

Iron concentrations approximate 0.3 percent. Electrophoretic mobilities of the *fast* and the *slow* components were measured in the Tiselius apparatus as -0.43 and $-0.30 \mu \text{ cm}^{-1}/\text{v cm}^{-1}$, respectively, at $\text{pH } 8.5$ (Veronal, 0.1μ) and as -0.32 and $-0.20 \mu \text{ cm}^{-1}/\text{v cm}^{-1}$, respectively, at $\text{pH } 6.0$ (phosphate, 0.1μ) (5).

The role of the heme proteins in the legume nodule has not been elucidated; it must be remembered that oxygenation may be fortuitous, and that the several components need not be physiologically unique.

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 2. This investigation was suggested and supported by D. L. Drabkin of the biochemistry department of the Graduate School of Medicine, University of Pennsylvania. The cooperation of the botany department of the University of Pennsylvania, and of the bacteriology department and the biochemistry division of the chemistry department of the University of Illinois, where parts of the research were conducted, is also gratefully acknowledged. The research has been supported, in part, by contracts between the Office of Naval Research and the University of Pennsylvania, between the National Science Foundation and the University of Illinois, and between the National Institute of Allergy and Infectious Diseases of the U.S. Department of Health, Education, and Welfare and the University of Illinois. More detailed descriptions of these studies are in preparation.
 3. Paul Feltman, formerly of the University of Illinois, assisted in developing these oxygenated preparations.
 4. E. Thorogood, *Chromoproteins from Soybean Nodules* (University of Pennsylvania, Philadelphia, 1955).
 5. These analyses were performed by J. H. Custer, USDA Agricultural Research Station, Eastern Utilization Research Branch, Philadelphia, Pa., through the courtesy of T. L. McMeekin and C. A. Zittle.
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Role of Glycolysis in Fatty Acid and Cholesterol Synthesis in Normal and Diabetic Rats

It has been repeatedly demonstrated that depressed glucose oxidation, as seen in such conditions as fasting or diabetes, results in marked derangements of fatty acid and cholesterol synthesis (1). That such disturbances in the synthesis of lipids are clearly secondary to the lack of glycolysis is shown by the fact that re-institution of carbohydrate breakdown, by whatever means, results in prompt return of lipid synthesis to normal (1). Despite these well-demonstrated relationships, neither the sites of such metabolic lesions in fatty acid and cholesterol synthesis nor the mechanism by which glycolysis repairs them is known.

It is now well established that glucose oxidation can take place by either of two

routes, the classical Embden-Meyerhof (EM) pathway or the more recently described hexosemonophosphate (HMP) shunt (2). While most glucose molecules are oxidized via the Embden-Meyerhof route (2), it seems possible that the hexosemonophosphate pathway might play a role in the *control* of cholesterol and fatty acid synthesis out of proportion to its quantitative importance in glycolysis.

Similar views concerning the control of fatty acid synthesis in normal animals have been suggested by the work of Brady in pigeon liver (3) and by the studies of Langdon in rat liver (4). A role of the hexosemonophosphate shunt in the lipogenic defect of the diabetic has not been previously established.

The present studies (5) were designed, first, to determine which of the two pathways of glucose oxidation is primarily responsible for the influence of glycolysis upon lipid synthesis in normal and diabetic animals and, second, to elucidate the mechanism by which such effects might occur.

Use was made of the facts, originally demonstrated by Wenner *et al.* (6) and confirmed by us (7), that, in tissue homogenates, glucose oxidation via the Embden-Meyerhof pathway is enhanced by the addition of diphosphopyridine nucleotide (DPN), while oxidation via the hexosemonophosphate shunt is stimulated by triphosphopyridine nucleotide (TPN). By adding the appropriate cofactor and simultaneously measuring the rates of lipid synthesis by acetate-1- C^{14} incorporation, the influence of each of these pathways upon fatty acid and cholesterol synthesis can, therefore, be determined.

As prepared, homogenates from normal animals were found to synthesize fatty acids at significant rates even in the absence of added coenzymes (Table 1). Stimulation of Embden-Meyerhof glycolysis caused a moderate increase in fatty acid synthesis (two- to threefold); in contrast, however, stimulation of the hexosemonophosphate shunt consistently produced a striking acceleration of from 30- to 100-fold. Stimulation of both pathways usually increased still further the rate of fatty acid synthesis.

The production of cholesterol would also seem to be greatly dependent upon the route of glycolysis. Whereas stimulation of the Embden-Meyerhof pathway alone produced negligible changes in the rate of synthesis of this sterol, hexosemonophosphate oxidation caused a marked increase of at least 40- to over 100-fold. In contrast to the case of fatty acid synthesis, however, addition of Embden-Meyerhof glycolysis to hexosemonophosphate oxidation depressed cholesterol synthesis relative to that seen when the shunt alone was stimulated.

It appears, therefore, that in the system under study, it is the glucose that is

Table 1. Effect of glycolytic pathway upon cholesterol and fatty acid synthesis. Two milliliters of cell-free homogenates of normal or alloxan diabetic rat liver, prepared with an equal volume of $0.1M \text{ K}_2\text{HPO}_4$ buffer, was incubated under an atmosphere of 95 percent O_2 and 5 percent CO_2 for 1 hour at 37°C . Glucose-6-phosphate ($18 \times 10^{-3}M$) served as the intermediate common to both pathways of glucose oxidation. Other cofactor concentrations were as follows: DPN, $0.7 \times 10^{-3}M$; TPN, $0.7 \times 10^{-3}M$; isocitrate, $18 \times 10^{-3}M$; acetate, $1.9 \times 10^{-3}M$. Fatty acids and cholesterol were isolated as previously described (8) and were assayed by liquid scintillation counting. EM, Embden-Meyerhof pathway; HMP shunt, hexosemonophosphate shunt.

Glycolytic pathway stimulated	Normal		Diabetic	
	Acetate- C^{14} recovered in		Acetate- C^{14} recovered in	
	Fatty acids (μmole)	Cholesterol ($\mu\text{mole} \times 10^{-2}$)	Fatty acids (μmole)	Cholesterol ($\mu\text{mole} \times 10^{-2}$)
Neither	0.5	< 3	< 0.1	< 3
EM	1	< 3	< 0.1	< 3
HMP shunt	29	212	73	302
Both	53	98	302	309
Neither	1	< 3	< 0.3	< 6
EM	3	12	1.2	< 6
HMP shunt	120	123	28	62
Both	224	73	263	52
Neither	4	7	< 0.3	< 6
EM	4	10	0.6	< 6
HMP shunt	133	962	30	754
Both	224	385	88	1066
Isocitrate + TPN	314	505	166	400

oxidized via the hexosemonophosphate shunt that is primarily responsible for the effects of glycolysis in enhancing both fatty acid synthesis and cholesterol synthesis in normal liver. It would also follow that, when glucose is being oxidized at an adequate rate, cholesterol synthesis might be controlled by the relative amounts of glucose that traverse each of the glycolytic pathways—that going by the hexosemonophosphate shunt stimulating the process, and that using the Embden-Meyerhof pathway depressing it.

The explanation for these effects was suggested by Langdon's finding that reduced TPN (TPNH), which is known to be produced by the hexosemonophosphate shunt but not by the Embden-Meyerhof pathway, is required in the synthesis of fatty acids at the point of conversion of crotonyl-CoA to butyryl-CoA (9). We would suggest that TPNH is also required or is at least rate-limiting in cholesterol biogenesis. A requirement for TPNH in both of these syntheses, then, makes it likely that it is this cofactor, and not some other product of hexosemonophosphate stimulation, that mediates the effect of hexosemonophosphate oxidation on lipid synthesis. This is probably the

case since another TPNH-generating system, D-isocitrate and TPN, is also capable of producing a marked stimulation of fatty acid and cholesterol synthesis (Table 1).

That the well-established defect in fatty acid synthesis seen in the diabetic state is likewise due to a lack of glucose oxidation via the hexosemonophosphate shunt is demonstrated in Table 1. The lesion observed in the intact animal and in the liver slice (1) is also demonstrable in liver homogenates (see also 10, 11). By stimulation of the hexosemonophosphate shunt, however, fatty acid synthesis in the diabetic can be restored approximately to the same level as it is in many normal livers. This increase represents a stimulation of diabetic lipogenesis of at least 100- to 700-fold. As in the normal liver, further enhancement of fatty acid synthesis is seen when both pathways are stimulated. For reasons which are not yet clear, cholesterol synthesis in the diabetic, while stimulated by hexosemonophosphate oxidation, was not relatively depressed by the addition of Embden-Meyerhof glycolysis.

That the defect in lipogenesis found in the diabetic liver is likewise primarily due to a deficiency of TPNH is supported by the fact that the alternate TPNH-generating system, D-isocitrate and TPN, will largely correct this lesion (Table 1). It would follow, therefore, that the primary diabetic block in fatty acid synthesis is at the site of action of TPNH—namely, at the reduction of crotonyl-CoA to butyryl-CoA (9). The location of this diabetic block at some point prior to the involvement of butyryl-CoA was previously indicated by the finding of Shaw, Dituri, and Gurin that butyryl-CoA can stimulate fatty acid synthesis in diabetic liver (11).

Finally, evidence that the conclusions drawn from these *in vitro* studies are applicable to the intact animal is the observation that diabetic acidosis is characterized by an accumulation of the ketone bodies, beta-hydroxybutyric and acetoacetic acids, the CoA derivatives of which are two of the fatty acid precursors preceding the blocked TPNH-requiring step.

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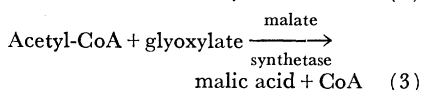
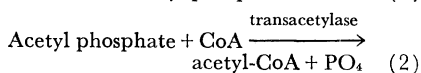
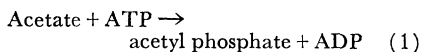
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Significance of the Malate Synthetase Reaction in Bacteria

An alternate pathway of malic acid synthesis has been discovered in our laboratories. An enzyme, named "malate synthetase," has been obtained from *Escherichia coli*, strain E26, that is grown on acetate. This enzyme converts equimolar concentrations of acetate and glyoxylate to malic acid (1).

Purification of malate synthetase by several ammonium sulfate precipitations and treatment with calcium phosphate gel and protamine sulfate resulted in a 50-fold purification with an approximate 30 percent yield. The final product was free of fumarase, Ochoa's condensing enzyme, isocitritase, and glyoxylate reductase. Experiments conducted with such preparations revealed that for each mole of acetyl phosphate and glyoxylate that disappears, 1 mole of malate is formed. With acetyl phosphate as substrate, coenzyme A (CoA) (2) and phototransacetylase are required. These can be replaced with acetyl-CoA. The formation of malic acid can thus be formulated as follows:



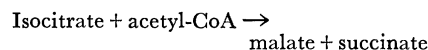
To demonstrate the initial reactants in reaction 3, acetyl-CoA was synthesized chemically from thiolacetate and CoA and was incubated in the presence of glyoxylate and malate synthetase. Analysis of the reaction mixture, after the incubation period, revealed that malate was quantitatively formed. Magnesium ions were routinely added, but it

has not yet been definitely ascertained that they are required.

The equilibrium of the over-all reaction is overwhelmingly in favor of malate synthesis. Attempts to demonstrate the reversibility of this reaction have failed thus far.

Malate synthetase appears to be an adaptive enzyme, for it was found only in cells that have been grown on acetate as the major carbon source. It has thus far been found in acetate-grown *E. coli*, *Aerobacter aerogenes*, *Corynebacterium creatinovorum*, and *Pseudomonas fluorescens*. When any of these organisms were grown on substrates other than acetic acid, malate synthetase could not be detected.

The significance of the occurrence of malate synthetase in nature (3) stems from the fact that it fills in a significant gap in our knowledge of carbohydrate metabolism in that it explains the long baffling problem concerning the mechanism by which bacteria can grow on two-carbon compounds such as acetic acid. Assume that cells, when first exposed to acetate as the sole carbon source, contain, as they do, minimal catalytic amounts of oxalacetate. This being the case, the first event to occur would be a combination of acetate and oxalacetate to form citrate. The latter could then be cleaved by way of isocitrate to succinate and glyoxylate. The glyoxylate formed would in turn condense with another molecule of acetate via the malate synthesis reaction, forming a new C_4 unit. The net result during this process, assuming no drainage to supply carbon skeletons for amino acid synthesis, would be a gain of one C_4 unit, as is shown in Fig. 1. Thus, by assuming the presence of even one molecule of oxalacetate, all acetate carbon could be converted to C_4 units as follows:



Further, since growth occurs on acetate as the sole source of carbon, intermediates are constantly being drained from the tricarboxylic acid cycle and utilized for amino acid synthesis. Under these conditions, rapid net synthesis of C_4 dicarboxylic acids is therefore required to provide the acceptor for the C_2 units entering the cycle. The malate synthetase reaction meets that need completely. This reaction, *in vitro*, at least, is rapid and proceeds almost exclusively in the direction of malate formation. In addition, as was pointed out earlier, the system is adaptive—that is to say, malate synthetase forms only when cells are grown on acetate as the sole carbon source. This suggests the enzyme is important primarily when C_2 intermediates are involved. Energy for synthesis during growth is, as illustrated in Fig. 1, un-