Reports

Oxygenated Ferroheme Proteins from Soybean Nodules

Twenty years ago, the discovery of a red pigment in legume nodules actively fixing atmospheric nitrogen provided a fresh approach to a problem of biology which merits far more than academic interest. Nodules, developing from roots underground in response to specific bacterial infection, to which bacteria they remain host, collectively constitute a plant organ responsible for all molecular nitrogen fixation by legume crops. Studies soon revealed that the nodule pigment is somehow associated with nitrogen fixation and, moreover, that it is an iron porphyrin protein remarkably like the vertebrate hemoglobins. But whereas in crude preparations the pigment could be reversibly deoxygenated, always during purification it became oxidized to the ferric state; thus upon purification it lost the ability to function as a stable oxygen carrier and lost, thereby, its qualification for classification as a hemoglobin. The instability thus evidenced in the presence of molecular oxygen, albeit during processing, cautions against prematurely categorizing the new pigment with the hemoglobins and so attributing to it a function, insufficiently demonstrated, which could be nonphysiological.

While most of these early studies were made on preparations of unresolved pigment, two components were ultimately fractionated by electrophoresis and analyzed (*1*). It was concluded that one native protoporphyrin protein of molecular weight approximately 20,000 and having approximately 0.3 percent iron had been isolated in its ferric state along with an artifact of twice this size.

The following account of the nodule pigment summarizes additional information from new studies, carried out over the past few years, culminating in the isolation of several oxygenated heme proteins from the soybean nodule and suggesting extensive research not only into nodule physiology but also into porphyrin-protein chemistry and physiology (*2*).

Three, and possibly four, ferro-protoporphyrin proteins can be isolated from fresh or frozen nodules of the soybean, *Glycine max,* two of these in abundance (*3*). An essential feature of isolation is rapid processing with controlled temperature and *p*H. Extraction is made by alkaline buffer in a blender, preferably under a stream of hydrogen or nitrogen; fractionation, by ammonium sulfate precipitation, preferably under H_2 or N_2, dialysis, and paper electrophoresis. The two major pigments, designated the *fast* and the *slow* from their respective rates of migration during electrophoresis, have been studied rather extensively.

Even 1-year-old frozen nodules and 1-year-old frozen ammonium sulfate pastes of crude pigment will yield ferro-pigments, but the dialyzed pigment mixture prepared for electrophoresis deteriorates rapidly. The isolated components disintegrate more slowly.

The first step recognized in pigment breakdown is the oxidation of the iron to its ferric state. Since the red, oxygenated pigments separate from their respective brown, oxidized, phases during electrophoresis, progress in oxidation is easily ascertained. In a fresh preparation, little (and, ideally, no) oxidized pigment is present; ultimately, no oxygenated pigment remains. Upon standing, the ferric-components progressively disintegrate to electrophoretically mobile, greenish fragments. Concurrently, denaturation and sedimentation of heme protein occurs.

Satisfactory methods of preservation have not been developed. Deterioration is retarded at low temperatures and in concentrated solutions, especially those containing ammonium sulfate. The use of reductants and antioxidants has not been favored because of the undesirability of complicating an already complex mixture.

The absorption spectra of the oxygenated ferro-pigments are similar, and are typical of oxygenated hemoglobins, with maxima at about 575, 540, and 412 mμ. However, whereas crystalline human oxyhemoglobin, as a reference standard, has an α/β (peak) ratio of 1.05 and an α (peak)/560 (minimum) ratio of 1.70, the freshly separated, oxygenated nodule pigments display diminished ratios of 0.99 and 1.35, respectively. Thus their spectra suggest, by analogy, the possibility of contamination with deoxygenated or, more likely, ferri-pigment; the ratios decrease daily and the spectra reveal a slow transition to the ferric state, with its weaker absorption, which is *p*H dependent, at about 570, 540, and 410 mμ. The maxima of the deoxygenated ferro-spectra lie at about 557 and 424 mμ.

The oxygenated ferro-pigments can be reversibly deoxygenated by chemical means or, similarly, the ferri-pigments can be reduced and oxygenated, but under restricted conditions—for example, when the reductant is promptly removed by electrophoresis. The easily reversible oxygenation of vertebrate hemoglobins has not been observed; instead, oxidation to the ferric state ensues. In alkaline media this oxidation could be mistaken for oxygenation simply because the ferri-pigment in its alkaline form absorbs light of almost the same wavelength as does the oxygenated pigment, though more weakly. Ease of oxidation appears in part to be due to the narrower range of *p*H stability clearly demonstrated by the nodule pigments.

As with ferri-hemoglobin, cytochrome *c,* or acid-base indicators in general, acid-base titration of the ferri-pigments can be followed spectrophotometrically (*4*); like cytochrome *c,* each pigment reveals several heme-linked acids: one of these dissociates reversibly with a *pK* of approximately 8.2, and four dissociate reversibly between *p*H 7 and *p*H 4 with more overlap than permits accurate *pK* analysis. Below *p*H 4, extensive molecular changes occur. Upon aging, the pigments are not titratable below *p*H 7, suggesting an impedance to electron-pair orientation about the porphyrin nucleus. Spectrophotometric titration also suggests a mesomerism which is seen to a lesser extent in the titration spectra of vertebrate hemoglobins.

The alkaline ferro-hemochromogen, the cyan-met, and the porphyrin dimethyl ester derivatives of the unresolved nodule pigment present typical protoporphyrin spectra. The protein moiety split from the hemin of the crude pigment recombines with vertebrate hemin and yields the composite electrophoretic pattern typical of the nodule pigment.

The molecular weights of the two major components are estimated, by dry-weight and heme-iron analysis, to be between 14,000 and 23,000 per heme unit.

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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 μ cm^{-1}/v cm^{-1}, respectively, at *p*H 8.5 (Veronal, 0.1 μ) and as -0.32 and -0.20 μ cm^{-1}/v cm^{-1}, respectively, at *p*H 6.0 (phosphate, 0.1 μ) (*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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References and Notes

1. N. Ellfolk and A. I. Virtanen, *Acta Chem. Scand.* 4, 1014 (1950); 6, 411 (1952).
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 reinstitution 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 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

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 0.1*M* K_2HPO_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 (mμmole)	Cholesterol (mμmole $\times 10^{-2}$)	Fatty acids (mμmole)	Cholesterol (mμmole $\times 10^{-2}$)
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