Metabolic Control of Creatine Biosynthesis

II. RESTORATION OF TRANSAOIDINASE ACTIVITY FOLLOWING CREATINE REPRESSON*

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In recent years, two novel metabolic control mechanisms have been discovered in unicellular organisms (cf. 1, 2). Both mechanisms employ the negative feedback principle, in which input is regulated by output. Each of these feedback controls is most effective when it operates on the first enzyme of a biosynthetic sequence. In the first type of control, the ultimate end product of the biosynthetic sequence inhibits the catalytic activity of the first enzyme of the sequence. In the second type of control, the ultimate end product exerts a negative feedback control of the concentration of the first enzyme of the biosynthetic sequence. It has been suggested that enzyme repression, as the second type of negative feedback is called, is of fundamental importance in controlling the levels of intracellular enzymes in bacteria. Indeed, evidence has been obtained which indicates that enzyme induction in bacteria involves reversal of an endogenous repression (4).

Since the known, well established examples of enzyme repression have in the past been confined to unicellular organisms, it has been suggested that this method of metabolic control might be restricted to rapidly dividing cells, such as bacteria. It is of decided selective advantage to such organisms to be able to adapt to widely different cultural conditions by efficient redeployment of the enzyme-synthesizing machinery. However, Szilard (5), although acknowledging the lack of experimental evidence, has proposed a general theory of enzyme repression applicable to higher animals as well as bacteria, and indeed has gone several steps further and proposed a general theory of antibody formation in animals based on the repression concept (6).

With the recent reports from our laboratory of clear-cut examples of enzyme repression in rats (7) and chicks (8), it would now appear that this means of metabolic control does in fact occur in higher animals. We would like to suggest that enzyme repressions might not only be involved in many of the homeostatic systems of higher animals, but they may also be of importance in establishing and maintaining the characteristic enzyme patterns of differentiated tissues. It would not be difficult to visualize instances in which loss of repressor control might result in dedifferentiation and neoplastic growth.

The specific instance of enzyme repression in higher animals which we have observed is the repression by dietary creatine of arginine-glycine transamidinase, the first enzyme of the biosynthetic pathway leading exclusively to creatine (Reaction 1).

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\text{Arginine + glycine} \rightarrow \text{guanidinoacetate + ornithine} \tag{1}
\]

\[
\text{Guanidinoacetate} + S\text{-adenosylmethionine} \rightarrow S\text{-adenosylhomocysteine} + \text{creatine} \tag{2}
\]

have found that chick liver transamidinase can be repressed to less than 2% of the normal activity after creatine feeding. This alteration of phenotype without change in genotype mimics physiologically a mutation at the locus responsible for transamidinase synthesis.

The question which naturally arises is, what is the mechanism of repression of transamidinase by dietary creatine? Experiments to date have not yet answered this question; however, certain of the possible mechanisms might be eliminated if the nature of the restorative process which follows creatine repression were known. For example, it is important to know whether restoration involves conversion of inactive transamidinase to an active form, or synthesis of the enzyme de novo. It was thought that nutritional experiments might provide one means of distinguishing between these two possibilities.

The primary purpose of this paper is to present information concerning the minimal nutritional requirements for significant restoration of chick liver transamidinase level following creatine repression. Additional experiments relating to the problem of transamidinase repression and recovery will also be described, and the physiological significance of transamidinase repression in the chick will be discussed.

EXPERIMENTAL PROCEDURE

In general, the experimental procedures employed were as described in the preceding paper of this series (8). Each reported value represents a homogenate, assayed in duplicate, of pooled lobes from the livers of two to five birds. Each experiment was performed in whole or in part several times to establish the validity of the results. The dietary components employed in the nutritional experiments, including all proteins and the salt-free acid-hydrolyzed casein, were purchased from the Nutritional Biochemicals Corporation. The specific activities of the homogenates are reported as μmoles of hydroxyguanidine formed per hour per g of liver (fresh weight) at 37°C.

RESULTS

Comparison of Transamidinase Levels during Fasting and Creatine Repression—We had previously noted that transamidinase in the chick is depressed during fasting or maintenance on

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\* Vogel (3) has defined repression as the control of enzyme synthesis by an end product. Since this definition implies that the mechanism of repression is known to some degree, we will employ the term repression only in a general sense to indicate negative feedback control of the concentration of an intracellular enzyme.
an inadequate diet (8). It appeared desirable, at the outset of a series of nutritional experiments on the problems of creatine repression and recovery from repression, to examine more closely the relative effects of fasting and creatine repression on the level of transamidinase in chick liver. The results of a time course experiment designed to provide this comparison are given in Fig. 1. Fasting lowers the transamidinase level, but not so much as the incorporation of 2% creatine in a diet of Purina Chick Startena powder. However, liver shrinkage in the fasted animals is a factor which should also be taken into consideration. Because of this, the total liver transamidinase activity in the fasted chicks is less than might appear from Fig. 1. In the absence of food, the concentration of liver transamidinase declines as the steady state equilibrium between synthesis and degradation of transamidinase molecules is displaced, because of a decrease in rate of synthesis. This decrease is caused both by a lack of dietary amino acids and an increased level of endogenous tissue creatine, which acts as a repressor. The time course of restoration of the transamidinase levels, when both groups are fed the same diet of Startena, is also of interest. During the first hours, the level per g of liver actually falls in the previously fasted chicks. During this time their livers increase markedly in size; the absolute amount of transamidinase per liver probably does not decrease. The slower rate of restoration of the creatine-fed chicks may be accounted for by the repressing action of excess creatine released from tissues in which it had accumulated during creatine feeding.

Inhibition of Transamidinase Restoration by Dietary Ethionine—The first experiment in a series designed to elucidate the mechanism of restoration of transamidinase activity following creatine repression was of a type relatively easy to carry out: an attempt to inhibit protein synthesis by administering an antimetabolite of an amino acid. Fig. 2 shows the results of such an experiment. It can be seen that transamidinase restoration is markedly retarded by the addition of 0.5% \(\text{L-ethionine}\) to a diet of powdered Startena, and that the inhibitory effect of ethionine can be reversed by 1.0% \(\text{L-methionine}\). This type of experiment is often cited as strong evidence for protein synthesis de novo.

Table I

<table>
<thead>
<tr>
<th>Diet</th>
<th>Specific activity of transamidinase</th>
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<tr>
<td>Experiment 1</td>
<td></td>
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<tr>
<td>90 Sucrose-10 Alphacel</td>
<td>1.3</td>
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<tr>
<td>10 Alphacel-90 casein</td>
<td>1.9</td>
</tr>
<tr>
<td>40 Sucrose-10 Alphacel-50 casein</td>
<td>9.3</td>
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<tr>
<td>40 Sucrose-10 Alphacel-50 casein-2 creatine</td>
<td>0.5</td>
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<tr>
<td>Experiment 2</td>
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<tr>
<td>40 Sucrose-20 Alphacel-40 casein</td>
<td>9.5</td>
</tr>
<tr>
<td>40 Dextrin-20 Alphacel-40 casein</td>
<td>8.2</td>
</tr>
<tr>
<td>40 Glucose-20 Alphacel-40 casein</td>
<td>9.2</td>
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Restoration of Transamidinase Activity on Various Synthetic Diets—It has previously been established that no recovery occurs in the absence of food (8). The next problem was to determine the minimal nutritional requirements for the recovery process. It can be seen in Table 1 that no significant recovery occurs when sucrose alone is fed, nor is casein effective by itself. However, the proper proportion of sucrose plus casein is quite effective in permitting the restoration of transamidinase activity following creatine repression. As an added control, creatine was added to the sucrose-casein diet to show that repression could continue on a synthetic diet, as well as on Startena. The other experiment summarized in Table I demonstrates that dextrin or glucose is equally as effective as sucrose as a carbohydrate source.

Many of our early experiments with synthetic diets were unsuccessful because the ratio of carbohydrate to protein which we employed was too high; for example, a recovery diet of 70 sucrose-30 casein is only one-half as effective as 40 sucrose-50 casein.

Fig. 1. Comparison of transamidinase levels during starvation and creatine repression. Chicks were 10 days old at start of the experiment; the control diet was Purina Chick Startena. A transamidinase activity of 100% represents a specific activity of 23 pmoles of hydroxyguanidine formed per hour per g of liver (fresh weight).

Fig. 2. Inhibition of transamidinase restoration by dietary ethionine, showing reversal of the inhibition by dietary methionine. Six-day-old chicks were fed Startena containing 2% creatine for 3 days before the start of this experiment.
casein. Similar optimal ratios were obtained with sucrose plus egg albumin or mixtures of egg albumin and casein. Once the optimal ratio was determined and the carbohydrate source settled, attention was directed to the effect of varying the composition of the protein component of the diet. Table II gives the results of one such experiment. Although neither zein nor hemoglobin can serve as the sole source of protein for transamidinase restoration, an equal mixture of these two incomplete proteins supports satisfactory recovery. Apparently a whole spectrum of amino acids is required for the recovery process. Proteins which do not support growth or nitrogen balance in the chick do not support transamidinase recovery, and vice versa.

The next step undertaken was to see whether casein could be replaced by a mixture of its component amino acids. The results of a typical experiment are given in Table III. It was found that acid-hydrolyzed casein, which is deficient in tryptophan, will not support recovery unless supplemented with tryptophan; niacin will not replace the tryptophan requirement. Acid-hydrolyzed casein plus tryptophan is as effective as enzyme-hydrolyzed casein, and almost as effective as casein itself.

The results described up to this point strongly suggest that restoration of transamidinase activity following creatine repression involves synthesis de novo of the enzyme, as is presumed to be the case in bacteria (9). It appears unlikely that such fastidious nutritional requirements would be necessary for conversion of an inactive transamidinase into active transamidinase. The results are compatible with our previously proposed mechanism of repression (8); however, they do not, of course, rule out the possibility that the repressor reacts irreversibly with pre-existing transamidinase molecules to form functionally inactive proteins which are then degraded by proteolytic enzymes. Nor do the data exclude the possibility that the presence of the repressor during synthesis of transamidinase modifies the tertiary structure of the enzyme and thus its catalytic activity. If such were the case, it is conceivable that only one of the catalytic sites of transamidinase might be rendered inactive. For example, the site specific for arginine might be altered, and the site specific for guanidinoacetate left unchanged. An experiment was therefore performed to test this possibility.

**Effect of Dietary Guanidinoacetate**

Comparative Study of Repression and Recovery with Two Different Transamidinase Assays—In the experiment shown in Fig. 3, the time course of creatine repression of transamidinase and subsequent restoration was followed by measuring the rate of guanidinoacetate-NH$_2$OH transamidination (solid line), as well as by measuring the rate of arginine-NH$_2$OH transamidination (broken line). It is apparent that both catalytic sites are affected to the same degree. It should be noted that ducklings were used in this experiment instead of chicks; the normal liver transamidinase level of ducklings is more than twice that of chicks, which facilitates employment of the weaker guanidinoacetate-NH$_2$OH assay (10). The fact that creatine repression can be demonstrated in yet another higher animal lends added physiological significance to this phenomenon.

Effect of Dietary Guanidinoacetate on Chick Liver Transamidinase—A popular current hypothesis is that enzyme induction involves reversal of an endogenous repression (9). Consequently, we tested a number of compounds for the ability to counteract creatine repression. At the relative concentrations we employed, we found no evidence for reversal of creatine repression by arginine, glycine, guanidinoacetate, methionine, or sarcosine. As a matter of fact, dietary guanidinoacetate is almost as potent as by measuring the rate of arginine-NH$_2$OH transamidination (broken line), and 6.5 when measured as the rate of guanidinoacetate-NH$_2$OH transamidination (solid line). The control diet was Purina Chick Startena.

**FIG. 3.** Comparative study of repression and recovery in ducks, with two different transamidinase assays. The Peking ducklings were 7 days old at the start of the experiment. Transamidinase activities of 180% represent a specific activity of 50.2 when measured as the rate of arginine-NH$_2$OH transamidination (broken line), and 6.5 when measured as the rate of guanidinoacetate-NH$_2$OH transamidination (solid line). The control diet was Purina Chick Startena.
as dietary creatine in repressing transamidinase in chick liver (Fig. 4). However, we think it probable that dietary guanidinoacetate does not repress rat kidney transamidinase (7). On the other hand, chick liver can readily convert guanidinoacetate into creatine (8), and it is well established that guanidinoacetate has a high priority for methyl groups in the chick; in fact, it has not been possible to create a creatine deficiency in chicks by limiting the supply of methyl groups, although deficiencies in arginine or glycine readily reduce creatine biosynthesis (12). We have not employed guanidinoacetate extensively in our repression studies, in spite of its effectiveness and low cost, because a dietary excess of guanidinoacetate would bleed off methionine required for protein synthesis. It would thus be difficult to distinguish between its action on transamidinase as a repressor precursor and as a general inhibitor of the synthesis of proteins containing methionine. When creatine is employed, these complications of interpretation are reduced in number, and the experimental situation is more clear-cut.

**DISCUSSION**

It was reported in the preceding paper of this series (8) that restoration of transamidinase activity, following removal of creatine from the diet, does not proceed in the absence of food. In this paper, experiments have been presented which suggest that the minimal nutritional requirements for restoration of liver transamidinase in the chick are sucrose (or glucose) plus essential amino acids. Sucrose alone, or a source of amino acids alone, will not support restoration of transamidinase; moreover, the recovery process is quite sensitive both to the relative portions of these two foodstuffs supplied in the diet and to the absence of dietary essential amino acids. Addition of an amino acid antimitabolite such as ethionine to an otherwise complete diet prevents restoration of transamidinase activity; methionine reverses the inhibitory effect of ethionine. Sucrose plus an incomplete protein such as zein or hemoglobin will not support transamidinase recovery, although a mixture of these two proteins is quite satisfactory. As a source of amino acids, acid-hydrolyzed casein requires supplementation with tryptophan to be effective; thus supplemented, it is the equal of enzyme-hydrolyzed casein, and approximately 80% as effective as casein itself. Actually, casein was employed in many of these experiments primarily because it is available in the various vitamin-free forms mentioned above. Mixtures of proteins of high biological value, such as casein and egg albumin, are superior to one of the proteins alone. Determination of the optimal amino acid balance for maximal rate of restoration must await additional experiments. The ability of dietary free amino acids, as represented by acid-hydrolyzed casein, to support restoration offers a unique opportunity to study the results of dietary imbalance. Instead of measuring growth, which is the integrated result of a large number of processes, the experimental assay would involve restoration of the tissue level of a single protein. We should like to emphasize that one has a precise tool here; the phenomenon of repression and recovery apparently involves only one enzyme, the concentration of which can be made to rise or fall while the animal remains in good nutritional condition. This ability to change phenotype without change in genotype should also prove to be useful for studies in other areas; for example, studies on the mechanisms of differentiation and maintenance of different enzyme patterns in the various tissues of adult animals. It is tempting to speculate that the reason that certain tissues in higher animals lack transamidinase, whereas other tissues have it in abundance, is that the former tissues have a higher steady state concentration of a repressing agent than do the latter.

With respect to what light the experiments reported in this paper can shed on the mechanism of creatine repression and recovery, we can say at this time only that none of our experiments thus far has contradicted the gross mechanism previously postulated (8) in which creatine, or a complex derivative (5) of creatine, modifies the steady state tissue concentration of transamidinase by inhibiting the biosynthesis of transamidinase molecules. Indeed, the evidence indirectly supports our earlier scheme. One must be cautious, however, in interpreting the data. Several recent reports suggest that the analogous problem of enzyme induction in higher animals, often thought to involve synthesis de novo, may involve cofactor availability (13), conversion of inactive enzyme to active enzyme (14), or depend upon nutritional status for unknown reasons (15, 16). We believe that another potential stumbling block in this area, the reliability of the enzyme assay (17, 18), is not involved in our experiments, inasmuch as we have confirmed our results with a number of the different assays available for arginine-glycine transamidinase. Moreover, it seems unlikely that a cofactor is involved in this reaction (19, 20). It may well be that the final elucidation of the mechanism of creatine repression must await solution of the problem of protein synthesis; indeed, intelligent use of this experimental system might aid in such a solution.

When a novel physiological phenomenon is discovered, its potential significance can in part be evaluated by noting its biological distribution. The fact that there are marked variations in the repressibility of a given enzyme in different bacterial species, and even in different strains or mutants of a single species (21), clearly must be accounted for. In view of this variation in susceptibility to repression among bacterial strains,
it is gratifying to find that transaminase is repressible in each of the three higher animals tested thus far: rats, ducks, and chicks. The chick is in many ways an ideal organism for studying both the mechanism and the physiological significance of the repression of arginine-glycine transaminase activity by dietary creatine. We have employed the chick in most of our studies for the following reasons: (a) Chicks are extremely responsive to dietary creatine; the concentration of transaminase in chick liver can readily be lowered to less than 2% of the normal level, at the same time leaving the levels of other liver enzymes unchanged.

(b) Both enzymes involved in creatine biosynthesis are located in one large, accessible organ, the liver. (c) Since liver is the physiological site of creatine biosynthesis in chicks, any controls which directly regulate creatine biosynthesis must act in this organ; on the other hand, the repression of transaminase in mammalian kidney (7), although it is undoubtedly of mechanistic significance, may or may not be of physiological significance.

(d) For the biosynthesis of creatine, the chick, unlike mammals, is dependent upon its diet to furnish all the creatine precursors: arginine and glycine, as well as methionine (12). This situation allows for greater experimental latitude in studying the mechanism of repression, analogous to the use of amino acid mutants by microbiologists interested in control mechanisms (25). Since, in contrast to mammals, the chick requires both arginine and glycine in its diet, the number of possible mechanisms of control of creatine biosynthesis to be considered in the chick is decreased by two; the availability of endogenously synthesized arginine or glycine, which might be a means of control in mammals, is less likely to be a factor when the supply of these amino acids is dependent upon the diet.

As for the physiological significance of the phenomenon of creatine repression, from the evolutionary standpoint it would have been of distinct survival advantage to the ancestors of the chick to be able to conserve the essential amino acids involved in creatine biosynthesis for the synthesis of protein, when creatine is supplied in the diet. Nowadays, of course, such carnivorous birds as vultures and sea gulls would be more likely to encounter creatine in their diets than would our domestic chicken. Gratifyingly enough, this teleological argument has experimental support. It has been established by several investigators that dietary creatine spares a portion of the arginine requirement of chicks (12). Since creatine does not inhibit the action of either of the two enzymes involved in its synthesis (8), but dietary creatine does repress the liver transaminase level, it would appear that the arginine-sparing action of creatine could be explained on the basis of transaminase repression. It is of interest that our quantitative data also support this interpretation. A bird feeding on muscle would be ingesting a diet containing 0.5% creatine, a concentration high enough to cause marked transaminase repression and decrease in conversion of glycine (and consequently other precursors) to creatine (8).

Although it cannot yet be established with certainty whether creatine repression of transaminase is a functional metabolic control mechanism, or only a vestigial control retained during the evolutionary process, the mechanisms of repression and recovery will remain of considerable theoretical interest. We believe that this system offers a unique opportunity to study the chemical nature of the actual repressing agent; for example, if creatine represents the metabolite moiety (5) of the repressor of transaminase, the limited metabolic fates of creatine should facilitate identification and isolation of such a compound.

SUMMARY

1. The concentration of arginine-glycine transaminase in chick liver can be repressed to very low levels by the addition of either creatine or guanidinoacetate to the diet. It would appear that guanidinoacetate is converted to creatine before the actual repressor compound is formed. Duck liver transaminase can also be repressed by dietary creatine; during repression, the catalytic sites specific for arginine and guanidinoacetate are both affected to the same extent.

2. A study has been made of the nutritional requirements for restoration of transaminase activity following creatine repression. It has been found that the proper proportions of sucrose and vitamin-free casein will support marked restoration. Glucose or dextrin can be substituted for sucrose. Although zein or hemoglobin alone will not substitute for casein, a mixture of these two incomplete proteins will support satisfactory restoration. The casein requirement can also be met with enzyme-hydrolyzed casein; acid-hydrolyzed casein is ineffective unless supplemented with tryptophan. Niacin will not replace the tryptophan requirement. When restoration is carried out on a complete commercial diet, added ethionine will strongly inhibit recovery; ethionine inhibition can be reversed by added methionine. The results of these nutritional experiments are compatible with the concept that restoration of transaminase activity following creatine repression involves synthesis de novo of the enzyme.

3. It is concluded that the physiological significance of the phenomenon of creatine repression of transaminase activity in birds, such as the chick and the duckling, is primarily to conserve the dietary essential amino acids, arginine, glycine, and methionine, for protein synthesis. Feedback control of the catalytic activity of the participating enzymes does not appear to be operative in creatine biosynthesis. Another role of repressive processes, conservation at the macromolecular level, cannot be properly evaluated in an organ the function of which also includes the synthesis and export of large quantities of plasma proteins. The possibility that the steady state repressor concentration of each differentiated tissue of a higher animal determines the characteristic transaminase level of that tissue is discussed.

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