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POSSIBLE ROLE FOR CARBAMYL PHOSPHATE IN THE CONTROL OF LIVER GLYCOGEN SYNTHESIS

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SUMMARY: Isotopic data suggested a possible correlation between the activation of liver glycogen synthesis and some unstable intermediate. Carbamyl phosphate was one candidate considered. L-Norvaline, an inhibitor of ornithine transcarbamylase, is known to increase intracellular carbamyl phosphate levels. L-Norvaline augmented the increase in glycogen synthesis caused by L-glutamine and L-alanine, and also induced considerable glycogen synthesis with ammonia as the nitrogen source.

One of the unsolved problems in liver glycogen metabolism is the mechanism by which certain amino acids greatly accelerate the rate of glycogen synthesis in hepatocytes from a gluconeogenic substrate in the presence of physiological levels of glucose (1). We have been looking for a possible common agent whose formation might be stimulated by known classes of activators of glycogen synthesis:(a)L-glutamine, L-alanine and L-asparagine, the most effective amino acid activators originally described by Katz et al. (1);(b) certain compounds, possibly heavy metal chelators, such as dipicolinate and n-butyl malonate whose effects we have earlier described (2); and (c)certain other amino acid combinations we have found effective (unpublished work) e.g. L-tryptophan plus L-histidine. In this paper we describe the activating effect of another compound, L-norvaline, on liver glycogen synthesis. L-Norvaline is an inhibitor of ornithine transcarbamylase (3), and therefore causes an accumulation of carbamyl phosphate in the liver cell.

METHODS: Hepatocytes were prepared from rats fasted for 24 hr as described earlier (5). The cells (40-50 mg dry wt) were incubated for 1 hr at 38° in 5 ml of buffered medium with a composition similar to Krebs-Henseleit buffer (6), but with the bicarbonate reduced to 5 mM, and 50 mM HEPES added, with the pH adjusted to 7.4. The gas phase was 100% O_2. The substrates added were glucose, L-lactate and dihydroxyacetone, all at 10 mM, plus additional nitrogenous substrates as noted. The bicarbonate content was reduced to obtain higher specif activities of $H^{14}CO_3^-$ in isotopic labelling experiments. Rates of activated glycogen synthesis, assayed as in (1), were essentially the same in this medium as in normal Krebs-Henseleit buffer. The incubations were terminated by (o.5 ml)

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injection of 2M acid ($HClO_4$, HCOOH or CH_3COOH) to the medium, and o.3 ml of 4N NaOH to a hanging plastic well, when $NaH^{14}CO_3$ was used. After $^{14}CO_2$ collectio for 90 min, the medium was washed out, made to 10.0 ml and centrifuged. In isotopic experiments, an aliquot (8 ml) of this was put on a 1x12 cm Dowex 50 (H^+, 100-200 mesh) column on top of a 1x25 cm Dowex 1 (acetate, 100-200 mesh) column, and the columns were washed with water to 80 ml. The Dowex 1 column was eluted with stepwise gradients of acetic and formic acids.

RESULTS AND DISCUSSION: In our search for an activator, one approach we have used is to add high specific activity $NaH^{14}CO_3$ to the medium, and to look for any new compounds which might correlate with the increased glycogen synthesis which occurs upon addition of the above classes of activators. Fig. 1 shows the earlier part of an elution profile of the Dowex 1 column from a hepatocyte incubation which included 10 mM L-glutamine as the activating agent. Some of these peaks have been identified (by various procedures, enzymatic analysis, use of standards, further paper chromatography, etc.). Peak IV is essentially all L-lactate, and peak V is 5-oxoproline. Peak VI is a mixture of L-malate and carbamyl-L-aspartate; these separate readily upon paper chromatography.

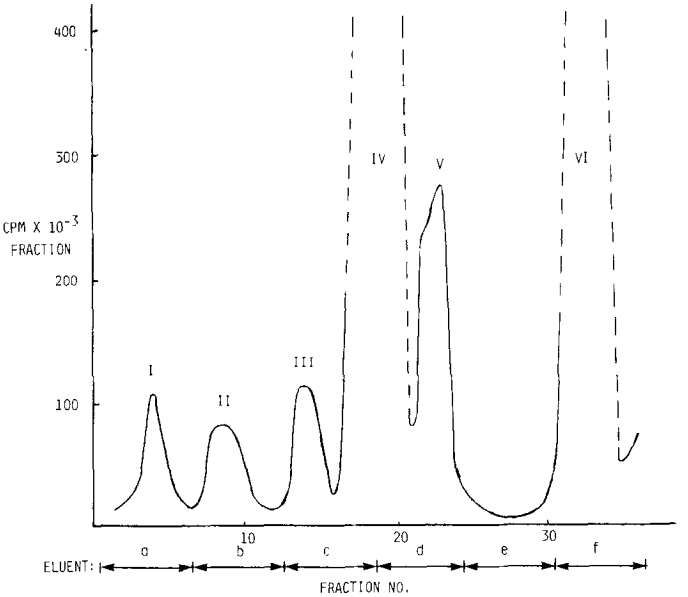

Fig. 1. Elution profile of the Dowex 1 column. Hepatocytes from fasted rats were incubated for 6o min with 10 mM glucose, 10 mM L-lactate, 10 mM dihydroxyacetone and 10 mM L-glutamine in a medium containing 5 mM $NaH^{14}CO_3$ (100 uCi). The incubation was terminated by injection of 0.5 ml 2N HCOOH to the medium, and further processed as in Methods. Fractions are 10 ml. Eluents: (a) 0.167 N CH_3COOH; (b) 0.5 N CH_3COOH; (c) 2 N CH_3COOH; (d) 4 N CH_3COOH; (e) 1 N HCOOH; (f) 2 N HCOOH.

Carbamyl-L-aspartate formation is markedly increased when L-glutamine is the glycogen synthesis activator used, while 5-oxoproline is most markedly increased when L-alanine is the activator. However, the peaks which show the best general correlation with glycogen synthesis are peaks II and III. These are low in control incubations of hepatocytes and appear upon addition of all of the classes of activators of glycogen synthesis. The sizes of these two peaks are highly variable from experiment to experiment, and we emphasize that the data shown illustrates a single experiment. Further, essentially all of the radioactivity in these two peaks is lost upon evaporation of the eluent. Within a given experiment, however, there has been some further indication of relevance to glycogen synthesis. Thus, the sizes of these peaks were decreased by graded doses of Zn^{2+} exactly to the degree that the Zn^{2+} decreased glycogen synthesis. What the data suggested was that we might be looking at a weak acid which is an unstable intermediate, or breakdown product(s) of this. One intermediate considered was carbamyl phosphate. Carbamyl phosphate itself is a fairly strong acid. It has a half life in water at 37° of about 40 min (7); it decomposes to HCO_3^- and NH_4^+, or to cyanate. Cyanate is a weak acid and has a half life of about 6 hr under neutral conditions.

To test for a possible role for carbamyl phosphate, we have used L-norvaline, which has been shown to raise intracellular carbamyl phosphate levels (4). L-Norvaline is little metabolized by hepatocytes (8), and inhibition of ornithine transcarbamylase is considered to be its primary site of action. Table I shows the effect of L-norvaline on glycogen synthesis in hepatocytes. L-Norvaline further stimulates glycogen synthesis in the presence of L-glutamine and L-alanine, known activators of glycogen synthesis. It also activates considerable glycogen synthesis in the presence of NH_4Cl, which is only slightly glycogenic by itself under the conditions used. The percent stimulation was largest with ammonia, but the absolute magnitude of the effect of L-norvaline was similar with all the nitrogen substrates. L-Leucine, another inhibitor of ornithine transcarbamylase (3), has effects quite similar to those of L-norvaline (results not shown). While the evidence is still indirect, we

Table I. Effect of L-norvaline on glycogen synthesis in rat hepatocytes

(10 mM) Nitrogen Substrate	(10 mM) L-Norvaline	Rate of Glycogen Synthesis
		umoles glucose/gm wet wt·hr
L-Glutamine	-	36 (±2)
L-Glutamine	+	44 (±3)*
L-Alanine	-	20 (±1)
L-Alanine	+	29 (±3)*
NH_4Cl	-	5 (±1)
NH_4Cl	+	14 (±2)*

Hepatocytes from 24 hr fasted male rats were incubated for 60 min with 10 mM glucose, 10 mM L-lactate and 10 mM dihydroxyacetone, with the additions as shown. The rate of glycogen synthesis in the absence of any nitrogen substrate was 3 umoles/ (gm wet wt·hr). Values are means (± SEM) of 5 experiments. * $P < 0.01$ in comparison with no L-norvaline.

consider it suggestive for a role for carbamyl phosphate in the control of liver glycogen synthesis. Exactly what this role might be can only be speculation at this stage. It might be as a precursor of another compound, e.g. carbamyl-L-aspartate or a pyrimidine. Or it might be that carbamyl phosphate itself is involved, e.g. as a competitive inhibitor (9,10) of glucose-6-phosphatase, causing glucose-6P accumulation and subsequent activation of glycogen synthase.

It is recognized that a correlation does not indicate a necessary causal relationship, but merely points to a suggestive hypothesis. We have previously shown that exogenous Zn^{2+} can markedly inhibit glycogen synthesis in hepatocytes(2) In this regard we note that Powers (11) has shown that Zn^{2+} is a potent inhibitor of mitochondrial carbamyl phosphate synthetase I, and that the enzyme can be activated by amino acids and by certain heavy metal chelators. A rigorous proof of the role of carbamyl phosphate, and determination of whether it may be the active agent itself or a precursor, will require considerable additional study.

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