myocardium. First, Mi-CK is inactivated rather selectively, as evidenced by no or only minor decreases in mitochondrial malate dehydrogenase or cytochrome oxidase activities (69). Second, increased concentrations of inorganic phosphate like those prevailing under ischemic conditions are known to favor Mi-CK release from the mitochondrial inner membrane and may thus disrupt the functional coupling between Mi-CK and adenine nucleotide translocase which is suggested to be important for metabolic channeling of high-energy phosphates out of the mitochondria (see Refs. 325, 675, 1059, 1124). Remarkably, the loss of Mi-CK activity and of total CK flux was found to correlate almost perfectly with the decrease in LV developed pressure ($r = 0.97$) (69, 420) and in the rate-pressure product ($r = 0.99$) (699), respectively.

Although in two studies, myofibril-bound MM-CK was found to be preserved despite a loss in total CK activity (509, 1062), its activity was reported to be depressed by others (304). Upon prolonged ischemia, redistribution of M-CK was observed in immunoelectron micrographs of the dog heart, with a progressive loss of M-CK from the myofibrillar A band (740). In addition to these findings, a transient decrease in M-CK mRNA level was observed in ischemic dog and rabbit hearts 0.3–6 h after ligation of the left anterior descending (LAD) coronary artery (630), and MB-CK activity was found to increase in both ischemic and surrounding nonischemic portions of the dog heart upon LAD occlusion (879).

Several lines of evidence suggest that CK inhibition during reperfusion is brought about by reactive oxygen species: 1) bovine, rabbit, and rat heart MM-CK as well as rat heart Mi-CK were inactivated by incubation with either (hypo)xanthine plus xanthine oxidase or H_2O_2 , with a concomitant loss of free sulfhydryl groups (48, 205, 351, 455, 622, 962, 997, 1154). Superoxide dismutase, catalase, desferrioxamine, reduced glutathione, dithiothreitol, and cysteine protected against inactivation. Rabbit MM-CK seems to be inactivated mainly by H_2O_2 , whereas both the superoxide and the hydroxyl radical have been implicated in the inactivation of bovine MM-CK. The xanthine oxidase activity required for half-maximal inactivation of bovine MM-CK was ~ 30 -fold lower than that found in rat myocardium. Rabbit MM-CK, when incubated for 15 min at 37°C , displayed half-maximal inactivation with $\sim 25 \mu\text{M}$ H_2O_2 . 2) Postischemic reperfusion in isolated rat hearts causes a decrease in total CK activity, an effect that can

be prevented by addition of superoxide dismutase to the perfusion medium (622). 3) In permeabilized muscle fibers of the rat heart, myofibrillar MM-CK was identified as the primary target of both xanthine oxidase/xanthine and H_2O_2 (632). Inactivation of CK was prevented by catalase or dithiothreitol. Under the same conditions, myosin ATPase activity was not affected, and there was also no indication for modification of myofibrillar regulatory proteins. 4) Myristic acid treatment increases catalase activity in rat hearts. In myristic acid-treated rats, recovery of heart function after ischemia and reperfusion was significantly improved, and the decrease in CK activity was either less pronounced than in controls or even absent (461, 462). 5) Neither CK inactivation nor production of reactive oxygen species is observed during ischemia, but during subsequent reperfusion (18, 48, 461, 462).

Inactivation of CK in the reperfused myocardium may be mediated in part by iron. Oxidative stress can induce the release of iron from storage proteins, making it thereby available for catalysis of free radical reactions. In fact, ferrous iron enhanced the inactivation of rabbit MM-CK by H_2O_2 or xanthine oxidase/hypoxanthine (504, 997). Micromolar concentrations of iron and iron chelates that were reduced and recycled by superoxide or doxorubicin radicals were effective catalysts of CK inactivation (see also Ref. 653). Korge and Campbell (503, 504) also obtained evidence that iron may directly inhibit CK activity and Ca^{2+} uptake into sarcoplasmic reticulum vesicles of the rabbit heart. Inactivation depended on the redox state and on modification of the reactive sulfhydryl group of CK and was prevented by dithiothreitol, desferrioxamine, and EDTA.

Protein *S*-thiolation, i.e., the formation of mixed disulfides between protein sulfhydryl groups and thiols such as glutathione, may also be a mechanism for regulation of metabolism during oxidative stress. In cultured cardiac cells exposed to diamide-induced oxidative stress, a significant proportion of CK became *S*-thiolated and was thereby (reversibly) inactivated (133). Whether and to which extent reactive oxygen species like the superoxide anion or H_2O_2 are implicated in the *S*-thiolation reaction (750), and whether *S*-thiolation of CK plays a protective rather than deleterious role (351), remains to be further clarified.

Recently, NO and peroxynitrite were found to inactivate CK reversibly and irreversibly, respectively, most probably by binding to the reactive sulfhydryl group of the enzyme (29, 315, 444, 497, 935, 1115). NO also inhibits CK-mediated Ca^{2+} uptake into SR vesicles and decreases the sensitivity of mitochondrial respiration to stimulation by ADP. Whereas NO has been implicated to be a reversible regulator of mitochondrial function, muscular oxygen consumption and energy metabolism, its reaction product with the superoxide radical, peroxynitrite, may display inhibitory effects that are not readily reversible (679,

1126). It will therefore be interesting to further study the effects of NO and peroxynitrite on CK activity, both in vitro and in the reperfused myocardium. In conclusion, there are several different possibilities how CK inactivation during reperfusion may be brought about. The detailed contributions of all these mechanisms to in vivo CK inactivation are worthy of future investigation.

A further peculiarity of the CK/PCr/Cr system in the reperfused myocardium is the "PCr overshoot phenomenon." After a period of ischemia (or anoxia), [PCr] recovers very quickly to supranormal values as soon as reperfusion is initiated (e.g., Refs. 17, 252, 478, 699, 852, 853, 963). In contrast, [ATP] recovers only slowly and incompletely. This indicates that the major determinant of the decreased contractility in the stunned myocardium is not a limitation in mitochondrial oxidative capacity and high-energy phosphate production, but rather a defect in energy utilization or signal transmission. Myristic acid treatment of rats, which increases catalase activity in the heart, suppressed the PCr overshoot phenomenon and improved mechanical and bioenergetic recovery (461, 462). Evidently, reactive oxygen species may play a critical role both in the PCr overshoot phenomenon and in the postischemic depression of cardiac contractility. It remains to be determined whether CK inactivation by reactive oxygen species at sites of ATP utilization (MM-CK bound to myofibrils, the SR, or the sarcolemma) is contributing to these phenomena. Interestingly, treatment of pigs with dobutamine during reperfusion after 15 min of coronary artery occlusion prevented both myocardial stunning and PCr overshoot but did not improve the recovery of [ATP] (478).

3. Implications for human pathological conditions involving ischemia

Left ventricular hypertrophy due to systemic hypertension in association with coronary ischemic heart disease has been recognized as a major risk factor for sudden death, postinfarction heart failure, and cardiac rupture (see Ref. 104). Therefore, it seemed desirable to investigate the combined effects of cardiac hypertrophy and ischemia on cardiac metabolism and contractility. Patients with severe left ventricular hypertrophy caused by valvular aortic stenosis, as well as rats or dogs with cardiac hypertrophy due to pressure overload or hyperthyroidism, proved to be particularly susceptible to hypoxia or ischemia (see Refs. 34, 104, 403, 766, 916, 1098). After 30 min of global ischemia, the rate-pressure product recovered to only 40% in hypertrophied hearts but to 83% in normal hearts (104). In hyperthyroid rats, systolic and diastolic dysfunction during hypoxia occurred in those hearts containing the lowest prehypoxic levels of PCr (403). In contrast to these findings, the cardiac PCr content was decreased in hypertensive rats by 14%; however,

during 12 min of hypoxia, the rates of PCr and ATP depletion as well as the changes in intracellular pH were indistinguishable between hypertrophied and normal hearts, even though diastolic dysfunction was more pronounced in the hypertrophied hearts (1098). Thus a correlation between ischemic susceptibility and the tissue concentration of PCr seems still questionable.

Another frequent human disease involving ischemia is acute myocardial infarction (AMI). In experimentally induced myocardial infarction in rats and pigs, energy reserve via the CK reaction was reduced substantially also in the residual intact (nonischemic, "remodeled") left ventricular tissue: [ATP] was unchanged or slightly decreased, whereas [PCr], [total Cr], total CK activity, MM- and Mi-CK activity, and flux through the CK reaction were reduced by 16–50% (see Refs. 265, 532, 533, 678, 698, 841). The B-CK, M-CK, and sarcomeric Mi-CK mRNA levels in the remodeled rat heart were considerably increased, decreased, and unchanged, respectively (698). In analogy to the results on the combined effects of cardiac hypertrophy and low-oxygen stress discussed above, MI hearts displayed impaired mechanical recovery following a period of hypoxia, thus suggesting that reduced energy reserve may contribute to increased susceptibility of MI hearts to acute metabolic stress. In remodeled left ventricular tissue of infarcted pig hearts, total CK activity and M-CK mRNA level were unchanged, whereas Mi-CK mRNA level as well as the protein contents of sarcomeric Mi-CK and M-CK were decreased by 30–53% (366). In contrast, B-CK protein content was increased by 77%. In humans, no difference in total Cr content was observed between noninfarcted regions of MI hearts and myocardium of healthy controls (87).

For clinical diagnosis of AMI, the release of CK from damaged ischemic tissue has been instrumental. After experimentally induced myocardial infarction, as much as 75% of the CK activity may be released from injured myocardial tissue (for reviews, see Refs. 327, 749, 789, 1120). Total plasma CK activity generally increases 4–8 h after onset of chest pain, peaks within 12–14 h, and returns to normal within 72–96 h. Because CK may also be released from other tissues, while MB-CK is normally found in highest concentration in the heart, the latter was an accepted marker of AMI for many years. Only recently, the conviction that under certain conditions, MB-CK may also be increased in tissues other than the heart (327) as well as the finding of even more specific and therefore more reliable myocardial markers (e.g., cardiac troponins I and T) (240, 508, 891) have reduced interest in MB-CK as an AMI marker. Mi-CK is also released from infarcted myocardium, in a time course similar to, but a peak activity that is considerably lower than that of MB-CK (411, 966). The apparent half-lives of MB-CK and Mi-CK in serum were estimated to be ~11 and 60 h, respectively.

Over the last decade, release of Cr itself has been

tested as an early marker for AMI diagnosis by Delanghe and co-workers (for references, see Refs. 174, 176). Because Cr is considerably smaller than an MB-CK molecule, it might be expected that it is released into the circulation at an earlier stage of tissue injury and may thus allow diagnosis of AMI more quickly. In fact, Cr levels in serum and urine transiently increased after AMI, with a mean time to peak concentration of 3–4 h and a mean half-life of Cr in serum of ~6 h. Because of the large variability between individuals and the expected low specificity for cardiac disease, serum Cr determinations are, however, unlikely to become a routine method for early AMI diagnosis.

4. *Increasing the functional capacity of the CK/PCr/Cr system as a strategy to improve ischemic tolerance?*

After all, can the knowledge gained in the preceding sections be used to devise strategies for preventing ischemic myocardial damage? Emphasis is given here on those treatments in which the beneficial effect may be brought about directly or indirectly by an intervention in the CK/PCr/Cr system.

As already mentioned above, the catecholamine dobutamine prevents both myocardial stunning and PCr overshoot in the pig heart (478). The calcium antagonist verapamil displayed favorable effects in both hereditary dilated cardiomyopathy (599) and ischemia (see Refs. 104, 531). Verapamil was shown to reduce infarct size and hemodynamic deterioration of the ischemically injured myocardium. It also slowed the rate of CK release into the circulation and preserved high-energy phosphate stores during both hypoxia or ischemia and reperfusion, possibly by reducing the energy demand of the heart (694, 701). When initiated early after myocardial infarction, treatment with the β -blocker bisoprolol prevented the changes in the CK/PCr/Cr system in intact LV tissue of the infarcted rat heart (533). In sham hearts, bisoprolol treatment resulted even in a 40% increase in total Cr levels above control. Possibly, these results are related to the beneficial effects of long-term β -blocker treatment in patients with chronic myocardial infarction. Similarly, long-term treatment with ACE inhibitors (captopril, enalapril, quinapril, or trandolapril) partially prevented the decreases in [ATP], [PCr], and [total Cr] in noninfarcted LV and RV tissue and, at the same time, diminished the reduction in cardiac output or LV developed pressure and attenuated the rise in LV end-diastolic pressure seen in nontreated infarcted rat hearts (381, 841). Unexpectedly, however, quinapril treatment also increased the susceptibility of both normal and infarcted hearts to acute hypoxia/reoxygenation injury (381).

All approaches toward decreasing the concentration

of reactive oxygen species in reperfused myocardium are expected to improve mechanical recovery of the heart and to prevent loss of CK activity (see above). In fact, perfusion with the lipophilic spin trap α -phenyl-*t*-butyl nitron improved recovery of contractile function, [PCr], and [ATP], and diminished CK release from ischemic and reperfused rat hearts (90).

Preceding ischemic episodes (“ischemic preconditioning”) reduced infarct size after 40–60 min of sustained ischemia in dogs and pigs (see Ref. 477). Ischemic preconditioning also slowed ATP and PCr depletion, tissue acidification, and ultrastructural damage during the final episode of ischemia. Furthermore, it increased post-ischemic ATP levels and [PCr]/[P_i] during the reperfusion period in the rat heart (see Refs. 346, 770, 988).

When AMI patients were followed for 2 yr, those with a peak serum concentration of Cr >0.1 mM after AMI displayed a considerably lower incidence of death and major cardiovascular complications (3 of 41) than those with a peak Cr concentration of <0.1 mM (16 of 44) (174, 177). This finding may be related to the ability of Cr to inhibit ADP- or collagen-induced platelet aggregation in concentrations as low as 0.15 mM. By possibly inhibiting intravascular thrombocyte aggregation, oral Cr supplementation (at doses and intervals still to be specified) may therefore become a simple and nonproblematic prevention of AMI having, most likely, minimal if any side effects.

In cultured cardiomyocytes of newborn rats, the intracellular concentrations of Cr and PCr were reported to rise with increasing [Cr] in the medium (876). Incubation with Cr, however, did not protect the cardiomyocytes against ischemic damage (640).

Clinical and laboratory trials have shown that PCr displays a variety of cardioprotective effects and is an effective agent for the treatment of AMI, congestive heart failure, and for myocardial protection during heart surgery (for overviews, see Refs. 140, 505, 836). PCr shows antiarrhythmic and antifibrillatory properties (607, 821). Experimental evidence suggests that this effect of PCr is due to inhibition of phospholipase A₂, thereby reducing the degradation of membrane phospholipids and depressing accumulation of lysophosphoglycerides (which are highly arrhythmogenic compounds) in ischemic myocardium. This finding is particularly relevant since arrhythmias turning into fibrillation and heart rupture are among the reasons for mortality from AMI.

In cardiac ischemia, PCr treatment preserved two-fold higher levels of intracellular ATP and PCr, decreased CK release as well as the size of the ischemic zone, and resulted in better recovery of developed tension as well as in a faster decrease in end-diastolic pressure during reperfusion. PCr also improved diastolic function parameters in patients with chronic ischemic cardiomyopathy (855). In addition, it provided functional protection against ox-

idative damage (i.e., lipid peroxidation) caused by perfusion of the isolated heart with H_2O_2 . Even though intravenous administration of PCr increased the intracellular concentrations of ATP and PCr in the heart of living rats (194), PCr is generally accepted not to be membrane permeable. Because in contrast to PCr, Cr plus P_i did not protect the ischemic myocardium, cardioprotection is believed to be brought about by extracellular effects of PCr. It may interact electrostatically with membrane phospholipids, thus decreasing the fluidity and possibly increasing the stability of the plasma membrane. The half-life of PCr in blood or plasma was estimated to be between 4 and 5 min (505) and ~ 1.3 h (194).

Close structural analogs like PCrn (821), creatinol *O*-phosphate (288), and *O*-benzyl-phosphocreatine (1024) were also shown to afford cardioprotection, possibly in the same manner as PCr. In contrast, P_i plus Cr, PArg (see Refs. 505, 836), *O*-benzyl-phosphoglycocyamine (1024), and *O*-benzyl-phosphocreatine ethyl ester (1025) were ineffective.

A substance displaying most likely still another mode of action is cCr. As already discussed in section VIII B, cCr competes with Cr for uptake into heart (and other tissues) where it is accumulated, together with its phosphorylation product, PcCr, in large concentrations at the expense of Cr and PCr. PcCr serves as a slowly hydrolyzable reservoir of high-energy phosphates for ATP regeneration. During cardiac ischemia upon long-term feeding of rats and chickens with cCr, the reduction in tissue [ATP], exhaustion of high-energy phosphates, and onset of rigor tension were all significantly delayed (419, 738, 808, 1028, 1076). Upon reperfusion, the number of hearts recovering mechanical function was significantly higher and the rate-pressure product comparable in cCr-treated rats relative to controls, despite a considerably longer period of ischemia (56 vs. 34 min) (738). Spontaneous defibrillation upon reperfusion occurred sooner in hearts of cCr-fed rats than in controls (178 vs. 346 s) (419).

Surprisingly, favorable effects were observed also after short-term cCr or PcCr treatment. Intravenous injection in dogs, rats, and rabbits of cCr or PcCr 30–120 min before 1) killing of the animals and heart removal, 2) aortic cross-clamping, or 3) coronary artery occlusion, all followed by a period of ischemia, resulted in a significant protection of the PCr and ATP pools (13, 218) and in a remarkable improvement of cardiac function upon reperfusion (13, 219, 383). In addition, cCr treatment reduced the cardiac production of neutrophil chemotactic factors (217, 218) as well as the accumulation of neutrophils in the heart after ischemia and reperfusion (217). Neutrophil accumulation in ischemic myocardium has been implicated to be involved in postischemic damage of the heart (see Refs. 217, 218). cCr has no inotropic or chronotropic effect on the dog heart (383).

Unexpectedly, cCr showed no beneficial effects in

Syrian hamsters with congestive heart failure. If anything, its effect was slightly negative when given in high amounts to animals already experiencing heart failure. Even though the reason for this discrepancy remains to be established, the results on normal hearts are encouraging and hint at the potential for cCr in organ transplantation. To close the gap between animal experiments and organ transplantation in humans, and because endothelial cells may be the primary target of reperfusion injury (548), we tested the effects of cCr on human umbilical vein endothelial cells (HUVEC) in cell culture. Despite the rather low levels of Cr, PCr, and CK activity in endothelial cells, preincubation with cCr for 24 h delayed ATP depletion during cold hypoxia, diminished release of lactate dehydrogenase during subsequent reperfusion, and better preserved the viability of the HUVEC in preliminary experiments (M. Wyss, T. Eberl, Y. Ishida, R. Margreiter, and R. Kaddurah-Daouk, unpublished results). Even though use of cCr seems particularly attractive at present in heart transplantation, beneficial effects on the preservation of other organs (e.g., skin flaps in reconstructive surgery, Ref. 147) should not be ruled out.

5. Conclusions

The findings discussed in this section provide a long list of arguments, although admittedly still not conclusive, for a correlation between the functional capacity of the CK/PCr/Cr system and ischemic tolerance of a given tissue. This has recently been corroborated by experiments on transgenic mice expressing high levels of BB-CK in liver, a tissue normally displaying only very low levels of CK activity (642). The concentrations of Cr and PCr in transgenic liver increased with the level of Cr in the diet. In transgenic liver with an initial [PCr]/[ATP] of 4.5, a delayed and less pronounced depletion of ATP as well as a smaller drop in pH were observed during 40 min of ischemia. Within 30 min of reperfusion, pH as well as P_i and ATP levels returned to preischemic values in transgenic liver, whereas only incomplete recovery of $[P_i]$ and [ATP] was seen in normal liver. In addition, during 90 min of hypoxia, release of lactate dehydrogenase was prevented in transgenic liver containing high levels of PCr. Protection against low-oxygen stress was not seen in normal liver containing elevated concentrations of Cr, or in transgenic liver low in PCr. Therefore, protection against low-oxygen stress and reperfusion injury depends on the presence of both CK and PCr. In conclusion, strategies toward improving the functional capacity of the CK/PCr/Cr system may represent effective means for improving the ischemic tolerance of a tissue. As shown above, cCr is a promising candidate for such a purpose and may improve cold preservation of organs, e.g., in heart transplantation.

D. Use of Cr Analogs as Antitumor Agents

The cytosolic BB-CK isoenzyme is expressed in a wide range of tissues such as brain, intestine, uterus, kidney, or prostate. It is also induced in a variety of tumors, including neuroblastoma, small cell lung carcinoma, colon and rectal adenocarcinoma, as well as breast and prostate carcinoma (see Refs. 41, 529, 559, 1157). In cancer patients, elevated B-CK expression is associated mainly with untreated or progressive metastatic disease. In several studies, elevated CK levels were an adverse prognostic indicator.

The gene encoding B-CK is subject to regulation by both an oncogene and a tumor suppressor gene. A remarkable similarity was identified between the human B-CK promoter and the adenovirus E2E promoter region (152). This region of the E2E gene has two overlapping promoter elements that are induced by the adenovirus oncogenic product E1a. A series of cotransfection and infection experiments in tissue culture demonstrated that both the enzymatic activity and the mRNA level of B-CK are induced by E1a (446). Mutational analysis revealed that the transforming domains of the E1a protein are required for B-CK induction.

The tumor suppressor gene p53 has been suggested to regulate normal cell growth by activating transcription of genes whose products suppress growth and tumor formation. Conceivably, p53 may also repress genes whose products function to initiate or sustain accelerated growth. As a matter of fact, B-CK expression is elevated in human small cell lung carcinomas, many of which contain mutations in p53 alleles. The effects of wild-type or mutant p53 on the expression of rat B-CK and M-CK were therefore determined in transient transfection experiments (1161). Wild-type p53 repressed the B-CK promoter in HeLa cells (cervical carcinoma cells transformed by human papilloma virus type 18), but not in CV-1 monkey kidney cells. Conversely, p53 activated the M-CK promoter in CV-1 but not in HeLa cells. Coexpression of the E6 protein from human papilloma virus type 16, which is known to promote p53 degradation, blocked the p53-mediated modulation of CK expression. These findings suggest that p53 exerts its effects through association with corepressors or coactivators that are distinctly expressed in different cell types (see, e.g., Ref. 981). Furthermore, they indicate that CK isoenzymes are among the few cellular genes that may be targets of p53 *in vivo*.

In a series of missense mutants with alterations in conserved region II of p53, the ability of the mutants to transactivate M-CK and/or to transrepress B-CK correlated well with their ability to inhibit transformation of rat embryonic fibroblasts by adenovirus E1a or activated Ras. Taken together, the regulation of B-CK expression by p53, E1a, and by a variety of hormones and components of signal transduction pathways suggests that this enzyme of

cellular energy metabolism is important for the metabolic events that take place during or after oncogenic activation.

If the CK system is involved in tumor growth through regulation of ATP production or modulation of as yet unknown processes, then molecules that disturb this system may have an impact on tumor growth or progression. Many Cr and PCr analogs that decrease the rate of ATP production through CK were synthesized, catalytically characterized, and evaluated for antitumor activity. Several were shown to be active both *in vitro* and *in vivo* against a broad spectrum of solid tumors characterized by high levels of CK expression (60) (see below). Therefore, the CK system emerges as a novel and interesting target for drug design, and Cr analogs potentially emerge as a new class of cancer chemotherapeutics that work through a unique mechanism of action.

cCr is an interesting lead compound. Its antitumor activity has been studied extensively. *In vitro*, there seems to exist a correlation between the CK activity of a tumor and its responsiveness to cCr (Fig. 13). Tumor cell lines expressing a high level of CK were inhibited by cCr, with 50% inhibition of their ability to form colonies in soft agar (CD_{50}) being achieved at cCr concentrations of 2–8 mM (559). On the other hand, tumor cell lines expressing low levels of CK were inhibited only at 10-fold higher concentrations of cCr or not at all.

cCr was also evaluated as an antitumor agent in a colony-forming system against freshly explanted human tumor cells (600). This was thought to more truly represent human disease than established cell lines that had been in culture for many years and hence underwent many changes. CK activity was evaluated in 192 tumor samples from 166 patients and ranged from 0.001 to 1.524 $U \cdot (mg \text{ protein})^{-1}$, with a median of 0.042 $U \cdot (mg \text{ protein})^{-1}$. The highest CK levels were found in mesotheliomas as well as in small cell lung and brain tumors, whereas the lowest levels were observed in kidney cancers, lymphomas, and non-small cell lung carcinomas. Interestingly, tumors with very low CK activity were not able to form colonies in soft agar, suggesting that the enzyme might be required for tumor establishment.

The effect of cCr was evaluated on 51 of the 192 tumor samples. At a concentration of 6.7 mM, cCr inhibited colony formation of 21% of these tumors. Activity was noted against bladder, brain, kidney, lung, lymphatic, ovarian, pancreatic, and uterine cancers. Standard chemotherapeutic agents were tested on the same tumor samples. No relationship was seen between tumor samples sensitive to cCr and those sensitive to standard chemotherapeutics such as alkylating agents, antimetabolites, DNA intercalators, platinum compounds, topoisomerase II inhibitors, and tubulin interacting agents, thus supporting that cCr works through a unique mechanism of action.

The specificity of cCr *in vitro* against tumor cell lines

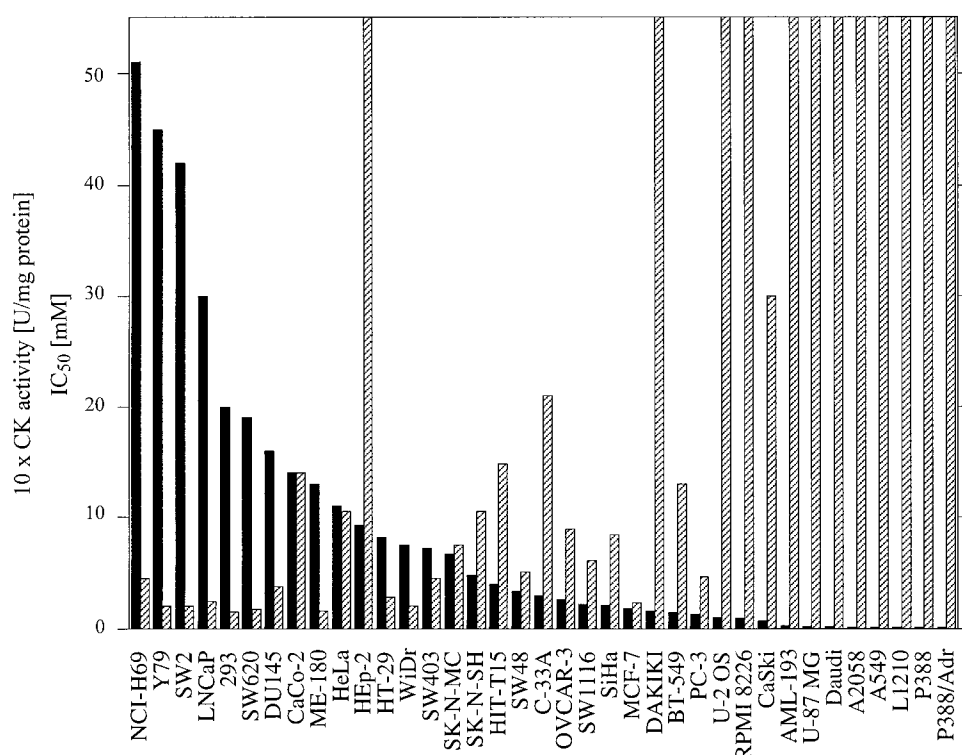


FIG. 13. Correlation between the CK activity of tumor cell lines and their responsiveness to cyclocreatine (cCr) (IC₅₀ of cCr for inhibition of colony formation of the cell lines in soft agar). For a description of the cell lines, see Lillie et al. (559).

expressing high levels of CK may be a result of the accumulation of cCr or of the synthetic phosphagen PcCr. Four tumor cell lines expressing high levels of CK, namely, ME-180 (human metastatic cervical carcinoma), DU145 (human prostate carcinoma), MCF-7 (breast adenocarcinoma), and OVCAR-3 (human ovarian adenocarcinoma), were sensitive to cCr and accumulated substantial amounts of PcCr [$0.33\text{--}0.63\ \mu\text{mol} \cdot (\text{mg cellular protein})^{-1}$] (559). In contrast, two cell lines expressing low levels of CK, U-87 MG (human glioblastoma/astrocytoma) and A2058 (human melanoma), were resistant to cCr and had undetectable levels of PcCr. All six cell lines, however, accumulated between 5 and $27\ \mu\text{mol cCr} \cdot (\text{mg cellular protein})^{-1}$.

To further test the correlation between accumulation of the synthetic phosphagen PcCr and growth inhibition, the unresponsive, low CK expressing melanoma cell line A2058 (see above) was transformed into a high CK expressing line, A2058-055. This was accomplished by transfecting the cells with a plasmid containing the human B-CK gene linked to the cytomegalovirus IE promoter and enhancer (559). The engineered line expressed >2,000 times the CK activity of the parent line A2058 and accumulated a large pool of the phosphagen PcCr. Remarkably, A2058-055, but not the parent line, was growth inhibited by cCr. These findings suggest that accumulation of the synthetic phosphagen PcCr is essential for inhibition of tumor growth.

Metastasis, the primary feature in fatal tumor pro-

gression, involves cell motility as a major determinant step (562, 953). Tumor cell motility is an energy-requiring process and depends on glycolysis. In in vitro studies in the high CK expressing cell lines A2058-055 and DU145, cCr was found to inhibit motility in response to different stimuli (676). In low CK expressing cell lines (A2058 and A2058-032), on the other hand, motility was not or only partially inhibited. Cr had no effect on the motility of the four cell lines when given alone but reversed the inhibition of motility by cCr. These results suggest that 1) the CK system may not only be important in solid tumor growth, but could also play an important role in the spread and invasion of these tumors, and that 2) cCr affects a key step utilized by several motility-stimulating systems.

The in vitro findings were complemented by evaluating the antiproliferative activity of cCr in vivo in a human tumor xenograft nude mouse model (559, 641). The growth of tumors derived from freshly injected ME-180 cells was significantly inhibited in athymic mice by feeding a diet supplemented with 1% cCr compared with animals fed a control diet ($149\text{ vs. }506\ \text{mm}^3$ by day 36). cCr also inhibited the growth of established ($>50\ \text{mm}^3$) ME-180 cell-derived tumors, as evidenced by tumor volumes on day 44 of 465 and $1,033\ \text{mm}^3$ for cCr-fed and control mice, respectively. Similarly, dietary cCr inhibited the growth of human neuroblastoma cell line-derived tumors in nude mice by up to 46%.

A variety of rat tumors were evaluated for respon-

siveness to cCr in a rat syngeneic model (641). Dietary cCr at 1% for 3–4 wk inhibited the growth of two rat mammary adenocarcinomas (Ac33tc and 13762A) as well as of MCI sarcoma by 20–50%. Cr at very high concentrations also inhibited the growth of some of the tumors, although in several other studies (60) (see also below), it was inactive.

To optimize the therapeutic potency of cCr, different dosages, routes of administration, and schedules were compared in the rat mammary tumor 13762 (991). The antitumor activity was scored as difference in the number of days required for treated tumors to reach a volume of 500 mm³ as compared with untreated animals. Tumor growth delay was more pronounced at the higher cCr dosage (1.0 vs. 0.5 g · kg⁻¹ · day⁻¹) and when cCr was administered intravenously rather than intraperitoneally. The earlier the cCr administration was initiated, the better growth inhibition was noted. The tumor growth delay with cCr was comparable to that achieved with clinically used anticancer drugs such as the antitumor alkylating agents CDDP [*cis*-diamminedichloroplatinum(II) = cisplatin] and cyclophosphamide, the antitumor antibiotic adriamycin, or the antimetabolite 5-fluorouracil.

Because cCr seems to be working through a unique mechanism of action, it might act synergistically with other agents. cCr was therefore tested both in vitro and in vivo in combination with several standard chemotherapeutic agents (991). The human small cell lung carcinoma cell line SW2 was only marginally responsive to cCr in vitro. When SW2 cells were incubated for 24 h with cCr and, additionally, during the fifth hour of cCr treatment with either one of four antitumor alkylating agents, CDDP, melphalan, 4-hydroperoxycyclophosphamide, or carmustine, a greater than additive killing of SW2 cells was observed (Fig. 14A). The antitumor alkylating agents used alone at the same concentrations were only moderately effective. In the rat mammary carcinoma 13762 model in vivo, cCr given in combination with either CDDP, cyclophosphamide, adriamycin, or 5-fluorouracil resulted in longer tumor growth delays than those achieved with any of the anticancer drugs alone (Fig. 14B). Favorably, no evidence of increased general toxicity was noted.

The effect of combination therapies was also tested with cCr and adriamycin on the human prostate tumor cell line LNCaP both in vitro and in vivo (379). Survival curves determined in cell culture revealed that LNCaP cells are only moderately sensitive to adriamycin, with 35% growth inhibition at a concentration of 8.4 μM. The combination of cCr and adriamycin produced additive to synergistic inhibition of cell proliferation, with the greatest effects being seen at the highest concentrations tested. For example, effects 10-fold greater than expected for additivity were observed for the combination of 6 mM cCr and 8.4 μM adriamycin. In vivo, cCr as a single agent

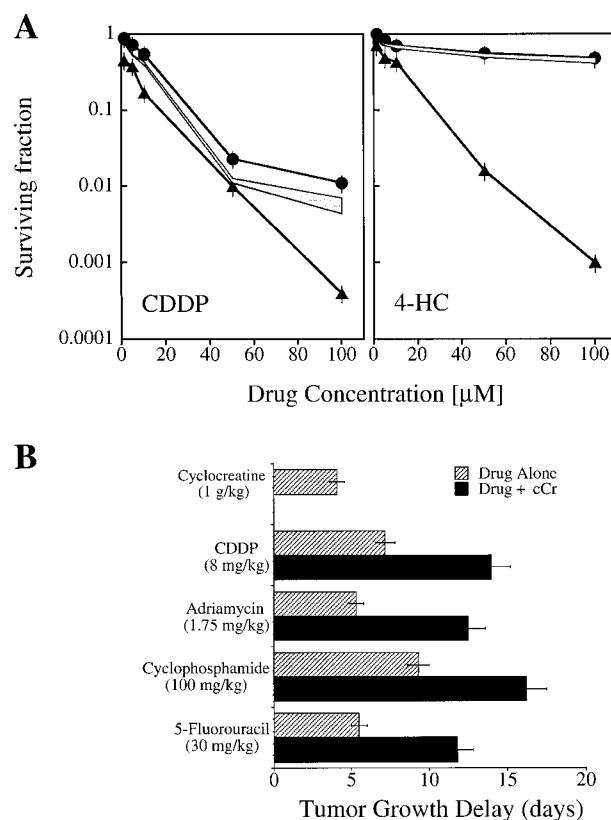


FIG. 14. Synergistic antitumor activity of cCr in vitro (A) and in vivo (B). A: human SW2 small cell lung carcinoma cells were treated with either cyclophosphamide (4-HC) or cisplatin (CDDP) alone (●) or in combination with cCr (▲). The shaded area represents the surviving fraction expected in case of an additivity of the antitumor effects. B: growth delay of rat 13762 mammary carcinoma produced by anticancer drugs in the presence or absence of cCr given intravenously. Tumor growth delay is defined as the difference in the number of days required for treated tumors to reach a volume of 500 mm³ as compared with untreated controls. [Modified from Teicher et al. (991).]

significantly inhibited the growth of LNCaP xenografts even when the tumor size was large. In contrast, adriamycin was not effective against larger tumors. Combined administration of cCr and adriamycin produced better tumor growth inhibition than either drug alone.

To more clearly understand the underlying basis of the cCr effects, the responsive ME-180 cervical carcinoma cell line was treated with a range of cCr concentrations, and the cell cycle distribution was examined after 0, 8, 16, and 24 h of drug treatment (602). FACS analysis revealed no major alterations in cell cycle distribution. A minor twofold accumulation in G₂-M was seen after 16 h but was not sustained. When synchronized ME-180 cells were analyzed, progression out of each phase (G₁, S, or M) was significantly reduced within the first 8 h of treatment with cCr. With continued treatment, progression was blocked. These results suggest that the predominant effect of the drug is to block progression out of all phases of the cell cycle.

To determine whether cCr was cytotoxic to cells during a specific phase of the cell cycle, ME-180 cells were blocked in G₁, S, or M. The synchronizing agent was then removed, and cells were allowed to grow in the presence or absence of cCr for 4 days. These experiments revealed that cCr is a phase-specific cytotoxic agent that kills cells preferentially in the S phase of the cell cycle. Preliminary studies showed no evidence for apoptotic cell death in response to treatment with cCr for up to 4 days.

From these findings, it can be concluded that the tumor growth inhibition exerted by cCr is due in part to both cytostatic and cytotoxic effects and that cCr causes a general block of progression out of all phases of the cell cycle. It has been proposed that such a general effect on cell cycle progression is a result of impacting a fundamental cellular target such as the rate of ATP synthesis. Compounds with anticancer activity that have been reported to block general cell cycle progression in some cell lines include interferon- γ (431) and genistein, a tyrosine kinase inhibitor (1032). Both compounds act through cell signaling pathways and are likely to have many effects on tumor cells.

The unique mechanism of antitumor activity of cCr coupled with its general effect on cell cycle progression may potentially explain why it acts synergistically with other anticancer chemotherapeutic agents that are cell cycle stage specific. Remarkably, normal cell lines that express high levels of CK such as brain and cardiac and skeletal muscle cells do not seem to be growth inhibited by cCr (603).

The cytosolic CK isoenzymes have been observed to associate with the cellular cytoskeleton. Evidence suggesting an association between CK on one hand and microtubules and intermediate filaments on the other hand has been provided by immunolocalization, *in vitro* binding, and functional studies (for references, see Ref. 601). Microtubules are known to be critical for many vital interphase functions, including cell shape, motility, attachment, intracellular transport, and cell signaling pathways. On the basis of these observations, the effect of cCr on the organization of microtubules in interphase cancer cells was investigated (601). Treatment of the cCr-responsive human tumor cell lines ME-180 and MCF-7 (see above) for 38 or 48 h with the minimum concentration of cCr that prevented proliferation caused the microtubules to become more randomly organized, an effect most apparent at the periphery of ME-180 cervical carcinoma cells. The microtubule changes were accompanied morphologically by cell flattening and by loss of the cell's bipolar shape. To address the mechanism causing altered microtubule structure, ME-180 and MCF-7 cells were challenged for 1 h with nocodazole, an agent that induces rapid depolymerization of microtubules similar to effects seen with colchicine. cCr induced the formation of an aberrant new population of microtubules that was more

stable when challenged with nocodazole than were normal microtubules. These microtubules were short, randomly organized, and apparently not associated with the centrosome.

For studying microtubule repolymerization, microtubules of ME-180 cervical carcinoma cells were dissociated by exposing the cells to nocodazole. This drug was then removed, and microtubules were allowed to repolymerize. The presence of cCr during the periods of preincubation, nocodazole treatment, and repolymerization gave rise to a more extensive array of microtubules than in the absence of cCr. The newly polymerized microtubules appeared to originate from the centrosome.

Interestingly, nontransformed cell lines that express low levels of CK and are not growth inhibited by cCr also lacked an effect of cCr on microtubule dynamics in nocodazole challenge experiments. This suggests a correlation between tumor growth inhibition, CK activity, the buildup of PcCr, and effects on microtubule dynamics, and that the antiproliferative activity of cCr may be due, at least in part, to its effects on microtubules. This assumption is supported by the fact that cCr induces microtubule stabilization after approximately the same period of time required for the inhibition of cell cycle progression, i.e., around 13 h.

cCr may therefore represent the first member of a second class of anticancer agents, in addition to the taxanes, that increases the stability of microtubules. Taxol, a member of the taxanes, stabilizes microtubules by binding directly to tubulin and lowering its critical concentration (see Refs. 179, 593). Like cCr, it induces the formation of microtubules that do not originate at the centrosome. Nevertheless, there are some differences between the effects of cCr and taxol. Although taxol stabilizes existing interphase microtubules, cCr seems to induce the formation of what appeared to be newly formed and highly stable microtubules. In addition, taxol induces extensive arrays of microtubules aligned in parallel bundles, an effect not noted for cCr. A synergistic tumor-killing effect was seen when cCr was combined with taxol (601). This further demonstrates that cCr and taxol have different modes of action.

The effect of cCr to induce the formation of stable microtubules may be hypothesized to be a result of decreasing the rate of ATP production via CK, which may secondarily affect the activity of proteins that regulate microtubule dynamics in tumor cells. Metabolic inhibitors that deplete ATP such as 2-deoxyglucose protect microtubules against depolymerization. It has been proposed that local changes in ATP concentration inhibit the phosphorylation of microtubule-associated proteins (MAP) which, in turn, control microtubule dynamics (63, 279). In addition, ATPases such as katanin (626) participate in microtubule disassembly and may be targets for cCr. Further experiments are needed to evaluate the effects of

cCr on the phosphorylation patterns of MAP, on the function of the katanin proteins, as well as on the ATP levels in responsive and nonresponsive tumors.

In another set of experiments, the potential relationships between cellular swelling and antitumor activity were evaluated (857, 858). C6 rat glioma multicellular spheroids were mapped by magnetic resonance spectroscopy, and sets of images demonstrated increased diffusion of water into the viable rim upon treatment with cCr. It was suggested that uptake of cCr is accompanied by cotransport of sodium, which leads to water accumulation (944). No cellular swelling was observed in multicellular spheroids of OC 238 human ovarian carcinoma cells exposed to cCr. Whereas the C6 line expressed CK activity and effectively built up PcCr, very little CK activity and no PcCr were found in the OC 238 cell line. Unexpectedly, both cell lines were growth inhibited by cCr, suggesting that induction of cellular swelling does not account for the antitumor activity of cCr. Also, there was no significant change in the nucleoside triphosphate pool in both cell lines, suggesting that growth inhibition may not be due to disturbances of general cellular energetics. The authors proposed that there might be more than one mechanism operative for cCr; it could potentially act on the cell surface or in a mitochondrial environment that is not visible by ^{31}P -NMR.

These conclusions were corroborated in part by a study in nude mice carrying a human colon adenocarcinoma (LS174T; CK activity 2.12 U/mg protein), where feeding for 2 wk with 2.5–5% Cr or 0.1–0.5% cCr significantly inhibited tumor growth (512). Both substances were equally potent, and the best correlation was observed between tumor growth inhibition and the total Cr or (Cr + cCr) concentration in the tumor tissue. The antiproliferative effect of Cr and cCr was not related to energy deficiency or to the proportion of PCr and PcCr but was associated with acidosis. Cr and cCr did not induce excessive water accumulation and had no systemic effects like induction of weight loss or hypoglycemia that might have caused tumor growth inhibition.

Ara et al. (28) evaluated the effects of Cr analogs on pancreatic hormones and glucose metabolism. Rats bearing the 13762 mammary carcinoma were treated with cCr (GPA; PCr) on *days* 4–8 and 14–18 after tumor implantation with or without the addition of sugars. Tumor growth delays increased from 9.3 (1.6; 7.6) days in animals not receiving sugars to 15.0 (6.3; 12.6) days in animals drinking sugar water. This could be a result of (direct or indirect) stimulation of tissue uptake of creatine/cCr by sugars, an effect that was noted previously (see sect. xi). Interestingly, blood glucose levels decreased over the course of the treatment, whereas the skeletal muscle glucose transporter protein GLUT-4 increased 1.5- to 2-fold. Plasma insulin concentration was decreased by 75–80%, plasma glucagon slightly elevated, and plasma

somatostatin increased three- to fourfold. These hormones are known to modulate tumor growth (for references, see Ref. 28), with insulin being an established growth factor for several tumors and somatostatin being a potent antiproliferative agent. Changes in hormonal profiles induced by Cr analogs may therefore be part of the mechanism of their antitumor action.

cCr has recently been evaluated in a phase I/II clinical study in terminal cancer patients. Safety and pharmacokinetic profiles were established (O'Keefe et al. and Schimmel et al., unpublished data). In a dose escalation study over a period of 10 wk, cCr was administered continuously by intravenous infusion at dose levels ranging from 10 to 150 mg/kg. Hypoglycemia and fluid retention were noted as dose-dependent and reversible side effects. The maximum tolerated dose was determined to be 80 mg/kg. No significant hematological, liver, or renal changes were observed. The good tolerance of high levels of cCr also in animal models and its unique mechanism of action make it a potentially attractive addition to cancer chemotherapy.

In addition to cCr, a series of other Cr and PCr analogs were evaluated for antitumor activity against the ME-180 cervical carcinoma, the MCF-7 breast adenocarcinoma, and the HT-29 colon adenocarcinoma cell lines (60). Several analogs, exhibiting thermodynamic and kinetic properties distinct from those of Cr and PCr, and thus impacting the rate of ATP production through CK (see sect. viiiB), inhibited the growth of the established tumor cell lines in culture with IC_{50} values in the low millimolar range. The compounds that were active in vitro were also shown to be active in vivo and substantially delayed the growth of subcutaneously implanted rat mammary adenocarcinomas. From the Cr analogs tested, cCr and phosphinic cyclocreatine were most active. The antitumor activity appeared to require rapid phosphorylation and buildup of new stable phosphagens that are less efficient than PCr in regenerating ATP. Compounds like GPA and hcCr became active after longer exposure times. This suggests that poor substrates will become active as antitumor agents if allowed sufficient time to build up their phosphorylated counterparts. Cr was inactive in these assays both in vitro and in vivo.

From the phosphorylated series of compounds, PCr and PcCr were most active, and several others were modestly active (60). Unlike the nonphosphorylated series, there was no obvious relationship between the antitumor activity of these compounds and their ability to generate ATP through CK. Furthermore, these phosphorylated molecules are poorly taken up by cells and may therefore modify a target distinct from CK, possibly at the membrane level. However, PcCr was shown to exhibit similar specificity and potency as cCr against high CK expressing tumor cells (859). Interestingly, CK activity seemed to be required at least in part for mediating the compound's

biological activity, leaving the exact mechanism of action to be elucidated.

Finally, other guanidino compounds were shown to have antitumor activity. Opheline, L- and D-lombricine (see Fig. 8 and sect. VIII A), 2-guanidinoethanol sulfate, and guanidinoethylphosphate markedly inhibited the growth of spontaneous mammary tumors in SHN mice, and it has been suggested that the guanidinoethyl moiety is the major determinant of antitumor activity in these molecules (1021). Nothing is known so far on the detailed mechanism of antitumor activity of these guanidino compounds.

In conclusion, CK along with its substrates Cr and PCr seems to be associated with the growth of many solid tumors and possibly their metastatic cascade. Further studies are needed to clarify the mechanism of action of the Cr analogs as antitumor agents. In addition to other hypotheses, the idea of Cr analog effects at the mitochondrial level, e.g., on the mitochondrial permeability transition pore (717) which is believed to be involved in programmed cell death, is intriguing and is currently being pursued.

E. Cr Analogs: A New Class of Antiviral Agents

Modulation of enzyme expression in the host cell is likely to be an important step for effective viral replication. Infection by DNA viruses like the human cytomegalovirus (HCMV) and adenovirus was shown to transcriptionally activate cellular genes, including B-CK (131, 446). In addition, the HCMV IE2 and adenovirus E1a gene products were shown to transactivate the B-CK gene up to 11-fold in cotransfection experiments (131, 446). These findings suggest an important role for B-CK in efficient viral replication. It is interesting to note that B-CK is also induced by many signals that stimulate cellular proliferation, including insulin-like growth factor I, steroid hormones such as estrogen and testosterone, or signal transducers such as phospholipase C and protein kinases A and C (see Refs. 123, 513, 526, 923–925). Modulating the CK system with Cr analogs may therefore influence the rate of ATP production and, hence, viral replication.

The effect of cCr on the replication of a series of DNA and RNA viruses was evaluated in both in vitro and in vivo assays (560). Among the DNA viruses tested, the human and simian cytomegaloviruses (HCMV and SCMV) and the varicella zoster virus (VZV) were most sensitive to cCr with ED_{50} values of 1–5 mM. cCr seemed to be more active against primate viruses than against mouse (MCMV), guinea pig (GPCMV), and pig herpes viruses. The inhibitory activity against herpes simplex viruses (HSV) types 1 and 2 was intermediate, with ED_{50} values of 8–12 mM. cCr displayed no or only minimal toxicity to the cell monolayers used in the infection assays, thus confirming that the antiviral effect of cCr is not due to inhibition of cellular growth.

The activity of cCr was tested against three HCMV isolates from AIDS patients that had developed different degrees of resistance to gancyclovir depending on the duration of treatment with the drug. Despite a ninefold difference in resistance to gancyclovir, the three isolates showed equal sensitivity to cCr. Likewise, two HSV strains differing in resistance to acyclovir by a factor of 10 displayed equal sensitivity to cCr, thereby suggesting that Cr analogs represent a new class of antiviral agents, with a mode of action distinct from currently used antiviral drugs.

The antiviral activity of cCr was also evaluated in two animal models in vivo, HSV-2 vaginitis and HSV-2 encephalitis. cCr-treated control animals showed no visible signs of toxicity and gained slightly more weight than the placebo- or acyclovir-treated controls. In the case of HSV-2-induced vaginitis in mice, cCr improved the survival rate, reduced the average lesion scores, and depressed the titers of recoverable vaginal virus by almost three orders of magnitude. The effect of cCr administered as 1% of the feed was comparable to acyclovir treatment at a dose of $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. When cCr and acyclovir were used in combination, even more pronounced effects were observed. In HSV-2-induced encephalitis in mice, cCr administration and acyclovir treatment improved the survival rate from 5% in the placebo group to 50 and 70%, respectively. Even though the number of mice analyzed was low, these findings are encouraging and call for continuing efforts to determine the potential and limits of cCr and other Cr analogs for antiviral therapy.

In contrast to herpes viruses, none of the RNA viruses tested (influenza virus types A and B, parainfluenza virus type 3, and vesicular stomatitis virus) was inhibited by cCr at concentrations as high as 28.4 mM. In summary, according to present knowledge, the antiviral activity of cCr appears to be limited to herpes viruses. The mechanism by which cCr exerts its antiviral activity is not fully understood. We have been able to determine that the effect of cCr is not on viral attachment, penetration, or uncoating, but rather on viral replication (Kaddurah-Daouk et al., unpublished data). The intracellular [ATP]/[ADP], the level of ATP, and the different nucleotide pools are all potential targets for modification by cCr, which could impact viral replication. The fact that cCr was effective against acyclovir- and gancyclovir-resistant strains suggests a novel mechanism of action and potential clinical use for the compound. The design, synthesis, and evaluation of further Cr analogs and drugs for CK modulation might generate a new useful class of antiviral chemotherapeutics.

In this context, it seems worth mentioning that Cr was shown to have anti-inflammatory activity comparable to phenylbutazone when evaluated in models of acute or chronic inflammation in the rat (475). Like standard non-steroidal anti-inflammatory agents, Cr also showed a sig-

nificant analgesic action. Some distant Cr analogs were also reported to have anti-inflammatory, analgesic, anti-hypertensive, local anesthetic, and platelet antiaggregating activities (78).

F. Significance of Cr and Creatinine for the Formation of Food Mutagens and Carcinogens

1. Cr and Crn as probable precursors of the cooked food mutagens and carcinogens of the amino-imidazo-azaarene class

The processing of foods, and in particular the frying and broiling of meat, is associated with the generation of mutagenic and carcinogenic principles. A whole class of new mutagens, the amino-imidazo-azaarenes (AIA), has been isolated over the past 20 years from cooked foods. Sources of AIA mutagens were broiled or fried fish, cooked chicken, beef and pork meat, beef extracts, beef flavors, fried eggs, as well as fumes from cooking meat (for reviews and references, see Refs. 119, 178, 215, 235–238, 296, 495, 540, 807, 902, 1009). The dietary origin of the AIA mutagens is supported by the fact that they are detected in the urine of healthy volunteers eating normal diet, but not of inpatients receiving parenteral alimentation (1031). According to their chemical structures, the currently known AIA mutagens can be classified into five groups, namely, the imidazo-quinolines (IQ and MeIQ; Fig. 15, structure 1; for systematic names, see Table 2), the imidazo-quinoxalines (IQx, 4-MeIQx, 8-MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, 4-CH₂OH-8-MeIQx, and 7,9-DiMeIQx; Fig. 15, structures 2 and 3), the imidazo-pyridines (PhIP, 4'-OH-PhIP, DMIP, and TMIP; Fig. 15, structures 4–6), the oxygen-containing AIA (Fig. 15, structure 7), and the naphthyridines (Fig. 15, structure 8).

The amount of mutagenicity and of AIA compounds formed during the cooking of meat depends on a number of factors, for example, on the cooking method, time, and temperature as well as on the proportions of fat, sugars, dietary fibers, and amino acids in the meat sample (see Refs. 119, 743, 807, 902, 903, 919, 1009). The generation of AIA can be minimized, e.g., by microwave pretreatment of meat, marinades, addition of soy protein, chlorophylls or antioxidants before cooking, or by using oil rather than butter for frying (see Refs. 119, 153, 435, 436, 465, 743, 839). Among the AIA compounds, IQ, 8-MeIQx, 4,8-DiMeIQx, and PhIP are the most important mutagens and together contribute ~80% of the mutagenicity (see Ref. 238). In terms of actual mass, the concentrations of IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, and PhIP were determined in a series of cooked foods (beef, chicken, hamburger, beef extracts, sun-dried sardines, and fish) to be between zero and 158, 72, 300, 28, and 480 ng · (g cooked food)⁻¹, respectively, whereby at least some of the upper limits represent overestimations due to methodological artifacts (for reviews and references, see Refs. 215, 235, 238, 495,

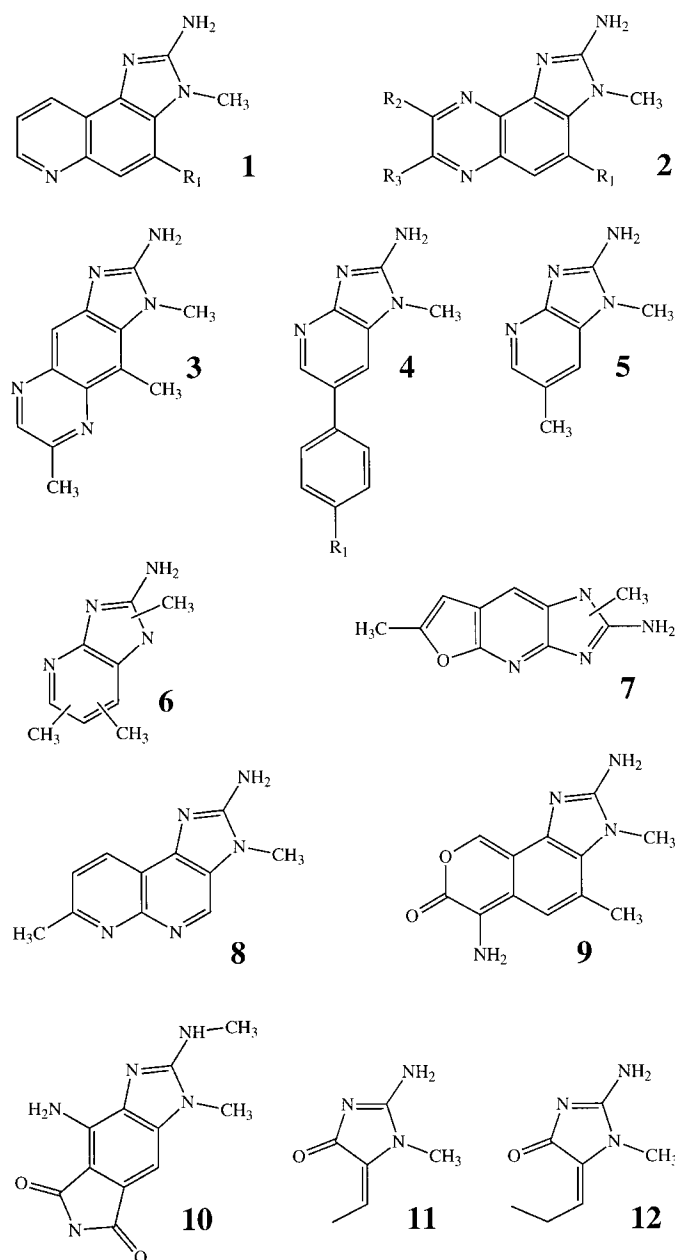


FIG. 15. Food mutagens derived most likely from Cr or Crn. 1) Imidazoquinolines (IQ): R₁ = H → IQ; R₁ = CH₃ → MeIQ; 2 and 3) imidazoquinoxalines (IQx): 2) R₁ = R₂ = R₃ = H → IQx; R₁ = CH₃, R₂ = R₃ = H → 4-MeIQx; R₁ = R₃ = H, R₂ = CH₃ → 8-MeIQx; R₁ = R₂ = CH₃, R₃ = H → 4,8-DiMeIQx; R₁ = H, R₂ = R₃ = CH₃ → 7,8-DiMeIQx; R₁ = CH₂OH, R₂ = CH₃, R₃ = H → 4-CH₂OH-8-MeIQx; 3) 7,9-DiMeIQx; 4–6) imidazopyridines (IP): 4) R₁ = H → PhIP; R₁ = OH → 4'-OH-PhIP; 5) 1,6-DMIP; 6) TMIP; 7) 2-amino-(1 or 3),6-dimethylfuro[2,3-c]imidazo-[4,5-b]pyridine; 8) naphthyridine mutagen; 9–12) mutagens detected so far only in model systems containing Cr or Crn: 9) 2,6-diamino-3,4-dimethyl-7-oxo-pyrano[4,3-g]benzimidazole; 10) Cre-P-1; 11) AEMI; 12) AMPI. For further details, see text; for references, see Refs. 215, 235, 237.

540, 896, 902, 903, 956). On the basis of the mean consumption figures in the Netherlands, it has been estimated that 15–20 μg of AIA are consumed per kilogram of hu-

TABLE 2. *Mutagenicity of selected amino-imidazo-azaarene food mutagens in the Ames test in the presence of S-9 mix toward the frameshift-sensitive Salmonella typhimurium strains TA98 and TA1538, and toward the base substitution-sensitive strain TA100*

Systematic Name	Abbreviation		Mutagen. TA1538, rev/ μ g	Mutagen. TA98, rev/ μ g	Mutagen. TA100, rev/ μ g
2-Amino-3-methylimidazo[4,5-f]quinoline	IQ	(1)	200,000–400,000	94,000–898,000	3,150–12,100
2-Amino-3,4-dimethylimidazo[4,5-f]quinoline	MeIQ	(1)	700,000–1,017,000	253,000–47,000,000	24,500–182,000
2-Amino-3-methylimidazo[4,5-f]quinoxaline	IQx	(2)	100,000–105,000	17,800–75,000	1,500
2-Amino-3,4-dimethylimidazo[4,5-f]quinoxaline	4-MeIQx	(2)	875,000–1,208,000	1,162,000	51,000
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline	8-MeIQx	(2)	70,000–99,300	35,700–417,000	1,500–14,000
2-Amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline	4,8-DiMeIQx	(2)	130,000–320,000	126,000–435,000	8,000–11,200
2-Amino-3,7,8-trimethylimidazo[4,5-f]quinoxaline	7,8-DiMeIQx	(2)	189,000	163,000–192,000	8,100–9,900
2-Amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-f]quinoxaline	4-CH ₂ OH-8-MeIQx	(2)		99,000	2,600
2-Amino-1,7,9-trimethylimidazo[4,5-g]quinoxaline	7,9-DiMeIQx	(3)		670	
2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	PhIP	(4)	1,800–4,000	1,700–3,520	0–140
2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-b]pyridine	4'-OH-PhIP	(4)		1.75	0
2-Amino-1,6-dimethylimidazopyridine	1,6-DMIP	(5)	8.0		
2-Amino- <i>n,n,n</i> -trimethylimidazopyridine	TMIP	(6)	100,000		
2-Amino-(1 or 3),6-dimethylfuro[2,3-e]imidazo[4,5-b]pyridine		(7)	~10,000		
Naphthyridine mutagen		(8)	~80,000		
2,6-Diamino-3,4-dimethyl-7-oxopyrano[4,3-g]benzimidazole		(9)	7,000	5,200	550
4-Amino-1,6-dimethyl-2-methylamino-1H,6H-pyrrolo[3,4-f]benzimidazole-5,7-dione	Cre-P-1	(10)		19,000	400
2-Amino-5-ethylidene-1-methylimidazol-4-one	AEMI	(11)			
2-Amino-1-methyl-5-propylideneimidazol-4-one	AMPI	(12)			

Numbers in parentheses refer to the compound number shown in Figure 15. For references, see Refs. 119, 170, 215, 236; see also Refs. 237, 239, 417, 440, 481, 493, 494, 521, 710, 907, 937, 994, 1065. rev, Revertants.

man diet (12). On the other hand, based on a dietary survey of the United States population, the combined daily intake of PhIP, 8-MeIQx, 4,8-DiMeIQx, and IQ was estimated to be only 1.4 μ g for a 70-kg person (540).

A large body of evidence indicates that Cr and/or Crn are important precursors of AIA mutagens. 1) In different fried bovine tissues as well as in meat extracts, beef flavors, bouillons, and gravies, mutagenicity correlated with the Cr and/or Crn content of the sample (for references, see Refs. 534, 807, 902). Fried meat, heart, and tongue contain high levels of Cr and Crn and displayed considerable mutagenicity. Liver and kidney, on the other hand, have only low levels of Cr and Crn and yielded no significant mutagenicity upon frying. Likewise, the mutagenicity of plant foods is at least 10-fold lower compared with animal (muscle) foods, which may be due to the absence of Cr and Crn in plants (934).

2) Addition of Cr or PCr to meat samples or to beef extracts before the cooking process increased mutagenicity up to 40-fold and AIA contents up to 9-fold (see Refs. 423, 481, 744, 902). Conversely, treatment of beef meat with creatinase before frying reduced the Cr content and the mutagenicity by 65 and 73%, respectively (1066). 3) Addition of Cr before cooking of meat had no influence on the number or on the relative proportions of AIA mutagens formed, but increased the level of all mutagens approximately to the same extent (see Ref. 744). This implies that Cr or Crn may be involved in the formation of

all AIA food mutagens. 4) The latter conclusion is supported by the fact that all currently known AIA food mutagens (Fig. 15, *structures 1–8*) share a 2-aminoimidazo moiety that resembles strikingly the structure of Crn. 5) Most AIA mutagens could be generated artificially in simple model systems containing Cr or Crn, amino acids, and sugars (see below). Omission of Cr and Crn from these model systems greatly reduced mutagenicity (e.g., Refs. 721, 904). 6) Finally, heating of model mixtures containing Phe and isotopically labeled Cr revealed that the 1-nitrogen, the methyl-carbon, and the amino-nitrogen from Cr all are incorporated into PhIP (236).

As an apparent exception to the rule, AIA mutagens (IQ, MeIQ, PhIP) have been detected in beer, wine, roasted coffee beans, and cigarette smoke as well as in urine of smokers of black tobacco, despite the absence of Cr and Crn in yeasts and plants (see Refs. 215, 424, 592). An amusing interpretation of these findings has been provided by Jägerstad et al. (424): "A possible explanation could be that animal urine, containing creatin(in)e, has been spread over the leaves of the tobacco plant through fertilization." As a more plausible alternative, other guanidines may participate in AIA formation (see below).

Artificial model systems have widely been used to study both the probable precursors and the reaction pathways leading to the formation of AIA mutagens. In these model systems, Cr or Crn was mixed with a variety of substances and incubated for different periods of time at

temperatures of 37–250°C, either dry, in aqueous solution, or in diethylene glycol-water mixtures which display increased boiling points of 128–150°C. With the individual components varied, almost all AIA food mutagens could be generated in model systems, namely, IQ, MeIQ, IQx, 8-MeIQx, 4,8-DiMeIQx, 7,8-DiMeIQx, 7,9-DiMeIQx, PhIP, and TMIP (for reviews and references, see Refs. 119, 236, 417, 424, 434, 492, 544, 710, 902).

Similar to the situation in cooked foods, a variety of factors influence mutagen yield in model systems, e.g., temperature, incubation time, concentration of antioxidants as well as the nature, concentration, and proportion of the precursors (for reviews and references, see Refs. 417, 424, 434, 465, 902). Maximal mutagen yield was achieved by mixing Cr or Crn with an amino acid and a sugar in a molar ratio of 1:1:0.5. Remarkably, almost the same ratio between these components is found in bovine muscle (534). In most instances, omission of any one component from the ternary mixture greatly reduced mutagenicity. Crn proved to be a more potent precursor than Cr. Because, in addition, conversion of Cr to Crn is favored at elevated temperatures, Crn rather than Cr is likely to be the actual precursor of AIA mutagens.

The amino acid in the ternary mixture determines not only the yield, but also the nature of the AIA mutagens produced. In mixtures with Cr(n) and glucose, Cys and Thr yield highest mutagenicity, followed by Lys, Ala, Ser, and Gly. In most of these ternary mixtures, 8-MeIQx and 4,8-DiMeIQx were identified as products. On the other hand, PhIP was only found in ternary mixtures containing Phe, Leu, Ile, or Tyr.

The influence of sugars on mutagen production is somewhat more complex. In most but not all mixtures of Cr(n) and amino acids, addition of a variety of sugars (glucose, fructose, sucrose, lactose) increased mutagen formation. Fructose proved to be more potent than glucose, and sucrose was more potent than lactose. As mentioned above, maximal mutagenicity was reached at a molar ratio of Cr(n), amino acid, and sugar of 1:1:0.5. When the sugar concentration was further increased, progressive inhibition of mutagen formation occurred. This inhibition was suggested to be due either to Maillard reactions that are expected to become more prominent at elevated sugar concentrations, or to inhibition of the conversion of Cr to Crn by sugars.

Model systems also produced new mutagens that have not been detected so far in cooked foods (for references, see Refs. 215, 905). From a heated mixture of Cr, Glu, and glucose, the AIA mutagen 2,6-diamino-3,4-dimethyl-7-oxo-pyrano[4,3-*g*]benzimidazole was isolated (Fig. 15, *structure 9*). The imidazo-quinoxaline 4,7,8-Tri-MeIQx was identified in a heated mixture of Ala, Thr, Crn, and glucose. Finally, pyrolysis of Cr monohydrate at 250–400°C gave rise to the mutagenic compound Cre-P-1 (Fig. 15, *structure 10*).

On the basis of the experiments with model systems, two alternative reaction pathways were proposed for the formation of imidazo-quinoline and imidazo-quinoxaline mutagens (for a detailed discussion, see Ref. 424). According to the first hypothesis, a pyridine or pyrazine and an aldehyde, both postulated to be formed from amino acids and sugars through Maillard reactions and Strecker degradations, react with Crn to yield an imidazo-quinoline compound. In support of this hypothesis, IQ has recently been isolated, although in low yield, from a heated mixture of 2-methylpyridine, Crn, and acetylformaldehyde (544). According to the second hypothesis, Crn first undergoes an aldol condensation with an aldehyde to yield an intermediary creatinine-aldehyde which, subsequently, combines with a pyridine or pyrazine to give an imidazo-quinoline compound. This hypothesis is supported by the identification of the postulated creatinine-aldehyde intermediates AEMI and AMPI (Fig. 15, *structures 11 and 12*) in heated mixtures of Crn and Thr, and by the generation of AEMI through direct reaction of Crn with acetaldehyde.

Common to both hypotheses are the postulates that pyridines or pyrazines are obligatory intermediates and that these pyridines or pyrazines are formed from sugars and amino acids through Maillard reactions (see also Ref. 465). The validity of these assumptions is, however, questioned by several lines of evidence: 1) imidazo-quinolines were formed in binary systems lacking sugars, namely, in mixtures of Cr(n) with Pro, Phe, Ser, Ala, or Tyr. 2) In mixtures of Cr with Pro or Ser, addition of glucose did not stimulate mutagen formation. 3) Upon addition of a variety of pyridines or pyrazines to model systems, the amount of mutagenicity at most doubled or did not increase at all. 4) Finally, in cooked meat, a poor correlation was observed between mutagenicity and the level of Maillard reaction products (see Ref. 534). On the other hand, in model systems containing Crn, Thr, and radioactively labeled glucose, label was in fact incorporated into 8-MeIQx and 4,8-DiMeIQx (see Ref. 902). Taken together, these results imply that different reaction pathways have to be considered and that sugars may be involved in but are not essential for the formation of imidazo-quinolines.

PhIP was identified in binary mixtures of Cr plus Phe, Crn plus Phe, and Cr plus Leu (see Refs. 424, 902, 989). When mixtures of Crn and Phe were dry-heated at 200°C, addition of glucose in a half-molar amount decreased rather than increased PhIP yield (989). Experiments with isotopically labeled Phe revealed that the whole phenyl ring, the 3-carbon atom, and the amino nitrogen of Phe are incorporated into PhIP (236). In the light of these findings, it comes as a surprise that in aqueous solution at pH 7.4 and at a temperature of 60°C, PhIP was only detected when sugars or aldehydes were added to mixtures of Crn plus Phe (591). It remains to be determined whether,

depending on the temperature or on other parameters, different reaction pathways must also be considered for the formation of PhIP.

Only a few studies have addressed the question of whether Cr(n) may be substituted by other guanidines in the formation of AIA mutagens. In binary mixtures with amino acids or ribose as well as in ternary mixtures with Gly and glucose, other guanidines (Arg, N^G -methyl-L-arginine, 1-methylguanidine, aminoguanidine, and guanidine) yielded at least eightfold lower mutagenicity than Cr (491, 613, 721). In ternary mixtures with Phe and glucose, however, 1-methylguanidine gave rise to even higher mutagenicity than Cr. Unfortunately, nothing is known so far about the nature of the mutagens formed in these model systems.

Patients with chronic renal failure are subject to an increased cancer risk. As is discussed in section IXH, the serum concentration of Crn is drastically increased in these patients, thereby creating "favorable" conditions for the formation of AIA. In fact, 8-MeIQx was detected in the dialysis fluid of all uremic patients examined (1134). The notion that this 8-MeIQx does not originate from meat consumption, but from de novo synthesis, is supported by the formation of AIA in a model system at as low a temperature as 37°C (591). The actual rate of in vivo AIA synthesis in uremic patients as well as its contribution to cancer development remain to be established.

In conclusion, the studies on cooked foods and on model systems provide valuable information on the structure and on potential precursors of AIA mutagens. On the other hand, we still await a breakthrough in the understanding of the reactions involved in the formation of the individual classes of AIA mutagens. This knowledge, in turn, may help to define new strategies for reducing this potential health risk.

2. Mutagenicity and carcinogenicity of AIA

For routine purposes, mutagenicity of AIA compounds is normally measured with the Ames test, using primarily the *Salmonella typhimurium* strains TA98, TA1538, and TA100. As can be seen in Table 2, AIA compounds are in general more mutagenic toward TA98 and TA1538, which are sensitive to frameshift mutations, than toward TA100, a strain that is sensitive to base substitutions. The high specific mutagenicities toward TA98 rank the AIA compounds among the most mutagenic substances currently known. For comparison, the carcinogens aflatoxin B1 and benzo[a]pyrene display specific mutagenicities of only 320–28,000 revertants/ μ g in *S. typhimurium* strains TA98 and TA100 (see Refs. 119, 215). In the Ames test, AIA compounds are not mutagenic as such but depend on metabolic activation by a rat liver S9 (9,000-g supernatant) fraction that contains activating

enzymes that are not present in *S. typhimurium* (see below).

The mutagenic potency of AIA compounds was corroborated in *Drosophila* as well as in a series of mammalian cell types such as Chinese hamster ovary and lung cells, mouse small intestinal stem cells, or mouse fibroblasts (see Refs. 119, 178, 215, 236, 794, 872). AIA compounds proved to stimulate DNA repair, measured as unscheduled DNA synthesis, as well as chromosomal damage, such as sister chromatid exchange and chromosomal aberrations. Furthermore, AIA induced preneoplastic lesions, e.g., foci positive for placental glutathione *S*-transferase (GST) and an increase in γ -glutamyl transpeptidase activity in rat liver, or aberrant crypt foci in the colon of rodents (see Refs. 215, 320, 343, 514, 731). In all these nonmicrobial systems, the mutagenic activity of AIA compounds was, in general, much lower than in the Ames test. In Chinese hamster lung cells, for example, IQ, MeIQ, and 8-MeIQx were even less mutagenic than benzo[a]pyrene. Whereas in *S. typhimurium*, PhIP is the least mutagenic AIA (Table 2), it usually displayed higher mutagenicity than other AIA in mammalian cell types. This is noteworthy since in many cooked foods, PhIP is the most abundant AIA.

The relationships between chemical structure and mutagenic activity were studied for a series of IQ analogs (see Refs. 178, 348, 907). Mutagenicity critically depends on the 2-amino group as well as on a methyl group either at the 1- or 3-position of the aminoimidazo ring. When a methyl group was attached to the 1-position (isoIQ), even higher mutagenicity was observed toward both TA98 and TA100 than when it was attached to the 3-position (IQ). Substitution of the 2-amino group with one or two methyl groups progressively decreased mutagenicity toward TA98 but increased (one methyl group) or only slightly decreased (two methyl groups) mutagenicity toward TA100. Mutagenicity also decreased greatly when the nitrogen atom in the quinoline part of the molecule was replaced by a carbon atom.

IQ, MeIQ, 8-MeIQx, and PhIP were shown to be carcinogenic, although, so far, only at concentrations much higher than those normally observed in cooked foods (see Refs. 215, 289, 413, 523, 723, 794, 797, 872, 886, 920, 1000, 1001, 1096). In mice, tumors were induced in liver, lung, forestomach, and hematopoietic system. In rats, tumors were observed in liver, Zymbal gland, skin, small and large intestine, clitoral gland, mammary gland, and in the oral cavity. Finally, in cynomolgus monkeys, only IQ was carcinogenic and induced metastasizing hepatocellular carcinomas. Apart from apparent species differences in the carcinogenic effects of AIA compounds, considerable differences were also observed between individual AIA. Although the liver is one of the main targets of IQ, MeIQ, and 8-MeIQx in both rats and mice, PhIP only induced colon, mammary, and prostate carcinomas in rats and

lymphomas in (adult) mice. Potential reasons for these discrepancies may be species- and tissue-specific differences in expression and/or substrate specificity of the mutagen-activating enzymes.

In neonatal mice, three single doses of AIA (IQ, 8-MeIQx, or PhIP) given on *days 1, 8, and 15* after birth induced liver tumors in high incidence within 8–12 mo (192). This is remarkable for three reasons: 1) the total AIA dose administered to these neonatal mice was 5,000- to 10,000-fold lower than AIA doses used in long-term carcinogenicity assays on adult rodents. 2) AIA are accumulated in mammary gland and milk of lactating rats and are also found in liver and blood of suckling pups as well as in the eye and liver of fetal mice (see Refs. 413, 618). These results suggest that the health risk imposed by AIA is more pronounced in neonates than in adults and that the detoxification mechanisms may not yet be fully developed soon after birth. 3) Metabolic activation of 8-MeIQx by hepatic microsomal fractions was undetectable in 6- to 11-day-old rats, then increased with age and reached a maximum in weanling animals (804). This latter finding raises questions about the mechanism of action of AIA in neonates (see below).

Limited data have been published on the activation of oncogenes in AIA-induced tumors (for reviews and references, see Refs. 587, 588, 682, 1013). In only one of five rat hepatocellular carcinomas induced by IQ, the *H-ras-1* and a second unidentified oncogene (neither *ras* nor *neu*) were activated (410). In the case of rat colon tumors, nine carcinomas and two adenomas induced by IQ and nine carcinomas induced by PhIP displayed mutations neither

in the *p53* gene nor in the *Ki-ras*, *Ha-ras*, or *N-ras* families. A high incidence of *Ha-ras*, *Ki-ras*, and *p53* gene mutations was, however, observed in rat Zymbal gland and mouse forestomach tumors induced by IQ, MeIQ, and 8-MeIQx. *Ki-ras* mutations were detected in 4 of 37 aberrant crypt foci induced by IQ in the rat colon. In four of eight PhIP-induced rat colon tumors investigated, a total of five mutations in the *APC* gene were detected. All five mutations involved deletion of a guanine base in a 5'-GGGA-3' sequence. On the other hand, *APC* mutations were seen in only 2 of 13 IQ-induced rat colon tumors and involved C-to-T and T-to-C base substitutions. Finally, 7 of 8 colon tumors and 9 of 15 mammary gland tumors induced by PhIP had microsatellite alterations in at least one locus, whereas no microsatellite mutations were observed in IQ-induced colon tumors.

In addition to their mutagenic and carcinogenic effects, PhIP and IQ induced cardiac damage in rats and nonhuman primates, respectively (see Refs. 872, 1001). In addition, IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, and 7,8-DiMeIQx were reported to inhibit monoamine oxidase (MAO)-A and MAO-B with K_i values of 140–250 and 240–450 μ M, respectively (604).

3. Metabolic activation and detoxification pathways of AIA and their impact on estimated AIA-derived cancer risks

As already mentioned, AIA are not mutagenic as such, but must be metabolically activated by a series of enzymes to exert their mutagenic effects (Fig. 16). The

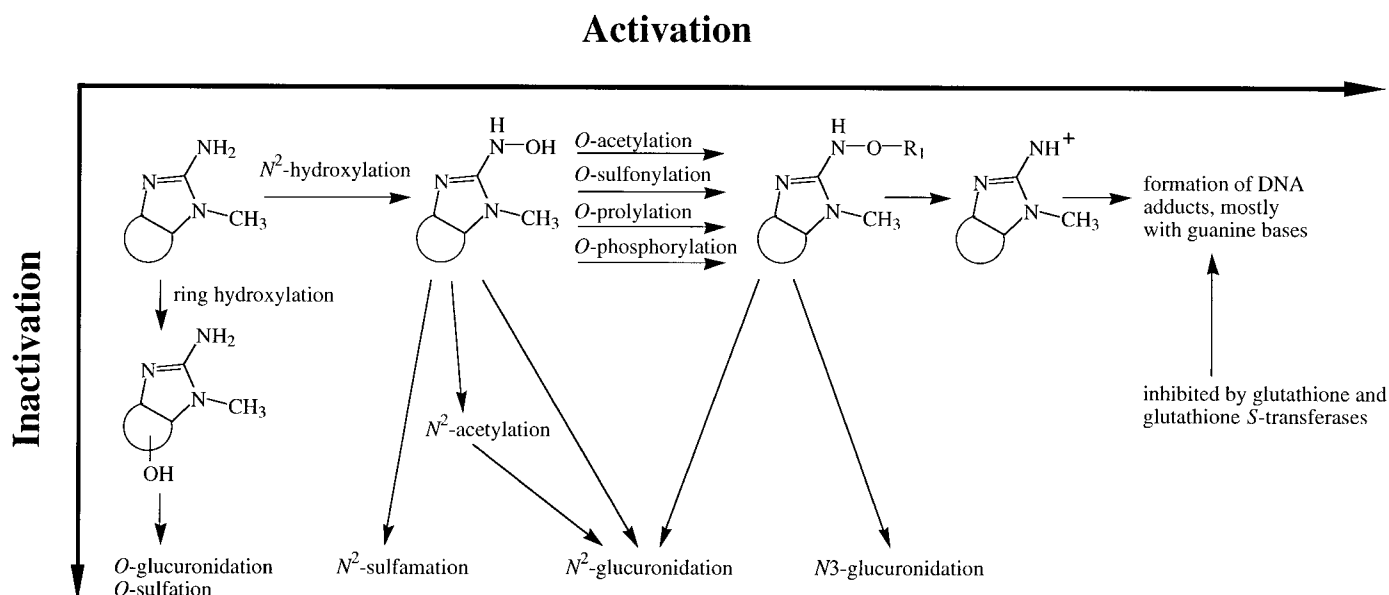


FIG. 16. Metabolism of amino-imidazo-azaarenes (AIA). The aminoimidazo part of the AIA is drawn correctly, whereas the remaining parts of the molecule are represented schematically by a ring. Reactions involved in the activation of AIA are drawn from *left to right*. Inactivation reactions, on the other hand, are arranged vertically. R_1 is OCOCH_3 for acetylation, OSO_3^- for sulfonylation, O-prolyl for prolylation, and OPO_3^{2-} for phosphorylation.

first reaction is *N*-hydroxylation of the exocyclic amino group, yielding the respective *N*-hydroxy-AIA. For most AIA, this reaction is catalyzed predominantly by the cytochrome *P*-4501A2 isoenzyme (296, 330, 872, 920). H_2O_2 in the physiological/pathological concentration range considerably stimulates metabolic activation of IQ by human and rat cytochrome *P*-4501A2 (19). PhIP, on the other hand, is hydroxylated at similar rates by cytochromes *P*-4501A1 and 1A2 (212, 330). In addition, a series of other cytochrome *P*-450 isoenzymes (see Refs. 158, 235, 345, 590, 872) as well as prostaglandin H synthase (670) were suggested to participate in *N*-hydroxylation of AIA, but their actual contribution to the metabolism of the respective AIA is only vaguely known.

N-OH-AIA can be activated further by *O*-acetylation, *O*-sulfonylation, *O*-prolylation, or *O*-phosphorylation (see Refs. 119, 159, 235, 743, 872, 920). In human liver, *O*-acetylation is mainly catalyzed by the polymorphic *N*-acetyltransferase (NAT2) isoenzyme and yields the respective *N*-acetoxy-AIA (345, 1121). In human mammary gland, on the other hand, *O*-acetylation is catalyzed predominantly if not exclusively by the NAT1 isoenzyme (828). *O*-Sulfonylation of *N*-OH-PhIP is catalyzed in human liver by the thermostable phenol sulfotransferase isoenzyme (TS-PST), whereas the contributions of thermolabile phenol sulfotransferase (TL-PST) and dehydroepiandrosterone sulfotransferase (DHEA-ST) seem to be negligible (126). Moreover, no *O*-sulfonylation was observed for *N*-OH-IQ and *N*-OH-8-MeIQx in human liver. While *O*-prolylation of *N*-OH-AIA is catalyzed by γ -prolyl-tRNA synthetase, the enzyme responsible for ATP-dependent *O*-phosphorylation has not yet been identified. All four activated AIA metabolites are likely to decompose to the highly reactive nitrenium ion (872; see also Ref. 348) which then reacts with protein, low- M_r thiols like glutathione or cysteine, or DNA.

Each AIA forms several different adducts with DNA (e.g., Ref. 110), but most of them involve guanine bases. So far, IQ, MeIQ, 8-MeIQx, 4,8-DiMeIQx, and PhIP were reported to form adducts with 2'-deoxyguanosine, by binding of the exocyclic nitrogen of the AIA to the C-8 of guanine (see Refs. 235, 617, 872, 886, 922). To a lesser extent, IQ and 8-MeIQx also formed adducts with the N^2 of guanine. The preference of activated AIA for guanine bases is in perfect agreement with the fact that most mutations induced by IQ, MeIQ, 8-MeIQx, and PhIP in the p53, Ha-ras, Ki-ras, APC, *supF*, *lacZ*, *lacI*, and *hprt* genes involve G-C base pairs (see Refs. 296, 682, 872, 1013).

In addition to these activation reactions, several detoxification pathways have to be considered, in particular *N*-glucuronidation, N^2 -sulfamation, ring hydroxylation followed by sulfation or glucuronidation, *N*-demethylation, and glutathione- plus GST-mediated suppression of AIA-DNA adduct formation (see Refs. 215, 235, 448, 521). *N*-Glucuronidation has been established for *N*-OH-IQ,

N-OH-8-MeIQx, and *N*-OH-PhIP. In the case of *N*-OH-PhIP, two distinct glucuronides, namely, *N*-OH-PhIP N^2 -glucuronide and *N*-OH-PhIP N^3 -glucuronide, were identified, with their ratio differing considerably between rat, dog, and human. Ring hydroxylation, like *N*-hydroxylation, is catalyzed by cytochrome *P*-4501A isoenzymes, although different isoenzymes may be involved in the two processes. In the case of PhIP, ring hydroxylation is catalyzed predominantly by cytochrome *P*-4501A1 and yields 4'-OH-PhIP, which displays no or only low mutagenicity. In addition, 5-OH-PhIP may be formed via a cytochrome *P*-450-independent pathway.

In *in vitro* studies, glutathione inhibited the covalent binding of *N*-acetoxy-IQ and *N*-acetoxy-8-MeIQx to DNA by ~40%, and further addition of human or rat GST isoenzymes had no extra effect (561). In contrast, covalent DNA binding of *N*-acetoxy-PhIP was only slightly inhibited by glutathione alone, but inhibition was greatly amplified by further addition of human GST isoenzyme A1-1 or rat GST isoenzyme 1-2. The human GST isoenzymes A1-2 and P1-1 as well as rat GST 12-12 had lesser effects. Analysis of incubation mixtures containing *N*-acetoxy-PhIP, reduced glutathione, and GST A1-1 failed to reveal glutathione conjugates of PhIP. Only oxidized glutathione and PhIP, in a molar ratio of ~1:2, were detected as reaction products.

The contribution of the different activation and detoxification reactions to the total metabolism of a given AIA differs to a large extent between individual AIA, between different tissues, and between species. It therefore goes without saying that our current knowledge on this matter is still incomplete. In humans, after the consumption of a test meal, 90.8 and 69.9% of 8-MeIQx and PhIP, respectively, were metabolized via cytochrome *P*-4501A2, 2.6 and 1.2% were excreted unchanged in the urine, and 6.6 and 28.9% were eliminated by other routes of metabolism (81). Metabolic activation of AIA by *N*-hydroxylation and *O*-acetylation occurs predominantly in the liver of rodents and primates. Accordingly, for all AIA except PhIP, highest DNA adduct levels are found in liver, followed by kidney, heart, and colon (160, 920). For PhIP, on the other hand, highest DNA adduct levels were detected in rat pancreas, followed by prostate, colon, lung, and heart, whereas liver contained 25- to 50-fold lower levels of PhIP-DNA adducts than the pancreas (447, 886). It is surprising that metabolic activation of PhIP nevertheless occurs predominantly in the liver and that *N*-hydroxy-PhIP and *N*-acetoxy-PhIP are the major metabolites that subsequently are transported via the blood circulation to those extrahepatic tissues that are subject to the mutagenic effects of PhIP (see Ref. 872). This apparent discrepancy suggests that an efficient detoxification mechanism is operative in the liver. Evidence supporting this notion in fact exists, indicating that protection may be brought about by glutathione and GST (539,

561). The contribution of *O*-acetylation, *O*-sulfonylation, *O*-prolylation, and *O*-phosphorylation to metabolic activation differs considerably between individual *N*-OH-AIA, tissues, and species, but all four reactions seem to be relevant (161). Genotoxicity of PhIP, in contrast to IQ, does not depend on acetyltransferase activity in Chinese hamster ovary cells, which is in line with the finding that *O*-sulfonylation of *N*-OH-PhIP is quantitatively more important than *O*-acetylation in the production of DNA adducts (see Refs. 235, 1121).

With some exceptions (see Ref. 872), a satisfactory correlation is observed for a given tissue between the capacity to metabolically activate AIA, the extent of AIA-DNA adduct formation, and the frequency of AIA-induced tumor development. For IQ, MeIQ, and 8-MeIQx, the principal site of metabolic activation is the liver. Accordingly, liver displays the highest level of DNA adducts, and tumors of the liver develop with high frequency in animals treated with these AIA. Metabolic activation of PhIP also occurs primarily in the liver, but most probably due to efficient detoxification (see above), only low levels of PhIP-DNA adducts are formed in this tissue. Consistent with this finding, PhIP induces liver tumors in neither mice nor rats. In the mammary gland of Fischer 344 (F344) rats, DNA adduct formation of *N*-OH-PhIP was ~3- and 17-fold higher than with *N*-OH-IQ and *N*-OH-8-MeIQx, respectively (159). Correspondingly, PhIP induced mammary carcinomas in F344 rats, whereas IQ and 8-MeIQx did not. Compared with rat and human, cynomolgus monkeys have a similar capacity to metabolically activate IQ. On the other hand, metabolic activation of 8-MeIQx and PhIP is considerably lower (212, 235). In line with this observation, IQ, MeIQ, and 8-MeIQx are potent carcinogens in mice and rats, whereas in cynomolgus monkeys, 8-MeIQx and PhIP induced no tumors so far.

Of particular interest with regard to human health perspectives is the question of the human cancer risk due to food-borne AIA. Although previous estimations arrived at maximum risks of up to 1 in 1,000 (see Ref. 215), a more recent calculation, based on an extensive literature review on the levels of AIA in cooked foods and on mean food consumption figures in the United States, yielded an incremental cancer risk due to AIA of $\sim 10^{-4}$ (540; see also Ref. 289). For several reasons, this number is still subject to considerable uncertainty. 1) The cancer risk was extrapolated from carcinogenicity data obtained in rats. However, due to differences in metabolic activation and detoxification pathways, human tissues may be more susceptible to AIA than rodent tissues (see, e.g., Ref. 235). 2) Because of (genetic) polymorphisms of AIA-activating enzymes, in particular of cytochrome *P*-4501A2 and NAT2 (see Ref. 235), some subjects may be considerably more susceptible to AIA than others. Accordingly, subjects with a phenotype of high cytochrome *P*-4501A2 and/or NAT2 activities have an increased risk of developing colorectal

cancer of potentially up to 1 in 50. 3) Chronic exposure to low levels of AIA may be more harmful than expected. As a matter of fact, combined treatment of rats with 5 or 10 mutagenic heterocyclic amines may result in synergistic rather than additive enhancement of mutagenic and carcinogenic effects (see Refs. 342, 344). Similarly, MeIQ was shown to enhance the mutagenicity of the drinking water mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (1090). 4) There are still only limited data available on the food levels of AIA. Only very recently, for example, PhIP levels in pan-fried, broiled, or grilled chicken were found to be much higher than previously suspected (896).

4. Nitrosation products of Cr and Crn as potential human carcinogens

An alternative pathway resulting in the formation of mutagenic and carcinogenic principles may be nitrosation of Cr, Crn, or methylguanidine (MG). Nitrate is reduced to nitrite by oral bacteria, and favorable conditions for nitrosation prevail in the stomach. A strong positive correlation was observed between the dietary nitrate (and nitrite) intake and gastric cancer mortality (340, 649). In the United States, a fourfold reduction in the calculated gastric nitrite load between 1925 and 1981 was associated with a threefold decrease in gastric cancer mortality.

Nitrosation of Cr, Crn, and MG has been studied mostly in *in vitro* systems. Crn is converted to *N*-methyl-*N*-nitrosourea (MNU) in four successive steps, three of which involve reaction with nitrite (651). MNU may also be formed from MG, with methylnitrosoguanidine and methylnitrosocyanamide as intermediates (see Ref. 649). MG, in turn, may be of dietary origin or, alternatively, may be formed *in vivo*, either from Crn via the reaction sequence proposed to proceed in uremic patients when serum [Crn] is increased (see sect. 1x*H*) or by an oxidation reaction of Cr or Crn catalyzed by iron or copper salts. Nitrosation of Cr successively yields sarcosine and *N*-nitrososarcosine (155). The latter, finally, may be dehydrated to *N*-nitrosodimethylamine.

Of the nitrosation products shown in Figure 17, Crn-5-oxime and 1-methylhydantoin-5-oxime were not mutagenic in the Ames test (651). Crn-5-oxime also displayed no carcinogenic activity (1113). MNU is a potent mutagen and direct-acting carcinogen, producing tumors in several species and in a variety of organs, particularly in stomach and CNS, but also in intestine, kidney, and skin (541, 987, 1088). In line with the observation that *O*⁶-methylguanine is the reaction product of MNU with DNA, MNU mostly induced base substitutions from GC to AT (see Ref. 1160). Compared with MNU, methylnitrosocyanamide displayed even higher mutagenicity in the Ames test, and it was there-

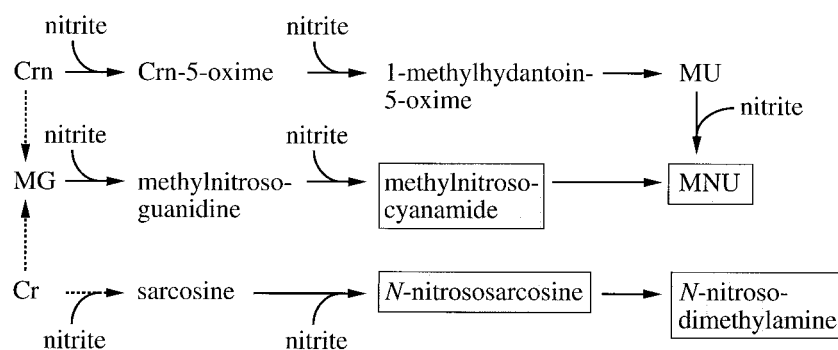


FIG. 17. Potential nitrosation products of Cr, Crn, and methylguanidine (MG). MNU, methylnitrosourea; MU, methylurea. Nitrosation products that were shown to be carcinogenic in animal experiments are boxed.

fore concluded that the active principle responsible for the mutagenicity of nitrosated MG is mainly methylnitrosocyanamide (223, 224). However, methylnitrosocyanamide is only moderately carcinogenic. *N*-nitrososarcosine is weakly carcinogenic in both rats and mice, causing esophageal and liver cell carcinomas, respectively (1113). *N*-nitrosodimethylamine is a highly toxic carcinogen and was suggested to be formed in the small intestine of uremic patients (550; see also Ref. 537).

Even though these findings might be taken to indicate that nitrosation products of Cr, Crn, and MG represent a significant health hazard, a series of arguments point to the contrary. 1) When nitrosation was studied under real and simulated gastric juice conditions, only MG yielded significant mutagenicity in the Ames test, whereas Cr, PCr, Crn, or guanidinoacetate gave no or very low mutagenicity (224). However, MG ingestion is likely to be ~100-fold lower than that of Cr and Crn (650). 2) Reaction of Crn with 0.4 M nitrite for 1 h at pH 1 gave only a 0.0003% yield of MNU (see Ref. 651). This must be compared with a nitrite level of 54 μ M in gastric juice of subjects from an area with a high gastric cancer incidence (see Ref. 650). 3) Nitrite participates in three steps in the reaction sequences from Crn to MNU and from Cr to *N*-nitrososarcosine. Given the low nitrite level of $\leq 54 \mu$ M, it is highly unlikely that these reactions proceed to a significant extent.

5. Conclusions

Circumstantial evidence suggests that Cr and Crn may add to human carcinogenesis by forming either AIA or nitroso compounds that covalently modify guanine bases and thereby result in DNA mutations. On the basis of current knowledge, however, Cr- and Crn-derived AIA and nitroso compounds may impose only a minor health risk. Nevertheless, because the formation and modes of action of these substances are still only incompletely understood, intensive research on this topic must continue. In the meantime, consumption of vitamin C, chlorophylls, or other dietary supplements, or a change in cooking habits, seem to be feasible ways to reduce the

potential health risk even further. Vitamin C was shown to inhibit nitrosation reactions by reducing nitrite to NO (see Ref. 649), and lactic acid bacteria, antioxidants, flavonoids, chlorophylls, food-coloring agents, isothiocyanates, and capsaicin reduce mutagenicity and carcinogenicity due to AIA mutagens (see, e.g., Refs. 20, 153, 235, 328, 363, 416, 465, 546, 589, 845, 872, 990, 1095, 1140).

G. Creatin(in)e Metabolism and Brain Function

Total CK activity and Cr content are lower in brain than in skeletal muscle or heart. Even though it might be concluded that, therefore, the CK system plays a less prominent role in brain physiology, there is ample evidence for close correlations between Cr metabolism and CK function on one hand and proper brain function on the other hand. In chicken and rat brain, the B-CK, M-CK, and Mi-CK isoforms were localized specifically to cell types for which high and fluctuating energy demands can be inferred (e.g., cerebellar Bergmann glial cells, Purkinje neurons, and glomerular structures) (450, 1081). Substantial evidence supports a direct coupling of CK (or ArgK) with growth cone migration (1087), with Na^+ - K^+ -ATPase and neurotransmitter release, as well as an involvement of CK in the maintenance of membrane potentials, calcium homeostasis, and restoration of ion gradients before and after depolarization (1081). CK and Cr were also suggested to participate in inhibition of mitochondrial permeability transition (64, 717), which is thought to be linked to both apoptotic and necrotic neuronal cell death.

Although the situation may be somewhat different in the rat and piglet brain (375, 1102), the PCr and total Cr concentrations as well as the flux through the CK reaction are significantly higher in gray than in white matter of the human brain (105, 573, 594, 784, 1086). These findings parallel the higher rate of ATP turnover in cerebral gray compared with white matter. Furthermore, electroencephalogram (EEG) activity increases considerably in the first 2–3 wk of life in the rat. Large increases in the response of rat cortical slice respiration to electrical stimulation, hyperthermia, or increased extracellular [KCl]

occur in particular between 12 and 17 days of age (see Ref. 377). Interestingly, in this same developmental interval, the proportion of Mi-CK and the flux through the CK reaction increase by a factor of 4 (377, 1018). On the other hand, both CK activity and flux through the CK reaction were shown to be decreased in aged rats and humans (see Ref. 909), and it is tempting to speculate that this may correlate with the cognitive decline in the elderly.

In rats in which EEG activity was varied over a fivefold range by either bicuculline stimulation or thio-pental inhibition, the forward rate constant of the CK reaction (k_f) measured by ^{31}P -NMR saturation transfer was linearly correlated with EEG intensity (849). A linear correlation was also observed in the brain between k_f and the accumulation of deoxyglucose-6-phosphate after intraperitoneal administration of deoxyglucose, which is a measure of glucose uptake and utilization in brain. In contrast, brain ATP concentration remained constant over the whole range of EEG intensities studied, and PCr concentration only decreased at high EEG intensities. These findings suggest that k_f is a more sensitive and reliable indicator of brain activity than [ATP] or [PCr]. In a similar study on the rat brain, the dihydropyridine calcium antagonist isradipine slowed ATP depletion during global ischemia, which was paralleled by a decrease in the flux through the CK reaction by $\sim 25\%$ (825).

By increasing the ATP-regenerating capacity via the CK reaction before oxygen deprivation, it might be possible to delay ATP depletion and, thereby, to protect the brain from ischemic or anoxic damage. As a matter of fact, in hippocampal slices of the guinea pig brain exposed to 5–30 mM Cr for 0.5–3 h before anoxia, PCr accumulation in the slices increased with Cr concentration and incubation time, synaptic transmission measured with electrophysiological methods survived up to three times longer during anoxia, and postanoxic recovery of both high-energy phosphates and of the postsynaptic potential was considerably improved relative to control slices (see Refs. 563, 730). Similarly, preincubation of rat neocortical slices with 25 mM Cr for ≥ 2 h had a pronounced protective effect on excitatory and inhibitory synaptic transmission during brief periods of hypoxia (579), and preincubation of rat hippocampal slices with 0.03–25 mM Cr slowed ATP depletion, prevented the impairment of protein synthesis, reduced neuronal death during anoxia in a dose-dependent manner, and delayed anoxic depolarization (43, 112). In brain stem slices of Cr-pretreated neonatal mice, and in slices of nonpretreated neonatal mice incubated for 3 h with 0.2 mM Cr, ATP depletion was delayed and hypoglossal activity enhanced and stabilized relative to controls during 30 min of anoxia (1105).

The susceptibility to seizures is highest in human term newborns and 10- to 12-day-old rats, and it has been suggested that the low Cr and PCr concentrations in the

metabolically immature brain may critically influence susceptibility to hypoxic seizures (376). As a matter of fact, injection of Cr into rat pups for 3 days before exposing them to hypoxia on postnatal *day 10* increased brain PCr-to-nucleoside triphosphate ratios, decreased hypoxia-induced seizures and deaths, and enhanced brain PCr and ATP recoveries after hypoxia.

As in skeletal muscle and heart (see sects. VIII A and IX C), long-term feeding of mice with the Cr analog cCr increased the total high-energy phosphate pool in the brain and slightly delayed ATP depletion during brain ischemia (1119), but did not improve the hypoxic survival time of the mice (30). The rate of cCr accumulation in the brain was relatively slow when the compound was supplied in the diet but could be increased by circumventing the blood-brain barrier, or when the compound was given intravenously (R. Kaddurah-Daouk, unpublished data). Feeding of mice with the Cr analog GPA resulted in the accumulation of PGPA, decreased the flux through the CK reaction in the brain *in vivo* by $\sim 75\%$, and enhanced survival during hypoxia (371, 372, 374).

Recently, GAMT deficiency was identified as the first inborn error of Cr metabolism in three children presenting with neurological symptoms (276, 867, 946–949). One of the male patients exhibited an extrapyramidal movement disorder starting at the age of 5 mo. At the age of 22 mo, he displayed severe muscular hypotonia and developmental delay. The EEG showed abnormally low background activity with multifocal spikes. Another patient, a girl aged 4 yr, presented a dystonic-dyskinetic syndrome, developmental delay, and epilepsy with myoclonic and atstatic seizures and grand mal. The third patient, a 5-yr-old boy, displayed global developmental delay and experienced frequent tonic seizures, associated with apnea. GAMT deficiency was identified as the underlying metabolic basis of the disease by finding considerably increased brain, cerebrospinal fluid, serum, and urine concentrations of guanidinoacetate; low Cr concentrations in plasma, cerebrospinal fluid, and urine; a virtual absence of Cr and PCr in the brain (0.2–0.3 vs. 5.1–5.5 mM in controls); strongly depressed Crn concentrations in serum and urine; as well as a drastically reduced GAMT activity in liver biopsies (0.5–1.35 vs. 36.4 nmol \cdot h $^{-1}$ \cdot g $^{-1}$ in controls). In addition, oral supplementation with Arg resulted in an increase in brain guanidinoacetate concentration but did not elevate cerebral Cr levels. The nucleic acid mutations causing GAMT deficiency were characterized and include base substitutions, insertions, and deletions (948).

Oral supplementation with 4–8 g Cr/day or 2 g \cdot kg $^{-1}$ \cdot day $^{-1}$ slowly normalized the concentration of Cr in the brain (276, 867, 946, 947). After 6 wk, brain Cr had reached almost 50% of its normal concentration, whereas after 25 mo on treatment, brain [Cr] was nearly normal. The slow increase in brain [Cr] is a further indication for

the limited permeability of the blood-brain barrier for Cr. Oral Cr supplementation also improved serum and urinary Crn concentrations, brain guanidinoacetate concentration, as well as EEG activity. Most importantly, Cr supplementation resulted in substantial clinical improvement, both with regard to muscle tone and extrapyramidal symptoms. Although the plasma concentration of Cr increased to supranormal levels (270–763 μM), the plasma concentrations of guanidinoacetate and homocysteine remained elevated even after 22 mo of Cr supplementation (949). This is noteworthy for two reasons: 1) it cannot yet be excluded that guanidinoacetate at the still elevated concentrations is neurotoxic and is responsible, instead of Cr deficiency, for the neurological symptoms associated with GAMT deficiency. 2) Increased serum concentrations of Cr due to supplementation should downregulate AGAT activity in kidney and pancreas and therefore result in subnormal guanidinoacetate concentrations. The opposite finding in a patient with GAMT deficiency suggests that the serum concentration of Cr may not be the sole signal for the downregulation of AGAT activity (see sect. iv).

Four points deserve further consideration. 1) Despite sharing the same primary defect, the three patients with GAMT deficiency, for unknown reasons, displayed strikingly different clinical symptoms. 2) Arginine restriction of the diet for 15 days while maintaining Cr supplementation tended to increase rather than decrease the plasma concentration and urinary excretion of guanidinoacetate (868). Therefore, early institution of Cr supplementation is so far the only successful therapeutic strategy in GAMT deficiency. 3) GAMT knock-out animals may become a valuable model for studying the relevance of the CK system at different developmental stages. Cr can be supplied through the diet during both pregnancy and postnatal development. At any developmental stage, Cr can be withdrawn from the diet and the accompanying changes in brain and muscle function studied. That Cr is in fact provided to the fetus in utero is supported by the lack of neurological symptoms in patients with GAMT deficiency during the first few months of life and by the demonstration of maternofetal transport of Cr in the rat (157). 4) Cr supplementation may prove beneficial in other diseases presenting with both neurological symptoms and reduced tissue concentrations of Cr. For example, Cr excretion was reported to be lowered in the hyperornithinemia-hyperammonemia-homocitrullinuria syndrome, which is characterized by clinical symptoms such as vomiting, lethargy, coma, seizures, ataxia, and various degrees of mental retardation (189). Arg or citrulline supplementation normalized Cr excretion and seemed to have favorable clinical effects.

After these lines of evidence for a close correlation between the functional capacity of the CK/PCr/Cr system and brain function, let us now turn to (human) brain

diseases where the relationships between the derangements of Cr metabolism and the pathological process are less clear. Glutamate is the major excitatory neurotransmitter in the vertebrate CNS. Whereas ATP-dependent uptake of glutamate into synaptic vesicles is well documented, it was only recently that glutamate uptake was found to be stimulated also by PCr (1127). PCr stimulated glutamate uptake into synaptic vesicles even in the absence of added ATP, with an EC_{50} of ~ 10 mM. A series of control experiments demonstrated that the effect of PCr is CK independent and, thus, not due to local regeneration of ATP. At a glutamate concentration of 50 μM , maximal PCr-stimulated glutamate uptake was significantly higher than that maximally stimulated by ATP. Mg^{2+} and Cl^{-} , which potentiate the stimulation of glutamate uptake by ATP, as well as inhibitors of ATP-dependent uptake had little effect on PCr-dependent uptake activity. In conclusion, ATP and PCr seem to stimulate glutamate uptake into synaptic vesicles via two different, albeit unknown, mechanisms, and both pathways are likely to contribute additively to total glutamate uptake.

Glutamate neurotoxicity has been proposed as a cause of neuronal death in a variety of diseases including Alzheimer's disease (AD). Although not in line with another study (85), an *in vivo* ^{31}P -magnetic resonance spectroscopy investigation of AD patients showed that PCr levels are low in mildly demented AD patients and then become increased as the dementia worsens (764; see also Ref. 767). Significant correlations were also seen in AD patients between $[\text{PCr}]/[\text{P}_i]$ and different neuropsychological test results (910). Brown et al. (98) found a decreased $[\text{PCr}]/[\text{P}_i]$ in both temporoparietal and frontal regions of the AD brain relative to controls, but an increased $[\text{PCr}]/[\text{P}_i]$ in the same brain regions of patients with multiple subcortical cerebral infarction dementia (MSID). AD and MSID are the two most common causes of cognitive decline in the elderly. It remains to be seen whether and how the CK system and the recently discovered role of PCr in glutamate uptake would impact dementia.

In recent years, it has been realized that oxidative stress may be a critical determinant of metabolic deterioration in a variety of neurodegenerative diseases, e.g., Alzheimer's disease, Parkinson's disease, Huntington's disease, or amyotrophic lateral sclerosis (see, e.g., Refs. 76, 210, 230, 428, 471, 785, 866, 912, 915, 957, 1023, 1139). The production of reactive oxygen species (ROS) and NO as well as the brain concentrations of malondialdehyde, protein carbonyls, and mitochondrial 8-hydroxydeoxyguanosine are increased in these diseases, suggesting stimulation of lipid peroxidation as well as oxidative protein and DNA damage. Among other effects, oxidative stress may compromise energy metabolism, which may lead to activation of excitatory amino acid receptors and to an increase in intracellular Ca^{2+} concentration. There is evidence that CK may be one of the main targets of

oxidative damage. CK isoenzymes invariably have a highly reactive sulfhydryl group in the active site (Cys-283 of human B-CK), oxidative modification of which results in pronounced or even complete inhibition of the enzyme (see Ref. 275). Most probably by attacking this reactive sulfhydryl group, ROS like the hydroxyl radical, superoxide radical, H_2O_2 , NO, or peroxynitrite inactivate CK (see sect. IXC). β -Amyloid peptide ($A\beta$), which forms the core of AD-associated senile (amyloid) plaques and is postulated to be a major determinant of AD, promotes protein oxidation and causes irreversible inhibition of CK at concentrations that are toxic to cultured neurons (355, 1139). Accordingly, CK activity is decreased in AD brain by up to 86% (see Refs. 9, 154, 908). A comparable increase in protein oxidation and decrease in brain CK activity was observed in Pick's disease and diffuse Lewy body disease (9). The mechanism of CK inhibition by $A\beta$ is not yet understood. $A\beta$ can fragment and generate peptide-derived free radicals that may then exert an inhibitory effect (355, 1139). Alternatively, Met-35 of $A\beta$ was reported to be oxidized to methionine sulfoxide presumably via a free radical oxidation process. In vitro, methionine sulfoxide in fact inhibits CK activity irreversibly (324). In the light of the unphysiologically high methionine sulfoxide concentration of 20 mM used, the physiological relevance of this finding must, however, be questioned. David et al. (154) observed that despite an 86% decrease in CK activity, the quantity of immunodetectable CK protein is virtually unchanged in AD brain [although Aksenova et al. (9) found a decrease in BB-CK content in frontal cortex], that inactivation is due most probably to modification at the active site of the enzyme, and that the modified, inactive CK, rather than being soluble in the cytosol, is mostly associated with particulate subcellular structures from which it can be extracted in part by detergents. In cultured rat hippocampal neurons, the antioxidant vitamin E was shown to protect from $A\beta$ -(25–35)-induced protein oxidation, CK inactivation, and neuronal toxicity (1139).

If impairment of high-energy phosphate metabolism plays a critical role in the progression of neurodegenerative disease, then compounds that increase the cerebral energy reserve may be neuroprotective. Recently, a series of experiments were conducted where Cr and some of its analogs were evaluated in animal models of Huntington's disease, Parkinson's disease, and amyotrophic lateral sclerosis. In the animal models for Huntington's disease, oral Cr or cCr supplementation produced significant protection against malonate lesions, and Cr protected against 3-nitropropionic acid (3-NP) neurotoxicity, whereas cCr did not (182, 616). Cr supplementation decreased the volume of lesions produced by subacute systemic administration of 3-NP to rats by >80%. With regard to the mechanism of action by which Cr exerts its neuroprotective effect, the following findings were made: 1) decreases in the concentrations of Cr, PCr, ATP, GDP, AMP, NAD,

and ADP plus GTP, which are known to be induced by 3-NP, were attenuated significantly in Cr-fed animals. 2) Cr protected against 3-NP-induced increases in striatal lactate concentrations in vivo. 3) Cr protected against malonate-induced increases in the conversion of salicylate to 2,3- and 2,5-dihydroxybenzoic acid, biochemical markers of hydroxyl radical generation. 4) Cr protected against 3-NP-induced increases in 3-nitrotyrosine concentration, a marker of peroxynitrite-mediated oxidative injury. 5) In cortical and striatal astrocytes in vitro, Cr pretreatment delayed increases in intracellular Ca^{2+} produced by 3-NP. It seems that by attenuating or delaying ATP depletion, Cr and some of its analogs prevent a pathological cascade that leads to free radical generation and eventual cell death. Alternatively, Cr and its analogs could regulate the mitochondrial permeability transition pore, a mitochondrial protein complex that was implicated with cell death (717).

In the MPTP model of Parkinson's disease, Cr or cCr supplementation produced significant protection against MPTP-induced depletion of dopamine and of its metabolites in the substantia nigra of mice (615). Both Cr and cCr also attenuated MPTP-induced degeneration of Nissl-stained and tyrosine hydroxylase-immunostained neurons. In a transgenic mouse model of amyotrophic lateral sclerosis with a G93A superoxide dismutase-1 mutation, oral supplementation with Cr dose-dependently improved motor performance and extended survival even more than the approved drug riluzole or any other agent tested to date (489). Cr protected the transgenic mice from loss of both motor neurons and substantia nigra neurons and from increases in biochemical indices of oxidative damage. In preliminary studies in Huntington's disease patients, Cr seems to favorably impact brain energetics and to reduce lactate production (Beal et al., unpublished data). These studies all suggest that Cr and its analogs may emerge as a new class of neuroprotective agents.

Multiple sclerosis (MS) is an inflammatory disease causing multiple plaques of demyelination, predominantly in white matter. The results published so far on the disturbances of Cr metabolism in MS brain are contradictory. Although the PCr/ATP ratio was reported to be decreased in one patient with a large lesion, but unchanged in four patients without lesions (106), it was suggested to be increased in another study (647). A 35% decrease in Cr concentration in MS plaques and normal-appearing white matter adjacent to plaques was observed in the postmortem MS brain (156). Total Cr was also decreased in MS plaques of children (100). On the other hand, total Cr and PCr concentration were found by Husted et al. (392) to be significantly increased in normal-appearing white matter of MS patients compared with controls. In this same study, total Cr was also increased in MS lesions. In an investigation of different brain regions of patients with relapsing-remitting MS, [Cr] was found to

be highest in areas far from lesions (748). PCr levels were found to correlate with the severity of the handicap and were greater in patients with a progressive course of the disease than in patients with relapsing-remitting MS (647). As far as muscle function is concerned, a similar or greater decrease in muscle [PCr] or pH during exercise was seen in MS patients relative to controls (473). PCr recovery after exercise was considerably slowed in MS patients, thus indicating impaired oxidative capacity. Clearly, the relationships between disturbances of Cr metabolism and MS are still unclear and deserve further attention.

Postmortem brain tissue of schizophrenic patients was reported to contain 5–10 times less water-soluble BB-CK and 1.5–3 times less Mi-CK compared with controls (490). The major part of BB-CK was water insoluble (particulate form of BB-CK) and required detergents for extraction and reactivation. Total CK activity in schizophrenic brain was decreased from 356 to 83 IU · (g wet wt)⁻¹. In another study, CK activity was depressed by up to 90% in different brain regions of schizophrenic patients (102). On the other hand, no abnormality was seen for the concentrations of total Cr or PCr in schizophrenic brain by some authors (763, 880), whereas total Cr concentration was suggested to be reduced bilaterally by Maier et al. (586), and PCr concentration to be increased by Williamson et al. (1107). The PCr concentration in the left temporal lobe of schizophrenic patients seemed to increase with the severity of psychiatric symptomatology (173, 466).

A series of ³¹P-magnetic resonance spectroscopy studies examined the changes of energy metabolism in brain and skeletal muscle of patients with migraine and cluster headache (for references, see Refs. 661, 662, 1097). Cluster headache is a disease that shares many common features with migraine. Abnormalities were observed in migraine with and without aura, during and between attacks, and in cluster headache during and outside the cluster period. They involve reduced levels of PCr, a decreased phosphorylation potential, as well as increased ADP concentrations and rates of ATP synthesis (V/V_{\max} values) in the brain. In resting muscle, findings were usually normal. Upon exercise, however, initial recovery of PCr was significantly delayed, implying defective resynthesis of high-energy phosphates in muscle. Defective mitochondrial function is likely to be the underlying cause, as supported by experimental evidence and by exclusion of brain ischemia as a pathogenic event during a migraine attack. Remarkably, giant mitochondria with paracrystalline inclusions were seen in skeletal muscle of a patient with migraine stroke (49). It has been suggested that the observed abnormalities of energy metabolism predispose migraineurs to develop an attack under conditions of increased brain energy demand (661).

Similar derangements of energy metabolism are observed in mitochondrial cytopathies, a heterogeneous

group of diseases in which a mitochondrial defect is established. Patients with chronic progressive external ophthalmoplegia (CPEO), mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonus epilepsy with ragged red fibers (MERRF), Leigh's syndrome, or Leber's hereditary optic neuropathy (LHON) all have a decreased PCr content, an increased calculated ADP concentration and V/V_{\max} , and a depressed phosphorylation potential in the brain (50, 216, 663). Ragged red fibers and cytochrome-*c* oxidase-deficient muscle fibers were seen in CPEO, but not in LHON patients (663). Remarkably, the metabolic changes were seen even before a clinical brain involvement was noted.

Among the mitochondrial myopathies, migraine and stroke-like episodes are described only in MELAS syndrome. This disorder affects children and is characterized by stunted growth, episodic vomiting, seizures and recurrent cerebral insults resembling strokes and causing hemiparesis, hemianopsia, and cortical blindness. In a male MELAS patient aged 25 yr presenting with the characteristic neurological symptoms and with ragged-red muscle fibers, Cr was given orally at a rate of 10 g daily for 14 days and 4 g daily thereafter (323). The patient and his family reported reduced headache, less weakness, better appetite, and an improved general well-being during treatment. In addition, muscle performance was significantly improved in an incremental exercise test, and electrocardiogram abnormalities vanished. In conclusion, all these findings suggest but do not prove similarities in the pathogenesis of migraine, cluster headache, and possibly also of mitochondrial cytopathies such as LHON, MELAS, or MERRF. Cr supplementation may alleviate some of the clinical symptoms in these diseases.

In the brain of rats with acute renal failure, PCr, ATP, and glucose levels are increased, whereas AMP, ADP, and lactate concentrations are decreased (see Ref. 261). These changes are associated with a decrease in both brain metabolic rate and cerebral oxygen consumption and are consistent with a generalized decrease in brain energy use. The best correlation of impaired motor nerve conduction velocity was found with the serum Crn level.

Involuntary movements, myoclonus, epileptic attacks, and severe burning feet sensations are found among renal failure patients on "appropriate" dialytic therapy. It has been hypothesized that some of the neurological symptoms may be caused by guanidino compounds [Crn, MG, GBA, guanidinosuccinic acid (GSA) and guanidine] that are considerably increased in cerebrospinal fluid and brain of patients with renal insufficiency (165, 166) (see also sect. IXH). A variety of guanidino compounds were reported to induce epileptic discharges and convulsions in rodents, e.g., guanidinoacetic acid, Cr, Crn, PCr, GBA, or MG (see Refs. 185a, 360, 362). Cr, Crn, and PCr induce tonic-clonic convulsions after intracister-

nal injection into rabbits (433). Crn also had convulsive activity after intracerebroventricular administration in mice (164). The relevance of these findings is questionable since intracisternal or intracerebroventricular injection cannot be compared directly with oral or intravenous administration of these compounds.

In mouse spinal cord neurons in primary dissociated cell culture, Crn, MG, guanidine, GSA, and some other guanidino compounds depressed GABA and Gly responses in a concentration-dependent manner, possibly by blocking the chloride channel (164, 167). The accompanying reduction in GABA- and Gly-dependent inhibition may lead to epilepsy. GSA, in contrast to Crn, MG, and guanidine, displayed significant effects at concentrations similar to those in cerebrospinal fluid and brain of uremic patients. The relative potencies with which the studied guanidino compounds depressed inhibitory amino acid responses corresponded with the relative potencies of the same compounds to induce epileptic symptomatology in behavioral experiments. MG, which is increased in uremia, may contribute to the neurological symptoms also by inhibiting acetylcholinesterase and/or $\text{Na}^+\text{-K}^+\text{-ATPase}$ (609, 610).

Some evidence suggests that guanidino compounds may exert their effects by influencing membrane fluidity. The lipid composition, including cholesterol concentration, is abnormal in epileptogenic whole brain tissue from cobalt lesions in animals, and dietary cholesterol appears to be inversely related to seizure susceptibility in animal models (see Ref. 696). Cholesterol administration normally is associated with a decrease in membrane fluidity (552, 705). Membranes from epileptogenic freeze-lesioned cat brain cortex displayed a lower order parameter (i.e., slightly higher fluidity) than control membranes (696). In contradiction to these results, guanidino compounds including MG, GSA, guanidine, and GPA decrease synaptosomal membrane fluidity of rat cerebral cortex, whereas anticonvulsant drugs, including diazepam, valproic acid, and phenobarbital, increase the fluidity of synaptosomal membranes in hippocampus and whole brain (see Ref. 361).

Guanidino compounds may not only be a trigger of epileptic seizures, but may also change in concentration during and after convulsions. Already in 1940, Murray and Hoffmann (680) noted that "in the instances of essential epilepsy studied, the basal content of 'guanidine' in the blood was found significantly high. All who presented convulsions of the grand mal variety showed a blood guanidine rise during the aura reaching a high point during convulsion." The levels of guanidinoacetate and Crn in cerebrospinal fluid increased at the onset of pentylenetetrazol-induced convulsion in the rabbit, while Arg started to decrease 2 h after the convulsion (360). Similarly, MG and guanidinoacetate levels in the rat brain were elevated for up to 3 mo after amygdala or hippocampal kindling,

whereas Cr and Arg showed no significant change (362, 885). In rodent, piglet, or dog brain, upon single and repeated seizures induced by either electroshock, fluoroethyl, or pentylenetetrazol, or in bicuculline-induced status epilepticus, PCr concentration in the brain decreased with seizure activity (see Refs. 198, 373–375, 849). The change in PCr was associated with a corresponding increase in Cr content so that total Cr concentration remained constant. Although it is widely accepted that the Cr-to-*N*-acetylaspartate ratio is significantly elevated in patients with temporal lobe epilepsy (e.g., Refs. 136, 146, 385, 761), it is not yet clear whether total Cr concentration in the brain is also increased (146) or unchanged (2, 761, 1034). In conclusion, the evidence for relationships between alterations in Cr metabolism and neurological symptoms in uremia is indirect and incomplete at present and, thus, needs further substantiation in the future.

Although guanidino compounds may have adverse effects on the nervous system in uremia, oral Cr (or cCr) supplementation is very unlikely to induce neurological complications in normal individuals, since only slight alterations in cerebrospinal fluid and brain concentrations of guanidino compounds may be expected. Cr and its analogs have been given to animals in high amounts and over several weeks and months with no neurological side effects. Likewise, oral Cr supplementation in humans with up to 30 g/day for several days as well as cCr administration in a phase I/II clinical study in gram amounts per day over an extended period of time also had no adverse neurological effects.

Finally, disturbances in Cr or guanidino compound metabolism were also seen in AIDS dementia (86); in patients with affective disorders, where, for example, Crn concentration in the cerebrospinal fluid was suggested to be negatively correlated with suicidal ideation and appetite (467, 704, 880); in hyperargininemic patients (595, 596); in the human brain after acute stroke (284); in brain tumors such as gliomas, astrocytomas, and meningiomas (488, 594); in the brain of dystrophin-deficient *mdx* mice (1014); in audiogenic sensitive rats (1103); or in rats intoxicated with the neurotoxins ethylene oxide or acrylamide (612).

In summary, brain function seems to be linked in a number of different ways with the CK system and with Cr metabolism, although the causal relationships in many cases are not yet known. Preliminary data suggest that both Cr and Cr analogs may have a therapeutic potential in brain disease. Cr supplementation, despite relatively slow uptake of Cr into the brain, may be indicated in diseases characterized by decreased brain concentrations of Cr or slowed PCr recovery. Cr and its analogs may also turn out to have therapeutic effects in neurodegenerative diseases associated with oxidative stress, such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis.

H. Creatin(in)e Metabolism and Renal Disease

The kidney plays a crucial role in Cr metabolism (see Fig. 4). On one hand, it is a major organ contributing to guanidinoacetate synthesis. On the other hand, it accomplishes urinary excretion of Crn, the purported end product of Cr metabolism in mammals.

In chronic renal failure (CRF) rats, the renal AGAT activity and rate of guanidinoacetate synthesis are depressed (520, 554). Accordingly, the urinary excretion of guanidinoacetate is decreased in a variety of renal diseases (10, 77, 412, 598, 969). Although the serum concentration of guanidinoacetate was also shown to be decreased in both uremic patients and renal failure rats (39, 412, 520, 598, 747), it was found, in a few other studies, to be unchanged (10, 165, 168, 638) or even slightly increased (163, 757). These conflicting results may be due to compensatory upregulation of guanidinoacetate synthesis in the pancreas, to different degrees of depression of urinary guanidinoacetate excretion, to unknown effects of peritoneal or hemodialysis, and/or to different stages of disease progression.

Similarly conflicting results were obtained for the serum concentration of Cr in uremic patients. It was found to be increased (163, 165, 412, 598, 757, 877), unchanged (165, 598), or even depressed relative to control subjects (168). The latter finding may be due to dialysis of these patients, which was shown to decrease the serum concentration of Cr (169, 877). Both the erythrocyte concentration of Cr (even after hemodialysis) and the urinary excretion of Cr may be increased in uremia (77, 412, 877), although in one study decreased urinary Cr clearance was observed (598). In striated muscle of uremic patients, the concentrations of PCr and ATP are decreased (716), whereas those of Cr and P_i are increased (99), thus suggesting that intracellular generation of high-energy phosphates is impaired.

The most consistent, and clinically most relevant, findings are an increase in the serum concentration and a decrease in the renal clearance of Crn with the progression of renal disease. Crn clearance (C_{Crn} ; in ml/min) is defined as

$$C_{Crn} = \frac{U_{Crn} \times V}{P_{Crn}}$$

where U_{Crn} and P_{Crn} are the urine and serum concentrations of Crn, respectively, and V is the urine flow rate (in ml/min). Both the serum concentration of Crn and Crn clearance have been, and still are, widely used markers of renal function, in particular of the glomerular filtration rate (GFR). The validity of this approach critically depends on the assumptions that Crn is produced at a steady rate, that it is physiologically inert, and that it is excreted

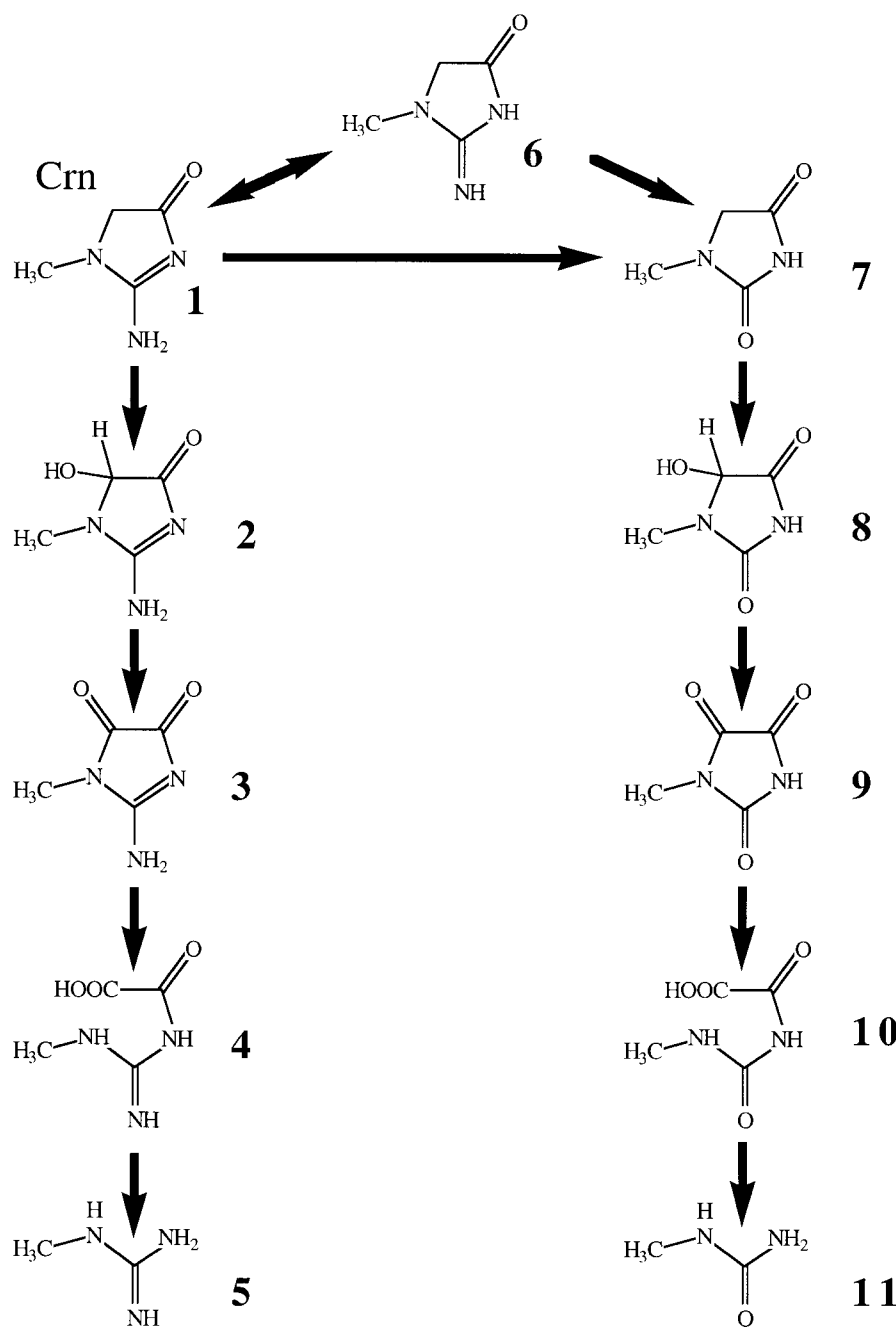
solely by glomerular filtration in the kidney. In recent years, these assumptions were shown to be invalid under uremic conditions, and several factors have been identified that may result in gross overestimation of the GFR (see, e.g., Refs. 108, 358, 536, 758, 791). For example, an increasing proportion of Crn in CRF is excreted by tubular secretion rather than glomerular filtration.

Another factor contributing to the overestimation of the GFR seems to be degradation of Crn in the human and animal body. Jones and Burnett (438) and Walser and co-workers (652, 1085) in fact showed, by calculating Crn balances, that 16–66% of the Crn formed in patients with CRF cannot be accounted for by accumulation in the body or by excretion in urine or feces. The most likely explanation for this apparent “Crn deficit” or “extrarenal Crn clearance” is Crn degradation. Whereas the normal renal Crn clearance is ~120 ml/min, the renal and extrarenal Crn clearances in CRF patients were calculated to be ~3–5 and 1.7–2.0 ml/min, respectively. Therefore, Crn degradation may be negligible in healthy individuals, which led to the postulate that Crn is physiologically inert, but it may become highly relevant under conditions of impaired renal function.

Several pathways for Crn degradation have to be considered. 1) Up to 68% of the metabolized Crn may be reconverted to Cr (652). To this end, Crn is most likely excreted into the gut where it is converted by bacterial creatininase to Cr which, in turn, is retaken up into the blood (“enteric cycling”) (439). This pathway may be a powerful means for limiting Crn toxicity (see below) and may also explain in part why the serum concentration of Cr is increased in many patients with CRF.

2) Bacterial degradation of Crn in the gut may not be limited to the conversion to Cr but may proceed further. 1-Methylhydantoin, Cr, sarcosine, methylamine, and glycolate were identified as degradation products when rat and human colon extracts or feces were incubated with Crn (439, 745). Upon incubation of colon extracts with radioactively labeled 1-methylhydantoin, however, no decomposition products were observed. These findings suggest at least two independent Crn degradation pathways: a) $Crn \rightarrow$ 1-methylhydantoin, catalyzed most likely by bacterial Crn deaminase; and b) $Crn \rightarrow Cr \rightarrow$ urea + sarcosine \rightarrow methylamine + glyoxylate \rightarrow glycolate, with the first two steps probably being catalyzed by bacterial creatininase and creatinase (see also Fig. 7). Last but not least, *Pseudomonas stutzeri*, which may be present in human gut, produces MG when incubated with Crn, both under aerobic and anaerobic conditions (1049). Accordingly, rat colon extracts proved to convert Crn to MG (437).

In support of these Crn degradation pathways, creatininase activity was recently shown to be increased considerably in the feces of patients with CRF (204). Crn degradation was lower in stool isolates of CRF patients



Methylguanidine

Methylurea

previously treated with antibiotics, or when the stool isolates themselves were incubated with antibiotics. Duodenal intubation demonstrated small bowel bacterial overgrowth associated with high concentrations of toxic methylamines. Consequently, CRF is accompanied by accumulation of Crn-degrading bacteria in the gut and/or by induction of creatininase activity in these bacteria.

3) A good deal of convincing evidence has been obtained for two oxidative Crn degradation pathways,

with the first leading to the formation of MG and the second to methylurea (Fig. 18). MG is not to be regarded as a metabolic end product, but may be degraded further (1145).

Both in vivo and in vitro studies have shown that Crn is converted to MG, with creatol, creatone A, and creatone B representing consecutive intermediates in this pathway (25, 271, 437, 687, 756). ROS, and in particular the hydroxyl radical, strongly stimulate the formation of

FIG. 18. Oxidative Crn degradation pathways favored in uremia and/or in inflamed skin tissue: 1) Crn; 2) creatol; 3) creatone A; 4) creatone B; 5) methylguanidine; 6) tautomer of Crn; 7) 1-methylhydantoin; 8) 5-hydroxy-1-methylhydantoin; 9) methylparabanic acid; 10) N⁵-methyloxaluric acid; 11) methylurea.

MG out of Crn (see Ref. 25). Accordingly, MG production was increased in vivo by hyperbaric oxygen therapy (979) and inhibited by superoxide dismutase (677), magnesium lithospermate B, as well as by tannin-containing rhubarb and green tea extracts that are thought to reduce the level of ROS (see Refs. 1141, 1142). Because *N,N'*-dimethylthiourea, an efficient hydroxyl radical scavenger, had no effect in normal rats on the conversion of creatol to MG, whereas it inhibited production of creatol and MG out of Crn in a dose-dependent manner (272, 1146), it may be assumed that ROS selectively affect the conversion of Crn to creatol. In vitro experiments contradict this view, in as far as the conversion of creatol to MG was stimulated by Fenton's reagent, which provides ROS (686).

Considerable uncertainty still exists on whether the individual steps of the pathway are enzyme catalyzed or not. In vitro studies revealed that the whole reaction cascade may proceed nonenzymatically (271, 294, 686). On the other hand, it might be anticipated that individual steps are catalyzed by bacteria in the intestinal tract (437). However, no difference in MG production and urinary excretion was observed between control and germ-free or antibiotic-treated rats (756).

Nagase et al. (684) provided evidence that rat liver, kidney, lung, muscle, red blood cells, and the gut flora synthesize MG. Control experiments on isolated rat hepatocytes revealed that nonenzymatic production can account for only a small proportion of MG synthesis from Crn in this tissue. Accordingly, rat liver peroxisomal enzymes were shown to catalyze the conversion of Crn to MG (978), and rat liver (microsomal) L-gulono- γ -lactone oxidase was identified to catalyze the reaction creatol + O₂ → creatone A + H₂O₂ (271). Although the *K_m* of this flavoprotein is 5 μ M for L-gulono- γ -lactone, but 12.8 mM for creatol, the *V_{max}* values for both substrates are similar. In rat kidney lacking L-gulono- γ -lactone oxidase, another flavoprotein, long-chain L-2-hydroxy acid oxidase, was shown to oxidize creatol to produce MG (see Ref. 746). Even though oxygen is consumed in this reaction, no H₂O₂ is liberated. In accordance with the lack of L-gulono- γ -lactone oxidase in both guinea pigs and primates including humans, evidence for nonenzymatic production of MG by human liver microsomes has been obtained (271).

An analogous reaction cascade results in the formation of methylurea out of Crn. 1-Methylhydantoin and 5-hydroxy-1-methylhydantoin were identified in the urine of uremic patients and rats and were shown to be derived from Crn (396, 686). Furthermore, upon oral administration of 1-methylhydantoin to rats, 5-hydroxy-1-methylhydantoin, methylparabanic acid, *N*⁵-methyloxaluric acid, and methylurea were detected in the urine (395). Because 1-methylhydantoin and 5-hydroxy-1-methylhydantoin were also identified in rabbit skin inflamed by vaccinia virus inoculation, but not in normal skin (394), the first step of this pathway, the conversion of Crn to 1-methyl-

hydantoin (Fig. 18, 1 → 7) may depend on bacterial Crn deaminase rather than on a nonenzymatic mechanism (Fig. 18, 1 → 6 → 7).

Even though the two oxidative Crn degradation pathways (Fig. 18, 1 → 5 and 7 → 11) seem to proceed via an identical reaction sequence and may both be stimulated by ROS, the urinary excretion of creatol (2) is threefold increased in uremic rats compared with normal controls, whereas that of 5-hydroxy-1-methylhydantoin (8) is four- to fivefold lower (396). The reason for this apparent inconsistency is unknown.

4) Some further Crn degradation pathways were proposed but have not been characterized in detail so far. Guanidinoacetate seems to be converted to guanidine in vivo (638, 684, 980). In normal rabbits, partial conversion of Crn to GBA was observed, whereas in a rabbit with decreased Crn clearance, GPA and Arg were suggested to be derived from Crn (83). Furthermore, in the presence of ROS in vitro, Crn was oxidized to glycoyamidine (688). Finally, Orita et al. (736), using ¹⁵N-labeled Arg, provided evidence for two distinct pathways for MG formation, with Crn acting as an intermediate in one of these pathways but not in the other. Several groups have shown, however, that this is actually not the case or that, at least, the direct formation of MG out of Arg is quantitatively irrelevant (294, 638, 684, 756, 1144).

As far as the serum and tissue concentrations as well as the urinary excretion rates of the potential Crn degradation products are concerned, they consistently indicate that the production of MG and creatol is increased in uremia (for references, see Refs. 21, 168, 169, 396, 479, 554, 598, 687, 722, 747, 1143). In CRF rats relative to controls, the concentration of MG was increased 3- to 18-fold in serum, blood cells, liver, muscle, colon, and kidney (735; see also Ref. 39). In brain, on the other hand, MG concentration was increased only twofold, indicating limited permeability of the blood-brain barrier for MG. The same conclusion can be drawn from experiments on the effects of intraperitoneal injection of guanidino compounds into rats, which suggested a low permeability of the blood-brain barrier for MG, Crn, and guanidinosuccinic acid (GSA) (1144, 1145, 1147). Guanidine, 4-GBA, and GPA may be increased in serum and cerebrospinal fluid of uremic patients (163, 165, 168, 757) as well as in serum, heart, skeletal muscle, brain, liver, kidney, and intestine of rats and mice with acute or chronic renal failure (10, 39, 554).

Crn and its degradation products are likely to be of critical importance with regard to uremic toxicity. Creatol and MG, in contrast to Crn, (further) deteriorate renal function when administered to normal rats or to rats with CRF (1147–1149). Mongrel dogs chronically intoxicated with MG, at concentrations similar to those in plasma of uremic patients, displayed many functional and pathological changes characteristic of uremia such as increased

catabolism, anemia, a decrease in the circulating platelet count, central and peripheral neuropathy, pruritus, arrhythmias and myocardial degeneration, congestion, and, in later stages of intoxication, anorexia, vomiting, increased salivation, and diarrhea (see Ref. 53). In vitro studies have shown that MG has a variety of potentially toxic effects by inhibiting, for example, mitochondrial oxidative phosphorylation, growth of cultured cells, or $\text{Na}^+\text{-K}^+\text{-ATPase}$ in the brain (for references, see Refs. 168, 735, 1147) or by affecting membrane fluidity (1138). Furthermore, MG, which is also present in various foods (for references, see Refs. 223, 294, 685), may be nitrosated intragastrically and may thereby become a potent mutagen (223) (see also sect. IXF).

Recently, MG was identified as a nonselective reversible inhibitor of NOS isoenzymes (see Refs. 341, 581, 930, 931, 1004, 1114). The three major isoenzymes of NOS, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), were inhibited with IC_{50} values of 90–370 μM (931, 1114). By inhibiting eNOS, intravenously administered MG increased mean arterial blood pressure in rats, thus raising the hypothesis that MG may contribute to the hypertension seen in patients with CRF (929, 930). Similarly, by inhibiting NOS in kidney and brain, MG may have an impact on the regulation of glomerular capillary pressure and on neurological functions, respectively.

Guanidino compounds including Cr, Crn, guanidine, MG, GAA, GPA, GBA, and GSA were also shown to have a series of other, potentially harmful, side effects (see also sect. VIII B). Insulin resistance is a common finding in CRF. Accordingly, insulin binding to erythrocyte receptors was shown to be decreased in uremic patients, but increased rapidly upon hemodialysis (814). Binding of insulin to erythrocytes is also depressed by 1 mM concentrations of Crn, Cr, and guanidinoacetate. Plasma of uremic patients as well as α - and β -GPA inhibit the hexose monophosphate shunt in red blood cells (453). GPA, GBA, GAA, and GSA inhibit the phytohemagglutinin-induced stimulation of proliferation of normal human lymphocytes (878). This inhibitory action may possibly explain the depression in total lymphocyte count and function as well as the greatly depressed cell-mediated response to protein antigens in uremia. Finally, guanidine, MG, Crn, Cr, GSA, and GBA have convulsive effects in animals and may thus contribute to the neurological symptomatology in uremia (see sect. IX G).

In conclusion, Crn degradation seems to be virtually irrelevant under normal conditions when Crn might even have a beneficial effect by acting as a hydroxyl radical scavenger. At greatly reduced GFR, on the other hand, when the serum concentration of Crn as well as oxidative stress are considerably increased, the formation of toxic Crn degradation products is favored and may contribute significantly to further disease progression. Because Crn

degradation is stimulated by ROS and in particular by the hydroxyl radical, the serum concentrations of creatol and MG as well as the creatol/Crn and MG/Crn ratios may not only serve as diagnostic indexes for the degree of CRF, but may also be used as measures of oxidative stress in uremic patients (25, 683, 687, 747, 1146).

XI. ANALYTICAL METHODS AND THEIR IMPLICATIONS FOR CLINICAL DIAGNOSIS

Ever since the suggestion of Popper and Mandel in 1937 (779) that the clearance of endogenous Crn approximates the GFR, investigation of serum and urinary [Crn] has been popular in clinical medicine. Even though the parallelism between Crn clearance and GFR turned out to be less strict than previously presumed (see sect. IX H), there is still a broad interest in improving analytical methods for measuring [Crn] and [Cr] in biological samples.

For many decades, chemical methods prevailed (551, 758, 932, 1071). Crn has been, and still is, measured mostly by the Jaffé reaction (422) in which Crn and picric acid under alkaline conditions form an orange-red-colored complex. Despite considerable effort, the detailed mechanism of color formation could not yet be resolved (see Refs. 551, 932, 958). The major disadvantage of the Jaffé reaction is its lack of specificity due to interference by a variety of metabolites, e.g., ketones and ketoacids, protein, bilirubin, and cephalosporins (e.g., Refs. 71, 443, 635, 926, 932, 958, 1071, 1110). Although a large number of modifications and improvements of the Jaffé reaction were proposed, none has eliminated all interferences. This disadvantage was outscored in the past by the usefulness of the Jaffé reaction in the clinical environment, by the lack of valid alternatives, and, in particular, by the low price of the required chemicals. Cr was frequently determined chemically by the α -naphthol-diacetyl reaction (1117), but this method is rather cumbersome and nonspecific as well.

Already in 1937, using crude bacterial extracts, Miller and Dubos managed to estimate enzymatically the Crn content of plasma and urine (see Ref. 55). But it is only in recent years that significant progress has been made toward developing and improving enzymatic Cr and Crn determination methods (see Refs. 758, 862, 926, 932). In keeping with the diversity of Crn degradation pathways (see Fig. 7), a series of alternative reaction sequences was proposed to be specific and accurate. 1) A first method is based on creatininase, creatinase, sarcosine oxidase, and a peroxidase. The hydrogen peroxide liberated in the sarcosine oxidase reaction is used by the peroxidase to produce a colored substance that can be measured spectrophotometrically or fluorimetrically (for references, see Refs. 432, 443, 926, 958, 1110). 2) In the reaction sequence of creatininase, CK, pyruvate kinase, and lactate dehydro-

genase, degradation of Crn (or Cr) is coupled stoichiometrically to the oxidation of NADH to NAD⁺, which can be measured spectrophotometrically (65, 80, 926, 1110). 3) Some Crn deaminase-based methods involve quantitation of the liberated ammonia either in a bromophenol blue indicator reaction (for references, see Ref. 758) or via glutamate dehydrogenase. In the latter case, Crn degradation is coupled stoichiometrically to NADPH oxidation (258, 333). These Crn deaminase-based methods have the advantage of comprising only two steps which may limit the risk of interferences. On the other hand, they involve quantitation of a volatile substance, ammonia, which might have a negative impact on the accuracy of the methods. 4) The last procedure involves five steps and, thus, is the most intricate one: Crn deaminase, 1-methylhydantoin amidohydrolase, *N*-carbamoylsarcosine amidohydrolase, sarcosine oxidase, and a peroxidase (715, 893). The second part of this sequence is, evidently, identical to that of *method 1*.

Even though enzymatic methods are deemed to be quite specific, they are still not free from interferences. For example, peroxidase detection systems suffer from bilirubin or ascorbate interference, thus necessitating incorporation of potassium ferrocyanide, bilirubin oxidase, and/or ascorbate oxidase in the assay (e.g., Refs. 70, 432, 715, 862, 926, 932, 1094). On the other hand, cytosine derivatives such as the antibiotic 5-fluorocytosine may act as substrates of Crn deaminase (see sect. VII G), a problem to be circumvented by the use of a more specific Crn deaminase either obtained from a natural source (298) or engineered biotechnologically. Further disadvantages of the enzymatic methods are the potentially lower storage stability and the (still) much higher price relative to the Jaffé reaction.

It goes without saying that the enzymatic methods also have distinct advantages. Although it was not possible to eliminate the interference problem for the Jaffé-based methods despite considerable effort over several decades, comparative studies have shown that already now, enzymatic methods produce less outliers and, in general, display a better performance than the Jaffé methods (see Refs. 70, 71, 258, 333, 443, 958, 1110). Even more importantly, molecular engineering offers great promise for further improving the properties of the enzymatic methods. For instance, mutagenesis approaches gave rise to more stable creatinase and sarcosine oxidase mutants (498, 709, 869, 871), and it is expected that more elaborate molecular design principles will allow us in the future to engineer enzymatic Crn and Cr determination methods virtually devoid of interference problems.

HPLC (see, e.g., Refs. 43, 111, 1137, 1158), capillary electrophoresis (186, 1015), and biosensors with immobilized enzymes (582) represent alternative approaches for Cr, PCr, and Crn determination that may be less prone to interference problems than the Jaffé methods. Improved

analytical tools for Crn and Cr determination are likely to find wide application not only in the diagnosis and monitoring of kidney function, but also in investigations and clinical evaluation of energy metabolism in various bodily tissues. Moreover, they may be instrumental in monitoring the efficacy of Cr supplementation in sports physiology (see sect. XI) and in the treatment of diseases responsive to Cr therapy. Even though, for instance, the nonspecific Sakaguchi reaction has been suggested to be useful for the screening of GAMT deficiency (867), more specific enzymatic tests for the quantitation of guanidinoacetate and/or Cr are likely to facilitate and improve the accuracy of such screenings.

XII. CREATINE SUPPLEMENTATION IN SPORTS PHYSIOLOGY

With increasing commercialization of the sports business, improving muscle performance by any means has become a critical issue. The efficacy of carbohydrate loading is widely accepted. Carbohydrates can be consumed in sufficient amounts via natural foods. On the other hand, stimulating muscle growth by, e.g., androgens is banned, since unphysiologically high concentrations of these hormones are required. Over the last few years, Cr supplementation as an ergogenic aid has boomed tremendously (for reviews, see Refs. 46, 103, 128, 305, 441, 674, 829, 1106). The success of British sprinters and hurdlers at the beginning of the 1990s has been associated with Cr supplementation (see Ref. 1106). Nowadays, there is widespread enthusiasm about the performance-boosting effects of Cr, which is seemingly used by many top athletes in explosive sports disciplines. Cr supplementation is popular, for example, among bodybuilders, wrestlers, tennis players, cyclists, mountain bikers, rowers, ski-jumpers, or cross-country skiers as well as among ski, rugby, handball, basketball, football, and ice hockey teams. It is not the principal goal of this section to share the widespread enthusiasm, but to critically discuss the scientific data published so far on Cr supplementation and its effects on muscle performance.

During high-intensity exercise, ATP hydrolysis is initially buffered by PCr via the CK reaction. Whereas PCr is available instantaneously for ATP regeneration, glycolysis is induced with a delay of a few seconds, and stimulation of mitochondrial oxidative phosphorylation is delayed even further. On the other hand, the PCr stores in muscle are limited so that during high-intensity exercise, PCr is depleted within ~10 s. Therefore, if it were possible to increase the muscle stores of PCr and thereby to delay PCr depletion, this might favorably affect muscle performance.

The normal muscle concentration of total Cr is ~125 mmol · (kg dry mass)⁻¹ (46). As early as in the 1920s, oral

Cr supplementation was recognized to increase the total Cr concentration in muscle (for references, see Refs. 144, 305, 368). Nowadays, Cr supplementation most commonly involves a "loading phase" of 5–7 days, where 20–30 g of Cr monohydrate are consumed per day, and a "maintenance phase" with 2–5 g Cr/day. These values must be compared with a daily Cr requirement of only 2 g (provided through the diet or by de novo biosynthesis) and with a Cr content of raw meat of ~4–5 g/kg. Because, in addition, Cr is partially converted to Crn during cooking, it goes without saying that it is virtually impossible by natural means to ingest 20 g Cr/day.

Cr loading with 20–30 g/day was shown to significantly increase the muscle concentrations of Cr, PCr, and total Cr while having no effect on ATP concentration (for reviews, see Refs. 674, 1106; see also Refs. 918, 1040, 1041). Large interindividual differences were noted, both as far as initial Cr concentration and responsiveness to Cr supplementation are concerned. Some subjects, particularly those with a high initial muscle concentration of Cr, did not respond to Cr supplementation. On the other hand, the greatest increase in total Cr content, e.g., from 114 to 156 mmol · (kg dry mass)⁻¹, was observed in subjects with a low initial Cr content (307, 338). Muscle concentrations of total Cr remain elevated for at least 30 days or 10 wk when supplementation is continued at a rate of 2 or 5 g/day, respectively (387, 1040). A similar increase in total muscle Cr concentration was achieved by Cr supplementation over 28 days at a rate of 3 g/day, suggesting favorable effects also in the near-physiological range (46, 387). Upon discontinuation of Cr supplementation, a 28-day washout period was sufficient to return the Cr and PCr concentrations to baseline values (232, 387, 1040).

Resting PCr concentration in the quadriceps muscle was found to be lower in middle-aged (58 ± 4 yr) compared with young subjects [30 ± 5 yr; 35.0 vs. 39.5 mmol · (kg wet wt)⁻¹], and middle-aged subjects also showed a lower PCr resynthesis rate between exercise bouts (913). Cr supplementation (0.3 g · kg⁻¹ · day⁻¹ for 5 days) increased both resting PCr concentration and PCr resynthesis rate more in the middle-aged than in the young group, so that the values became identical. In addition, Cr supplementation significantly increased time to exhaustion in both groups combined from 118 to 154 s. These results suggest that the ergogenic potential of Cr supplementation even increases with age.

Cr supplementation increased the urinary excretion of Crn, which is in line with elevated muscle concentrations of Cr and PCr (145, 225, 387, 823, 1040, 1041). Upon discontinuation of Cr ingestion, urinary Crn excretion slowly returned to the presupplementation value.

A few studies addressed the question of whether Cr accumulation can be influenced by dietary or other factors. In fact, exercise further stimulates Cr uptake into

muscle. When subjects performed 1 h of strenuous exercise per day with only one leg, Cr supplementation increased the mean muscle Cr content from 118 to 149 mmol · (kg dry mass)⁻¹ in the control leg, but to 162 mmol · (kg dry mass)⁻¹ in the exercised leg (338; see also Ref. 522). Supplementation and exercise resulted in a total Cr content in one subject of as much as 183 mmol · (kg dry mass)⁻¹, suggesting that the upper limit of total muscle Cr concentration of 155–160 mmol · (kg dry mass)⁻¹ proposed by some authors (see Refs. 46, 305, 441, 674, 1106) can be surpassed at least under certain conditions. Simultaneous ingestion of relatively large amounts of carbohydrates (glucose and simple sugars) also augments Cr retention in muscle (302, 303). This effect seems to be mediated indirectly by insulin, which increased in plasma almost 20-fold within 20 min of carbohydrate ingestion (see also Ref. 943). In vitro and in vivo work on rat skeletal muscle, mouse myoblasts, and humans suggests that insulin may stimulate Cr uptake (see sect. IVB). That the composition of the supplement may in fact be critical for the ergogenic effect of Cr has been demonstrated in a study showing no significant (or considerably smaller) improvements in exercise performance with Cr supplementation alone; however, addition of glucose, taurine, and electrolytes to the Cr supplement promoted significant increases in both one repetition maximum bench press and vertical jump performance, as well as a decrease in 100-yard sprint times (952). It should be noted that the ergogenic action of Cr seems to be counteracted by high-dose ingestion of Cr during exercise that also resulted in postexercise distress and even syncope (1038).

Not all of the ingested Cr can be retained in the body. While a high proportion of Cr is usually retained in the initial days of Cr supplementation, urinary Cr excretion progressively increases with continued ingestion of Cr (62, 116, 338, 583, 823, 1040). Urinary excretion of Cr can, however, be reduced significantly by simultaneous ingestion of carbohydrates (302, 303). Because guanidinoacetate and Cr compete for the same reabsorption mechanism in the kidney, Cr supplementation also stimulates urinary excretion of guanidinoacetate (368). Finally, as discussed in sections IVA and IXA, an increase in the plasma concentration of Cr, from a normal level of ~25–100 μM to up to 1.8 mM 1 h after Cr supplementation (303, 338, 776, 1035), will downregulate endogenous Cr biosynthesis by repressing AGAT expression. This downregulation is fully reversible (see Ref. 1077).

In most studies, Cr supplementation was accompanied by a significant increase in body weight. Although in most cases this weight gain was in the range of 1–2 kg, higher values of 3–5 kg have occasionally been reported (for reviews, see Refs. 442, 674, 1106; see also Refs. 116, 302, 303, 387, 418, 583, 627). The underlying basis for this weight gain is still unclear. It may be due to stimulation of muscle protein synthesis (255, 406, 1153) or to increased

water retention in the initial days of Cr supplementation (387, 442). Because the proportion of fat tended to decrease and lean tissue weight increased with Cr supplementation (209, 312, 510, 511, 952, 1040), the increase in body weight most likely reflects a corresponding increase in actual muscle mass and/or volume, a point that is particularly relevant for bodybuilders.

Because the contribution of PCr hydrolysis to ATP regeneration is expected to be most relevant in (supra-) maximal exercise, in particular at the time when [PCr] normally becomes limiting (365, 386), a favorable effect of Cr supplementation on muscle performance is most likely in short-term high-intensity exercise. On the other hand, it is less evident how Cr supplementation should improve endurance performance. In fact, a variety of studies have shown an improvement in muscle performance upon Cr supplementation mostly in high-intensity, short-duration, repetitive exercise tests, but to some extent also in exercise tests of intermediate duration of 30–300 s (for reviews, see Refs. 441, 1106; see also Refs. 1, 225, 336, 583, 627, 911, 913, 952, 1038, 1041). In series of 10 repeated 6-s bouts of high-intensity exercise on a cycle ergometer interspersed with 30-s passive rest periods, subjects were better able to maintain a pedal speed of 140 rev/min after a 6-day period of Cr supplementation (30 g/day) than after placebo ingestion (Fig. 19) (44). In a protocol of 6×6 s of repeated cycle sprints departing every 30 s, Cr supplementation (20 g/day over 5 days) significantly increased total work completed over the six sprints, work performed in *sprint 1*, as well as peak power (162). In a series of two or three 30-s bouts of maximal isokinetic

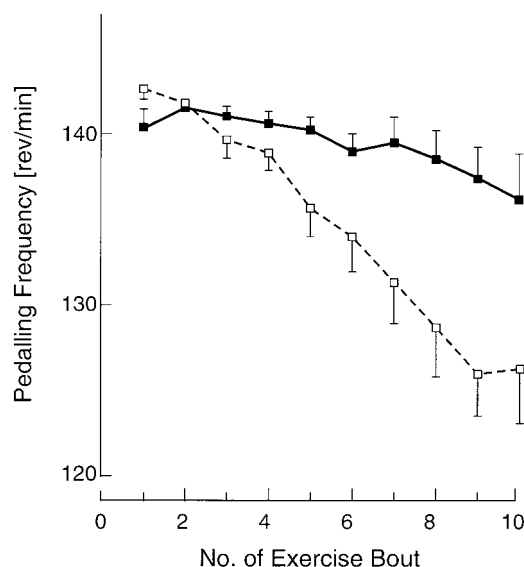


FIG. 19. Effect of Cr supplementation on muscle performance in 10 6-s bouts of high-intensity cycling at an intended frequency of 140 rev/min. Mean rev/min for the interval 4–6 s of each individual exercise bout are shown before (□) and after (■) 6 days of Cr supplementation. [Modified from Balsom et al. (44).]

cycling, separated by 4 min of recovery, Cr ingestion at a rate of 20 g/day for 5 days significantly increased peak and mean power output in *exercise bout 1* as well as total work output in *bouts 1* and *2*, all by 4–8% (66, 113). The increases in both peak and total work production over the two exercise bouts in the study of Casey et al. (113) were positively correlated with the increase in muscle concentration of Cr plus PCr. Cr supplementation (20 g/day for 5 days) increased the performance in a maximal continuous jumping test by 7% during the first 15 s and by 12% during the second 15 s (84). In the same study, Cr supplementation also improved the time of intensive running up to exhaustion in an all-out treadmill run lasting ~60 s by 13%. Similarly, time to exhaustion of the leg at 80, 60, 40, and 20% of the maximum voluntary force of contraction (lasting ~20–160 s) was significantly increased in resistance trained subjects by Cr supplementation (10 g/day for 5 days); in addition, Cr supplementation increased maximum voluntary force of contraction by ~10% (583). In a study on a limited number of weight-trained subjects, performance in the Wingate bike test was improved 15–23% (209). Muscular strength, as evaluated by a one repetition maximum (1 RM) free weight bench press test, was increased 6.5%, and the number of lifting repetitions at 70% of the respective 1 RM load even by 35%. Body weight and calculated fat free mass were significantly increased by Cr supplementation in these weight-trained athletes despite a significantly lower daily energy, carbohydrate, and fat intake. No explanation is currently available for this latter finding. In resistance-trained men, Cr supplementation (25 g/day for 6 days) resulted in a significant increase in muscle performance during multiple sets of bench press (higher number of repetitions until exhaustion at 10 RM) or jump squat exercise (increased peak power output) (1068). In a protocol involving three interval series of 3×30 , 4×20 , and 5×10 maximal voluntary contractions of the knee extensors on an isokinetic dynamometer, with rest intervals of 2 min between series and 20–60 s between individual bouts, dynamic torque production was increased 10–23% after 6 days of Cr ingestion ($0.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) (1039). This ergogenic action of Cr was, however, completely abolished by simultaneous caffeine intake, even though caffeine had no effect on the stimulation of Cr accumulation into muscle in response to Cr supplementation. In trials of 4×300 -m or $4 \times 1,000$ -m runs with 3- and 4-min rest intervals between repetitions, Cr supplementation (30 g/day for 6 days) significantly reduced the running times for the final 300-m or 1,000-m runs as well as the total time for $4 \times 1,000$ m (339). Best 300-m and 1,000-m times in this series decreased significantly by 0.3 ± 0.1 and 2.1 ± 0.6 s, respectively. Cr supplementation (20 g/day for 5 days) significantly increased the work performed in three maximal kayak ergometer tests of 90-, 150-, and 300-s duration by 7–16% while having no effect on peak power (627). Finally, Cr

supplementation at a rate of $0.25 \text{ g} \cdot (\text{kg body mass})^{-1}$ over 5 days significantly improved the 1,000-m performance of competitive rowers by an average of 2.3 s (823). Regression analysis revealed a positive relationship of borderline statistical significance between performance and Cr uptake.

It should not be ignored that some conflicting reports suggested no improvement in muscle performance with Cr supplementation (for a review, see Ref. 1106; see also Refs. 62, 141, 426, 918, 1035). Several explanations can be put forward for the discrepant findings which, however, will have to be tested: e.g., too short or too long (possibly causing downregulation of the Cr transporter) a period of Cr supplementation, insufficient daily Cr dosage, or a high proportion of nonresponders among the subjects analyzed. In the study by Cooke et al. (142), a potentially favorable effect of oral Cr supplementation on power output during bicycle ergometry may have been masked by the high standard errors of the data. The lack of a favorable effect of Cr supplementation on supramaximal running on a motor-driven treadmill for 3–6 min until exhaustion, in a 6 km terrain run (45), in 700-m maximal running bouts (993) and in a series of 1-min supramaximal cycling bouts (232) may be due to the longer duration of exercise which inevitably results in a decrease in the relative contribution of PCr hydrolysis to total work output. Notably, Cr supplementation even resulted in a significant increase in the run time for the 6-km terrain run (45). The absence of a favorable effect of Cr supplementation on repeated 10-s cycle ergometer sprint performance (51) or on 60-m sprint performance (798) lacks an explanation and is in direct contrast to the studies that have shown an ergogenic effect of Cr supplementation in high-intensity, intermittent exercise (see above).

Inconclusive data have been published on the effect of Cr supplementation on swimming performance. An improvement in performance parameters was seen in some studies (312, 765), but not in others (103, 673, 999). In the study of Thompson et al. (999) on trained swimmers, the daily ingestion of only 2 g Cr for 14 days may have been insufficient to increase the muscle stores of total Cr. On the other hand, the tendency toward worse 25- and 50-m performance times of competitive swimmers after Cr supplementation (20 g/day over 5 days) was suggested to be due to an increase in hydrodynamic resistance which is associated with the body weight gain caused by Cr ingestion (103, 441, 673). In elite competitive male swimmers, Cr supplementation (9 g/day for 5 days) had no effect on performance in a single 50-yard sprint but significantly improved performance in a repeated sprint set of 8×50 yards at intervals of 90 s (765). A study on junior competitive swimmers performing three 100-m freestyle sprint swims with 60-s recovery between heats indicated that Cr supplementation (21 g/day for 9 days) may improve performance already in *heat 1* relative to the

placebo group and decreases swim time in *heat 2* (312). In conclusion, the results published so far suggest that Cr supplementation has either no or even an adverse effect on single-sprint swim performance but may have a slight, favorable effect on repetitive swimming performance.

Cr supplementation (20 g/day for 5 days) caused an acceleration of PCr recovery after intense electrically evoked isometric contraction of the vastus lateralis muscle in humans (Fig. 20) (306, 307; see also Refs. 47, 913). The most pronounced effects were observed in those subjects in whom Cr supplementation caused the largest increase in muscle Cr concentration. Both Bogdanis et al. (75) and Trump et al. (1017) demonstrated that PCr resynthesis during recovery from intense muscular activity is critical to the restoration of muscle power at the onset of a next bout of maximal exercise. In a test protocol consisting of two 30-s cycle ergometer sprints interspersed with 4 min of recovery, a high correlation was seen between the percentage of PCr resynthesis and the percentage recovery of power output and pedalling speed during the initial 10 s of *sprint 2* (75). Consequently, an increased rate of PCr recovery after Cr supplementation

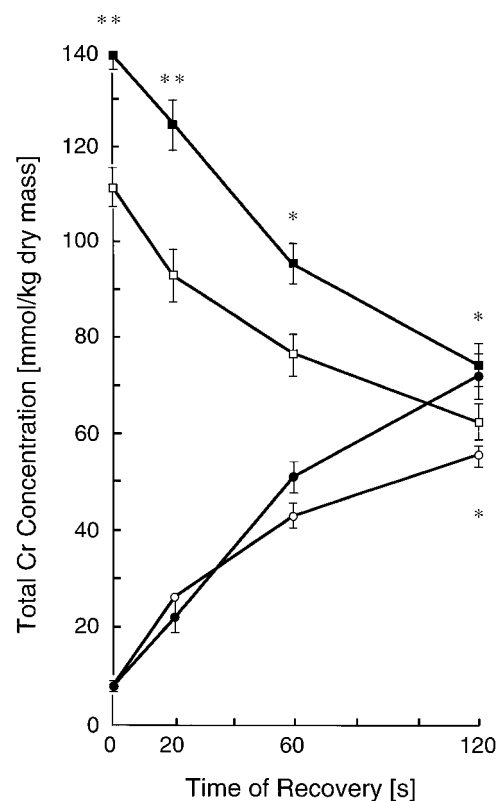


FIG. 20. PCr resynthesis (○, ●) and accompanying decrease in free Cr concentration (□, ■) in human muscle biopsy samples obtained after 0, 20, 60, and 120 s of recovery from intense, electrically evoked isometric contraction before (○, □) and after (●, ■) 5 days of Cr ingestion. Only subjects with an increase in total Cr concentration in vastus lateralis muscle of $\geq 19 \text{ mmol} \cdot (\text{kg dry mass})^{-1}$ after Cr ingestion demonstrated an accelerated rate of PCr resynthesis. * $P < 0.05$. ** $P < 0.01$. [From Greenhaff et al. (307).]

may be relevant for muscle performance during high-intensity, intermittent exercise and might allow harder training units, mostly in explosive sports disciplines, but possibly also in endurance sports. Hespel and co-workers (1051, 1041), however, reported that in their studies, Cr supplementation (20–25 g/day for 2–5 days) had no effect on PCr resynthesis rate but accelerated muscle relaxation during intermittent brief isometric muscle contractions, which also may contribute to the ergogenic action of Cr. More research is required to solve this controversy.

To further study the metabolic effects of Cr supplementation, plasma ammonia and hypoxanthine concentrations were evaluated as measures of adenine nucleotide degradation, and plasma as well as muscle lactate concentrations as measures of anaerobic glycolysis. In short-duration, high-intensity, anaerobic exercise lasting <30 s, Cr supplementation sometimes decreased plasma accumulation of ammonia and hypoxanthine as well as the loss of muscular ATP during and after exercise, despite no change or even an increase in total work performed (see Refs. 113, 308, 441). In these exercise tests, blood lactate levels and accumulation of lactate in muscle were either decreased (see Refs. 47, 441) or not affected by Cr supplementation (see Refs. 1, 51, 308, 441, 765). On the other hand, Cr supplementation increased blood lactate accumulation in maximal kayak ergometer tests of 150- and 300-s duration where it was associated with a higher work performance (627), and in a treadmill run of 3–6 min until exhaustion, with this latter finding not being readily explainable (45). In a series of supramaximal 1-min cycling bouts, no changes were caused by Cr supplementation in the intramuscular concentrations of ATP, ADP, AMP, IMP, ammonia, glycogen, and lactate as well as in blood pH, lactate, and ammonia concentrations before and after exercise (232). During more prolonged, submaximal exercise, performed by continuous running at 10 km/h on a treadmill at predetermined workloads of 50, 60, 65, 70, 75, 80, and 90% of maximal oxygen uptake for 6 min each, as well as during subsequent recovery, Cr supplementation (20 g/day for 5 days) had no effect on respiratory gas exchange (oxygen consumption, respiratory exchange ratio, carbon dioxide production, or expired gas volume), blood lactate concentration, or heart rate (954).

In mildly hypertriglyceridemic and hypercholesterolemic subjects, Cr supplementation (20 g/day for 5 days, followed by 10 g/day for 51 days) reduced the plasma concentrations of total cholesterol, triacylglycerols, and very-low-density lipoprotein-C by 5–26% while having no effect on the concentrations of low-density lipoprotein-C, high-density lipoprotein-C, and Crn (208). In addition, Cr supplementation slightly but significantly increased plasma urea nitrogen and urinary uric acid excretion in women and showed a trend to decrease the blood glucose level in men (208, 1040).

In a study on the effect of intravenous injection of PCr (0.3 g at 24 h and 30 min before exercise) on the cycle ergometer performance of untrained volunteers, total work and anaerobic threshold were increased by 5.8–6.8% in a protocol involving a stepwise increase in physical exercise until exhaustion (1069). On the other hand, accumulation of lactate and lactate dehydrogenase in the blood was decreased. PCr administration also improved exercise tolerance during prolonged submaximal exercise at 70% of the individual's maximal oxygen uptake. Favorable effects of PCr administration on muscle performance were also observed in other investigations on trained cyclists, other athletes, or patients during recovery from leg immobilization (see Ref. 128). Whether intravenous injection of PCr and oral Cr supplementation share the same mechanism of action is at present unclear.

The studies on humans have recently been complemented by analogous experiments on rats. Cr supplementation for 10 days at a rate of $3.3 \text{ g} \cdot (\text{kg diet})^{-1}$ significantly increased the concentrations of Cr, PCr, and total Cr in both plantaris and soleus muscle, with no further increase when supplementation was continued for another 18 days (92). While having no effect on CK activity and myosin heavy chain distribution, Cr supplementation increased citrate synthase activity in soleus muscle of sedentary rats and, in combination with high-intensity run training, caused a modest hypertrophy as well as a 30% increase in citrate synthase activity in plantaris muscle. In both run duration (60 m/min, 15% inclination) and repetitive interval treadmill performance tests (30-s intervals of high-intensity running separated by 30-s recovery periods), Cr supplementation alone had a modest ergogenic effect. However, the combination of run training and Cr supplementation resulted in a marked enhancement of performance that may be due to an increase in both anaerobic (PCr stores) and aerobic capacity (citrate synthase activity). On the other hand, Cr supplementation (0.2 g daily for ~10 wk) decreased mitochondrial β -hydroxyacyl-CoA dehydrogenase activity in rat soleus muscle by 47% (983), and endurance performance was unchanged or tended to be compromised in Cr-fed rats (983, 1073, 1074). Cr supplementation ($400 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ subcutaneously for 7 days) increased the blood plasma concentration of urea, particularly in endurance-trained rats, but did not significantly affect glycogen levels in rat liver and skeletal muscle before or 24 h after exhaustive swimming (734; see also Ref. 983). In a preliminary study, excessive Cr supplementation (4% in the diet plus 50 mM in the drinking water) over an unrealistically long period of 3–6 mo seemed to decrease the expression of the Cr transporter in rat quadriceps muscle, whereas GPA administration (2.5% in the diet plus 1% in the drinking water) may have a slight opposite effect (317). Both an increased plasma urea level and decreased Cr transporter expression would be undesirable so that these aspects

have to be studied in detail in humans. It will be particularly relevant to investigate the kinetics of downregulation and reexpression of the Cr transporter during and after Cr supplementation. According to current knowledge, a "loading phase" of 5–10 days should be sufficient to obtain maximal benefit. Thereafter, the ration should be reduced considerably or even completely to allow reexpression of the Cr transporter and, thereby, to prepare the muscle for a next Cr loading phase.

To conclude, Cr supplementation may improve muscle performance in three different ways: by increasing the muscle stores of PCr which is the most important energy source for immediate regeneration of ATP in the first few seconds of intense exercise; by accelerating PCr resynthesis during recovery periods; and by depressing the degradation of adenine nucleotides and possibly also the accumulation of lactate during exercise. The results published so far provide both a rational explanation and promising results for an ergogenic action of Cr in intermittent, supramaximal exercise. On the other hand, there is hardly any indication so far that Cr supplementation might also increase endurance performance (see also Refs. 46, 225, 674, 1038, 1106). In addition to its application in humans, Cr supplementation may also be useful as an ergogenic aid for racing horses, racing camels, greyhounds, or huskies (e.g., Ref. 577).

Even though Cr supplementation is commonly regarded as safe, no proper clinical study has been conducted yet to evaluate the compound's safety profile in humans. Side effects of Cr supplementation have occasionally been described (for a review, see Ref. 442): mild asthmatic symptoms and gastrointestinal distress (208, 1038), muscle cramps and muscle strains (128, 985), and heat intolerance. Muscle cramps and heat intolerance may be related to the increased water retention in muscle during the initial days of Cr supplementation. During this period, therefore, subjects should take care to be properly hydrated and to avoid strenuous exercise (128, 442). This is especially true for subjects attempting to rapidly reduce body mass, e.g., in sports like wrestling, judo, karate, or weight-lifting where athletes often compete in a weight class below their usual body mass. As a matter of fact, Cr supplementation during the period of weight loss proved to even have an adverse effect on muscle performance (733). Anecdotal reports of adverse events to the Food and Drug Administration included rash, dyspnea, vomiting, diarrhea, nervousness, anxiety, fatigue, migraine, myopathy, polymyositis, seizures, and atrial fibrillation (Food and Drug Administration Special Nutritionals Adverse Event Monitoring System Web Report October 20, 1998; <http://vm.dfsan.fda.gov/cgi-bin/aems.cgi?QUERY=creatine&STYPE=EXACT>).

As already suggested in section IXA, subjects with impaired renal function and those at risk should avoid Cr supplementation. While in normal subjects Cr supplemen-

tation only slightly increases serum [Crn], a considerably more pronounced increase may be seen in patients with renal dysfunction that is potentially associated with an increase in Crn-derived uremic toxins (see sect. IXH). Accordingly, Cr supplementation had no effect on serum markers of hepatic and renal function as well as on routine clinical chemistry in healthy volunteers (208, 776), whereas Koshy et al. (507) reported on a 20-yr-old man developing interstitial nephritis while on Cr supplementation, and Pritchard and Kalra (786) described the case of a 25-yr-old man with focal segmental glomerulosclerosis where rapid deterioration of renal function was linked to Cr supplementation. Upon discontinuation of Cr supplementation, renal function parameters recovered. Clearly, both short- and long-term side effects of Cr supplementation must be studied more carefully in the future.

Despite the current popularity of Cr supplementation, it must be kept in mind that it is virtually impossible by natural means to ingest 20–30 g Cr/day. Therefore, and also based on the lack of proper investigations on the potential side effects of this compound and on its mechanism of action, discussions should continue on whether Cr supplementation is a legal ergogenic aid or whether it should be regarded as a doping strategy (605, 1106).

XIII. CONCLUSIONS AND PERSPECTIVES

As outlined in this review, research on Cr and Crn metabolism has received a fresh impetus in recent years. For example, the DNA sequencing and three-dimensional structure determination of enzymes involved in Cr metabolism—in particular the identification and characterization of the Cr transporter—the potential link of the CK/PCr/Cr system to a variety of diseases, together with the emerging potential therapeutic value of Cr analogs, the widespread use of Cr as an ergogenic dietary supplement, or the identification of cooked food mutagens derived from Cr and Crn have stimulated activities in many different areas of research. It seemed therefore timely to summarize the current knowledge to point out the many complex relationships between the different areas of Cr research, and thereby to provide a starting point for future multidisciplinary efforts to unravel the still existing mysteries associated with Cr and Crn.

Although, at the molecular level, many details are currently known, the integration and regulation of Cr metabolism in mammals is still only incompletely understood. It seems that the Cr transporter, a saturable uptake mechanism for Cr found in the plasma membrane of tissues unable to synthesize Cr, plays a central role in regulating intracellular Cr and PCr concentrations (sect. IV). Because this protein was discovered only in 1993 (319), research on its relevance for Cr metabolism is still

in its infancy. An interesting question to be addressed is the potential downregulation of the Cr transporter by extracellular Cr in both health and disease. Preliminary data suggest that such regulation could indeed exist (317, 571).

Despite being investigated for many years already, CK has just recently evoked renewed interest. The availability of CK knockout mice, the first description of a three-dimensional structure of a CK isoenzyme, as well as the potential regulation and inactivation of CK by AMP-activated protein kinase, NO and ROS represent major steps forward to more clearly understand the physiological functions of the CK/PCr/Cr system in health and disease (see sect. VII D). In addition, it has been realized that the CK and adenylate kinase systems can, to some extent, mutually replace each other in mediating intracellular transport of high-energy phosphates, which may explain why targeted disruption of CK genes in transgenic animals and other strategies directed at compromising the functional capacity of the CK/PCr/Cr system often have relatively mild effects.

The ergogenic effects of Cr have prompted its widespread use among athletes. Short periods of supplementation with high doses of Cr (20–30 g/day over 5–7 days) significantly improved work performance in a variety of short-term, intermittent, supramaximal exercise tests (see sect. XI). On the other hand, an improvement in endurance performance with Cr supplementation seems at present unlikely. Two questions in this area await clarification: 1) Is the increased short-term performance due to improved power indexes of the existing muscle mass, or simply to a stimulation of muscle growth? The latter possibility must be favored at present on the basis of the increase in body weight normally associated with Cr supplementation. 2) Should Cr supplementation be considered a natural ergogenic aid or an illegal doping strategy by the legal sports authorities? Although high amounts of Cr are tolerated by athletes, conclusions about the safety of Cr supplementation are still premature and await further studies. The rare and often anecdotal reports on potentially adverse side effects of Cr supplementation need to be studied carefully. Crn, which slightly increases in concentration upon Cr supplementation, is the precursor of a uremic toxin, methylguanidine (see sect. IX H). Cr and Crn, when present in food and cooked at high temperatures, seem to be precursors of food mutagens that are carcinogenic in different tissues of rodents and monkeys (see sect. IX F). Even though the relevance of these pathways may be called in question, definite conclusions cannot be drawn until sound studies on the long-term complications of Cr supplementation become available.

Potential relationships between disturbances in Cr metabolism and diseases of skeletal muscle, heart, brain, and kidney were presented in sections IX A–IX C, IX G, and

IX H. Although some of the relationships may be accidental, it is nevertheless striking in how many cases a close correlation between the functional capacity of the CK/PCr/Cr system, tissue integrity, and metabolic capacity was observed. If these correlations are corroborated in the future, Cr supplementation may become a versatile therapeutic tool for treating such diseases. In addition, Cr analogs may develop into an interesting new class of therapeutic agents possibly having novel mechanisms of action that are not yet fully clarified.

Cr supplementation was shown to be of benefit to patients suffering from gyrate atrophy of the choroid and retina, mitochondrial cytopathies and other neuromuscular diseases (see sect. IX A), and GAMT deficiency (see sect. IX G). Clearly, these results are preliminary and need to be confirmed.

cCr displays significant antitumor activity when used alone and synergistic effects when used in combination with currently used anticancer drugs (see sect. IX D). Because cCr has a mode of action different from those of other anticancer agents, it is a particularly promising candidate for combination therapies. Cr analogs also displayed antiviral effects (sect. IX E) and had an impact on blood glucose levels (sect. VIII B). Both long-term and short-term treatment of experimental animals with cCr yielded considerable protection against ischemic damage of skeletal muscle, heart, and brain. Therefore, Cr analogs like cCr may be instrumental for increasing the survival time of organs during (cold) preservation, an issue that is particularly relevant at present for heart transplantation. Finally, Cr and cCr had potent protective effects in several animal models of neurodegenerative disease, e.g., in the MPTP model of Parkinson's disease, in a transgenic mouse model of amyotrophic lateral sclerosis, or in malonate- and 3-nitropropionic acid-induced models of Huntington's disease (see sect. IX G).

The recent discovery of a number of new aspects of Cr metabolism has created new interest in the physiological and pathological relevance of the CK/PCr/Cr system, as well as in novel ergogenic and therapeutic applications that may be derived from these findings. In addition, despite more than 150 years of research since the discovery of Cr (121), Cr and its metabolism as a whole are likely to provide us with many new, unexpected surprises also in the future.

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