ADP-Rib or oligo(ADP-Rib) was completely abolished by heating the preparation at 60°C for 5 min. It had an apparent pH optimum around 6.0.

These results show that a nuclear preparation from calf thymus contained an enzyme which splits the ribose-ribose bond of poly-(ADP-Rib). The purification and characterization of this enzyme is now in progress.

REFERENCES

**Lysine, a Precursor of Carnitine in the Rat**

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Vichai Tanphachitr, Donald W. Horne, and Harry P. Broquist

From the Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37208

**SUMMARY**

Rats, fed a 20% gluten diet in which lysine is the limiting amino acid, exhibited a reduction of growth, anemia, and hypoproteinemia. Carnitine concentrations in skeletal muscle and heart of such lysine-deficient rats were significantly lower than in rats supplemented with 0.8% lysine, but were higher in the liver. Following intraperitoneal administration of lysine-deficient rats, radioactive from DL-[6-14C]lysine, but not DL-[2-14C]lysine, was significantly incorporated into carnitine in the liver and skeletal muscle. These data thus establish a role for lysine in carnitine biosynthesis in the rat. A hypothetical scheme postulating transformations of lysine to yield carnitine is considered.

We have recently reported (1, 2) that Neurospora crassa lysine auxotroph 33833 grown on a carnitine-free synthetic medium incorporates radioactivity from DL-[6-14C]lysine and DL-[4,5-14H]lysine without dilution into carnitine. In contrast, radioactivity from DL-[2-14C]lysine or DL-[2-2H]lysine was not found in biosynthesized carnitine under comparable conditions (2).

Considering the possibility that methylated derivatives of lysine, including e-N-trimethyllysine, participate in carnitine biosynthesis. Indeed in two experiments e-N-trimethyllysine metabolism in which case the broad outlines of carnitine biogenesis stemming from lysine metabolism might include the following transformations: lysine → e-N-trimethyllysine → γ-butyrobetaine → carnitine. In isotopic experiments reported herein with lysine-deficient rats, conditions are such that carnitine synthesis can be shown from lysine with labeling patterns consistent with the above scheme.

**MATERIALS AND METHODS**

**Diet and Animal Care**—Forty male, 24-day-old rats (Sprague-Dawley strain) were placed on the diet described by Gupta et al. (5) which is a 20% gluten, cornstarch, corn oil, salts, and vitamins diet further supplemented with histidine, methionine, threonine, and tryptophan. All ingredients were purchased from Nutritional Biochemicals. The sole limiting amino acid in this diet is l-lysine, present at a level of 0.37% in the complete diet as determined by microbiological assay of an acid hydrolysate of the gluten protein. No detectable carnitine was found in the basal diet, using the assay described below in which the limit of detection was <0.1 μg of carnitine per g of basal diet. The diet of 20 of the above animals was further supplemented with 0.8% L-lysine monohydrochloride. The unsupplemented and lysine-supplemented groups were kept in pairs in suspended cages with screen bottoms and given food and water ad libitum throughout the test period.

**Analytical Procedures on Blood Samples**—After 8 weeks the animals were anesthetized (sodium pentobarbital, Abbott). Heart blood was obtained, using heparin as an anticoagulant, for the determination of plasma protein and various hematological parameters. Hemoglobin was determined by the cyanmethemoglobin method (6). Hematocrit was measured by a microhematocrit method (capillary tube, 32 × 0.8 mm, Drummond Scientific Company, Broomall, Pennsylvania). Plasma protein was determined by a refractive index method (7) (T. S. meter model 10400, A. O. Instrument Company, Buffalo, New York).

**Carnitine Assay of Rat Tissues**—Tissues were homogenized (20 volumes of CHCl3:CH3OH, 3:2, v/v) and centrifuged (10,000 × g, 30 min). Supernatant solutions were retained and the residues washed (10 volumes of CHCl3:CH3OD) and recentrifuged. The combined supernatant solutions were concentrated to dryness in vacuo, the residues suspended in 3 to 5 volumes of 0.1 N KOH, heated (50°C, 30 min) to release “bound carnitine” (8), and were then acidified (pH 3, 2 N HCl) and extracted with ether to remove lipid materials. The aqueous extracts was neutralized (NaHCO3) and assayed for carnitine with the carnitine acetyltransferase assay (9) which measures the carnitine-dependent release of CoA from acetyl CoA with ELL-

man's reagent at 412 nm. The enzyme was obtained from Sigma.

Radioactive Materials and Methods—DL-[6-14C]lysine and DL-[2-14C]lysine were purchased from Schwarz BioResearch and New England Nuclear, respectively, and were judged to be pure by appropriate chromatographic criteria. Radioactive samples were counted in a Packard Tri-Carb liquid scintillation counter using 10 ml of a mixture composed of 0.3% 2,5-diphenyloxazole and 20% absolute ethanol in toluene.

RESULTS AND DISCUSSION

The data, upper portion of Table I, illustrate that rats fed a 20% gluten diet for 56 days develop a severe lysine deficiency as evidenced by poor growth, anemia, and hypoproteinemia. Although there was no mortality in the unsupplemented group throughout the test period, the body weight of these rats after 56 days averaged only 32% of the 0.8% lysine-supplemented group. The anemia and hypoproteinemia that resulted in the unsupplemented group are statistically significant and constitute important indicators of lysine deficiency as described in the classical paper of Harris, Neuberger, and Sanger (10).

Of great interest was the finding, Table I, that the carnitine content of the skeletal muscle and heart of the lysine-deficient rats was significantly lower than that of the controls. Such findings might be attributed to (a) a generalized impairment of protein synthesis with concomitant inefficient synthesis of carnitine, or (b) a markedly reduced lysine pool in the tissues, a portion of which is required as a substrate for carnitine biosynthesis. Evidence that (b) is the more likely alternative is presented below. The finding that the carnitine in the liver was actually somewhat higher in the lysine-deficient rats than the controls, Table I, may reflect an increased demand for intrahepatic synthesis of carnitine to meet the needs of the other tissues under these stringent nutritional conditions.

In the experiment of Fig. 1A, a rat that had been on the lysine-limiting 20% gluten diet for 99 days was administered DL-[6-14C]lysine as described in the figure legend. An extract of rat liver was subsequently prepared and examined for radioactivity and carnitine following separation of lysine and carnitine by ion exchange chromatography. Fig. 1A clearly shows coincidence of radioactivity derived from DL-[6-14C]lysine with carnitine fractions. Such fractions, tubes 47 to 50, when pooled, desalted, and submitted to thin layer chromatography by procedures described elsewhere (2) could not be distinguished from authentic carnitine.

The above experiment was repeated, Fig. 1B, with a similar lysine-deficient rat but differed in that DL-[2-14C]lysine was the tracer. No incorporation of radioactivity into carnitine was detected in this instance (Fig. 1B). Extracts of skeletal muscle from the rats utilized in the experiments of Fig. 1, A and B, were also analyzed by these procedures for carnitine deriving from the labeled lysines. Again only DL-[6-14C]lysine labeled carnitine in this tissue. These experiments thus show that the lysine-deficient rat can synthesize carnitine, if lysine is provided, and that a fragment of the lysine molecule containing carbon atom 6, but not carbon atom 2, is utilized in this process.

The specific activity of biosynthesized l-carnitine of liver and skeletal muscle deriving from DL-[6-14C]lysine was 0.003 μCi per μmole, indicating a dilution of about 500-fold of the preferred DL-lysine. This is attributed to dilution by endogenous carnitine.

**Table I**

<table>
<thead>
<tr>
<th>Determination</th>
<th>Additives to 20% gluten diet (g)</th>
<th>Col. 1 × 100</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>None</td>
<td>52.0 ± 0.4</td>
<td>50.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.8% lysine</td>
<td>97.1 ± 2.2</td>
<td>307.5 ± 6.5</td>
</tr>
<tr>
<td>Hemoglobin, g%</td>
<td>None</td>
<td>11.3 ± 0.3</td>
<td>13.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>0.8% lysine</td>
<td>39.7 ± 0.8</td>
<td>43.8 ± 0.6</td>
</tr>
<tr>
<td>Plasma protein, g%</td>
<td>None</td>
<td>4.8 ± 0.2</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.8% lysine</td>
<td>77 ± 5</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>Carnitine, μg/g</td>
<td>None</td>
<td>114 ± 3</td>
<td>159 ± 8</td>
</tr>
</tbody>
</table>

<sup>a</sup> P values were obtained by comparing rats consuming the unsupplemented 20% gluten diet with those supplemented with 0.8% lysine, using the t test.

**Fig. 1.** A, elution profile of radioactivity (—) and carnitine (——) following ion exchange chromatography of rat liver extract derived from a lysine-deficient rat administered DL-[6-14C]lysine; B, as in A but rat had received DL-[2-14C]lysine. Two rats receiving the unsupplemented 20% gluten regimen for 99 days were placed in separate metabolic cages and maintained on this diet throughout the experimental period. Rat A (weight, 143 g; hemoglobin, 12.7 g%; hematocrit, 40%; plasma protein, 5.1 g%) received 41 μCi of DL-[6-14C]lysine (diluted to a specific activity of 3.87 μCi per μmole) intraperitoneally at 12-hour intervals for 3 days (total dose, 246 μCi). Rat B (weight, 139 g; hemoglobin, 11.5 g%; hematocrit, 37%; plasma protein, 5.2 g%) received in the same manner a total of 246 μCi of radioactivity as DL-[2-14C]lysine (specific activity, 3.87 μCi per μmole). Five days after the final administration of radioactivity, the animals were sacrificed and an extract of liver was prepared (cf. materials and Methods). Four milliliters of liver extract (equivalent to 2.7 g wet liver) were applied in each instance to ion exchange columns and developed under conditions appropriate for the separation of carnitine and lysine (cf. legend of Fig. 1 in Reference 2 for experimental details) and subsequent fractions were analyzed for radioactivity (0.1-ml aliquots of 1.8-m1 fractions) and carnitine (cf. Materials and Methods). Carnitine fractions, tubes 47 to 50; lysine fractions, tubes 52 to 55.
in these tissues synthesized from the residual lysine in the 20% gluten diet. Even under these nutritional conditions, it is estimated that 0.074% of the administered DL-[6-14C]lysine was incorporated into "total liver and muscle carnitine" of Rat A (Fig. 1A). This is a significant figure, particularly when it is considered that carnitine is presumably needed only at a catalytic level. In N. crassa 393933 auxotrophic for lysine, the lysine → carnitine conversion is about 0.2% (1, 2). The fact that lysine was seemingly not incorporated into carnitine in the rat in early experiments of Wolf and Berger (11) is understandable in the light of the present findings, since their rats (11) were consuming a lysine-rich 24% casein diet and received about 13 times less radioactive lysine for a shorter time period (24 hours).

Lysine deficiency in the rat has many features in common with starvation (10), the end result classically being regarded as "protein malnutrition." In this context the significance of the work reported herein may be in focusing on a previously unrecognized aspect of lysine deficiency, namely that leading to a diminution of carnitine synthesis in the tissues, a situation which could potentially result in adverse effects on fatty acid and energy metabolism as well.

REFERENCES

An Adenosine 3',5'-Monophosphate-inhibited Protein Kinase from Physarum polycephalum*

(Glenn D. Kuhn)
From the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88001

SUMMARY
A potent protein kinase present in the acellular slime mold, Physarum polycephalum, which catalyzes the phosphorylation of casein by ATP has been found to be inhibited by cyclic adenosine 3',5'-monophosphate (cAMP). The rate of the kinase-catalyzed phosphorylation of casein was completely inhibitable at a concentration of cAMP above 1 × 10^{-7} M. A cAMP-activated protein kinase, which is readily separable from the cAMP-inhibitable enzyme, is also present in this organism. The discovery of both of these enzymes in the same organism suggests a more complicated regulatory role for cAMP, via its effects on protein kinases, than previously proposed.

The initial discovery by Walsh, Perkins, and Krebs (1), in rabbit skeletal muscle, of a protein kinase that is activated by cAMP provided impetus for extensive investigations into the distribution of this enzyme in various eukaryotic and prokaryotic cells (2-12). Several of these enzymes have been purified and evidence of a common control mechanism explaining the interaction of cAMP with cAMP-activated protein kinases has recently been presented for enzymes isolated from rabbit skeletal muscle (13), beef heart (14), and bovine adrenal cortex (15).

The ubiquitous occurrence throughout the animal kingdom of these cAMP-activated protein kinases has afforded strong support for the proposal that all of the diverse effects produced by cAMP are a consequence of its capacity to activate different protein kinases (6, 7). Collectively, and in the absence of direct evidence to the contrary, these reports have generated the belief that a general feature of cellular regulation involving cAMP has been established (16, 17).

The present communication reports the first apparent evidence contrary to the above proposal. A potent protein kinase, which is completely inhibitable to nanomolar concentrations of cAMP, has been discovered in the acellular slime mold, Physarum polycephalum. An apparently distinct cAMP-activated protein kinase, readily separable from the cAMP-inhibitable enzyme, has also been detected in the same organism.

Purification of Protein Kinases—Shake cultures of P. polycephalum were grown in 100 ml of semidefined medium plus hematin, as described by Chin and Bernstein (18). Inoculum for plasmoidal cultures was prepared by inoculating shake flasks with 3 ml of 3-day stock cultures and incubating the flasks for 18 to 24 hours to a protein level of about 2 mg per ml of medium. To obtain plasmodia for enzyme extraction, the mold was allowed to settle in the culture flask, and plasmodia were collected with a large bore pipette, centrifuged briefly at 6000 × g for 30 sec, and washed by again settling through cold, glass-distilled water. After removal of excess wash water by centrifugation, the wet-packed plasmodia were resuspended in approximately twice their volume of buffer as described below.

To prepare the partially purified cAMP-inhibited protein kinase, plasmodia were suspended in 5 mM potassium phosphate buffer (pH 7.0), containing 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (Calbiochem, B grade) (Buffer 1). All succeeding procedures were carried out at an ice bath temperature of approximately 4°C. The mold was ruptured either by brief homogenization for 30 sec in a Potter-Elvehjem homogenizer or by repeated passing of the plasmodia through a syringe assembly with a No. 18 needle until the suspension assumed a homogeneous consistency, followed by a single passage through a French pressure cell at 12,000 psi. Cell debris was removed by centrifugation at 27,000 × g for 20 min. The resultant clear, yellow supernatant was adjusted to pH 5.0 with acetic acid and a copious precipitant was removed by centrifugation. Approximately 70% of the crude extract protein was precipitated by this step. The supernatant was immediately readjusted to pH 7.0 with 1 M imidazole solution. This supernatant fraction contained the

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1 The abbreviations used are: cAMP, cyclic adenosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate.
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