STUDIES ON THE BIOSYNTHESIS OF HEMIN IN SOY BEAN NODULES*

by

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INTRODUCTION

The observation that root nodules of leguminous plants contain a hemoprotein is of interest since the prosthetic group of this chromoprotein is indistinguishable from that of hemoglobin[1,2]. Hemin synthesis in animal tissues involves the utilization of certain intermediates of the Krebs tricarboxylic acid cycle[3,4]. Since it has been shown recently that the Krebs cycle and oxidative phosphorylation occur in plant and animal tissues[5,6,7], it is conceivable that similar intermediates may be involved in the biosynthesis of the tetrapyrrole nucleus in plant and animal systems. In this investigation we have endeavored, (1) to determine whether soy bean root nodules are able to incorporate the carbon atoms of glycine and acetate into the tetrapyrrole skeleton of hemin and, (2) to characterize the root nodule system as to some of its requirements for *in vitro* operation.

EXPERIMENTAL

Materials

Soy bean root nodules. Nodules were obtained from the roots of soy bean plants whose seeds had been inoculated with "Nitragin"** at the time of planting. The roots were harvested when the plants were about six weeks old. In order to avoid the loss of nodules, the field was flooded*** so that the plants could be pulled out with ease. After the roots were washed free of the adhering soil, the nodules were removed, immediately frozen in a carbon dioxide-acetone bath and stored at about $-15°$ until used. In a few instances fresh nodules were employed.

Isotopic materials. Glycine-1-^{14}C and 2-^{14}C, and acetate-1-^{14}C and 2-^{14}C were used with a specific activity ranging from 17–25 μc per mg†. Sodium carbonate-^{14}C with a specific activity of 28 μc per mg was employed††.

Methods

Measurement of radioactivity. Samples of approximately 2 mg each were weighed out on copper foils and spread uniformly with a suitable solvent over the entire surface of the foil. The samples were then dried, and counted in a flow counter in the proportional region using methane as the

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** This material was a mixture of legume bacteria prepared by The Nitragin Company, Inc., Milwaukee, Wisconsin.

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† Labeled glycine and acetate were obtained from the Nuclear Instrument and Chemical Corporation, Chicago, Illinois.

†† Obtained from the Isotope Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee.

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flow gas. A plateau and background count for each of the chambers used were determined at least once a day.

Preparation and incubation of nodule homogenates. Portions of approximately 25 g (wet weight) of thawed nodules were ground in a Waring blendor or with mortar and pestle. The medium was 0.2 *M* phosphate buffer pH 7.1 in 0.3 *M* sucrose and in some instances phosphate buffer alone; approximately the same results were obtained with both media.

Aliquots equivalent to 5 g of nodules were incubated in modified Warburg vessels[8] at 25° for 210 minutes. The vessels were equipped with two sidearms containing 1 ml of 2 *N* sulfuric acid and 1 ml of 2 *N* potassium hydroxide respectively. Experiments carried out at temperatures ranging from 18–37° indicated that the temperature optimum for a 210 minute incubation period was approximately 25°.

All additions of substrates, cofactors and inhibitors to the incubation media were made after the grinding operation with the exception of sodium fluoride which was added to the media before grinding the nodules in order to inhibit phosphatases and peroxidases.

Isolation of hemin. After completion of the incubation, the potassium hydroxide was removed from the Warburg vessel and stored under xylene, and the incubation mixture was centrifuged. The residue was washed twice with 25 ml of 50% (v/v) acetone-glacial acetic acid, and the washings were combined with the supernatant fluid obtained in the above mentioned centrifugation. This mixture was poured into an equal volume of acetic acid containing 1 mg of hemin. This hemin was added to facilitate isolation and purification and did not influence the pattern of results obtained. Hemin was isolated and purified as previously described[9]. In some instances 0.1 *N* HCl was used instead of acetic acid for the hydrolysis of the hemoprotein. Usually 2 to 5 mg of hemin were isolated in each experiment. In order to ascertain that the porphyrin component present in the hemin isolated was protoporphyrin IX, a sample of pyridine hemochromogen was converted to protoporphyrin IX dimethyl ester according to the procedure of GRINSTEIN[10]. The millimolar ^{14}C-activity of the protoporphyrin IX dimethyl ester was identical with that of the pyridine hemochromogen.

Isolation of the evolved carbon dioxide. Carbon dioxide was collected in 2 *N* potassium hydroxide present in one side arm of the incubation vessel. The carbonate was precipitated with a saturated solution of barium hydroxide. The barium carbonate thus obtained was washed three times with water, once with methanol and once with acetone, and then dried *in vacuo*. Samples of this salt were used for measuring radioactivity.

RESULTS AND DISCUSSION

The results to be reported are average values obtained from 3 to 7 experiments in each particular case. The error in these experiments is less than ± 20% if not stated otherwise. The values obtained are reproducible to within 85% or better if samples of nodule homogenates are compared under similar conditions. The variation within the group of three to seven experiments mentioned did not exceed 25%. The experiments show that some of the reactions leading to hemin formation in plant and animal systems have similar metabolic characteristics, except in their response to the addition of sodium fluoride.

Effect of Substrates and Cofactors. The alpha carbon atom of glycine and the carbon atoms of acetate were incorporated into hemin to approximately the same degree; *i.e.* approximately $40 \cdot 10^3$ disintegrations per min per mmole per μc of glycine or acetate per 5 g of nodules as is shown in Table I. The carboxyl carbon atom of glycine was incorporated to a considerably smaller extent. This finding is different from that obtained in animal tissues which do not utilize a measurable amount of the carboxyl carbon atom of glycine for hemin synthesis[11]. The slight but measurable activity found in hemin isolated from nodule homogenates incubated with carboxyl labeled glycine might be interpreted as incorporation *via* an indirect metabolic route or by carbon dioxide fixation or both.

In order to ascertain the relative importance of CO_2 fixation in this process, the homogenates were incubated in the presence of ^{14}C-labeled carbonate. The results obtained using labeled carbonate and those obtained using glycine or acetate are compared in Table I.

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TABLE I

THE INCORPORATION OF THE CARBON ATOMS OF GLYCINE AND ACETATE AND OF THE CARBON ATOM OF SODIUM CARBONATE INTO HEMIN AND THEIR CONVERSION TO CARBON DIOXIDE

Labeled compound	*% Radioactivity* in hemin*	*% Radioactivity* in the evolved CO_2*
Glycine-2-^{14}C	100	100
Glycine-1-^{14}C	10	160
Acetate-2-^{14}C	120	120
Acetate-1-^{14}C	90	85
Sodium carbonate	**	$1.2 \cdot 10^4$

* The % radioactivity is expressed as the % of the value obtained using α-^{14}C labeled glycine.
** Less than 1 %.

The results show that the alpha carbon atom of glycine and the carbon atoms of acetate were incorporated into hemin to approximately the same degree, while only a very slight amount of carbonate carbon was utilized. The data for the evolved carbon dioxide indicate that the activity in the evolved carbon dioxide arising from carbonate was 100 times greater than that arising from the carbon atoms of glycine and acetate.

Tables II and III show the effects of various substrates, cofactors, and combinations of these substances on the incorporation of the alpha carbon atoms of glycine and acetate into hemin*.

The Krebs cycle acids studied, substances containing sulfhydryl groups, and adenosine triphosphate (ATP) alone inhibited to a slight degree or had no marked effects, whereas coenzyme A (CoA) had a marked inhibitory effect. Most combinations of the compounds studied had also a depressing effect, whereas combinations of CoA and succinate, or of oxaloacetate, CoA, ATP, and acetate using labeled acetate had a stimulatory effect. Magnesium as well as acetate and glycine produced a marked stimulation as illustrated in Table IV which shows the results obtained upon addition of various concentrations of glycine and acetate. The enhancement observed on addition of glycine to soy bean nodule homogenates when labeled glycine was employed is in agreement with that reported by SHEMIN *et al.*[12] using duck blood, and DRESEL AND FALK[13] using hemolyzed avian erythrocytes. Similar results were obtained on addition of glycine when labeled acetate was used.

The augmentation produced by acetate deserves some comment since members of the citric acid cycle such as alpha-keto-glutarate and oxaloacetate were inhibitory and succinate was without effect. That added succinate was without effect might be explained by assuming that there is a deficiency in the enzyme system which is necessary for the activation of succinate. The enhancing effect of acetate on the other hand might be due to the conversion of acetate to active succinate[14]. Thus in the latter case activated succinate would be responsible for the effect observed for acetate, whereas in the former the added succinate lacks activation, and therefore cannot be utilized. This interpretation is applicable to experiments in which either labeled acetate or labeled glycine was used. The stimulation observed when an excess of glycine was added might be interpreted to mean either the involvement of a coenzyme derivative which plays a common role in the activation of glycine[15] and transamination reactions,[16] or enzyme saturation. After

* The same general pattern of results was observed using carboxyl labeled glycine and acetate but for the sake of brevity these results are not reported.

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TABLE II

EFFECT OF SUBSTRATES AND CO-FACTORS ON THE INCORPORATION OF THE α-CARBON ATOM OF GLYCINE INTO HEMIN IN SOY BEAN NODULE HOMOGENATES

ml of 1.0 M succinate added	*ml of 0.1 M α-keto-glutarate added*	*ml of 0.1 M oxaloacetate added*	*ml of 0.1 M pyruvate added*	*mg of coenzyme A*** *added*	*mg of adenosine triphosphate added*	*ml of 1.0 M glucose added*	*ml of 1.0 M malate added*	*ml of 1.0 M magnesium chloride added*	*mg of glutathione added*	*ml of 1.0 M malonate added*	*ml of 0.1 M cysteine added*	*Hydrogen sulfide added*	*mg of diphospho-pyridine*** nucleotide added*	*Radioactivity in hemin % of control value*
—	—	—	—	—	—	—	—	0.2		—		—	—	112
—	—	—	—	—	—	—	0.2	0.2		—		—	—	150
—	—	—	—	—	—	5	—	—		—		—	—	69
—	—	—	—	—	4	—	—	—		—		—	—	100
—	—	—	—	5	4	—	—	—		—		—	—	48
—	—	—	2	5	4	—	—	—		—		—	—	62
—	—	—	—	—	—					—		—	5	95
—	2	—	—	—	—	—	—	—		—		—	—	78
—	—	—	2	—	—	—	—	—		—		—	—	50
—	—	2	—	—	—	—	—	—		—		—	—	45
—	—	—	—	—	—	—	1	—		—		—	—	45
1.0		—	—	5	—		—		—	—	—	—		140
1.0		—	—	5	4		—		—	—	—	—		145
1.0		—	—	5	4		—		—	5	—	—		60
—		—	—	—	—		—		10	—	—	—		70
—		—	—	—	—		—		—	—	5	—		65
—		—	—	—	—		—		—	—	—	+		67
1.0		—	—	—	—		—		—	—	—	—		86
—		—	—	—	—		0.5		—	—	—	—		38*
—		2	—	5	4		—		—	—	—	—		78
—		2	2	5	4		—		—	—	—	—		24*

* The radioactivity was so low that a reliable measurement could not be carried out with an error of less than 25%.
** Obtained from Pabst Laboratories, Milwaukee, Wisconsin. The preparation was 70% pure.
*** Obtained from Schwarz Laboratories, New York, New York.
+ Hydrogen sulfide was present in the media.

TABLE III

EFFECT OF SUBSTRATES AND CO-FACTORS ON THE INCORPORATION OF THE α-CARBON ATOM OF ACETATE INTO HEMIN IN SOY BEAN NODULE HOMOGENATES

ml of 1.0 M succinate added	*ml of 0.1 M α-keto-glutarate added*	*ml of 0.1 M oxaloacetate added*	*ml of 0.1 M pyruvate added*	*mg of coenzyme A added*	*mg of adenosine triphosphate added*	*ml of 0.1 M $MgCl_2$ added*	*ml of 0.1 M cysteine added*	*% of the control radioactivity in hemin*
–	–	–	–	–	4	–	–	118
–	–	–	–	5	–	–	–	31
–	–	2	–	–	4	–	–	143
–	–	–	2	5	4	–	–	35
–	–	2	2	5	4	–	1	34
–	1	–	–	–	–	–	–	82
–	2	–	–	5	4	–	–	180
–	–	–	–	–	–	1	–	140
1	–	–	–	–	–	–	–	90

TABLE IV

EFFECT OF THE CONCENTRATION* OF GLYCINE AND ACETATE ON THE INCORPORATION OF THE α-CARBON ATOM OF GLYCINE INTO HEMIN IN SOY BEAN NODULE HOMOGENATES

Molar concentration of sodium acetate added	*Molar concentration of glycine added*	*Radioactivity in hemin % of control value*
0.01	0.00	50
0.01	0.005	75
0.01	0.01	95
0.01	0.02	122
0.01	0.03	120
0.01	0.05	100
0.00	0.02	95
0.002	0.02	96
0.004	0.05	106
0.01	0.02	134
0.02	0.02	120

* Approximately 1.0 mg of glycine containing 12 μc of labeled glycine were also added in each experiment.

the addition of a larger amount of glycine more of this coenzyme derivative for the activation of glycine would be required while at the same time the amount of this derivative available for transamination reactions would be decreased. Thus, a greater quantity of keto acids would be available for the formation of the intermediate which is derived from the Krebs acid cycle and is required for hemin formation. Conversely the addition of an excess of alpha-ketoglutarate may result in the utilization of the coenzyme which is required for the activation of glycine. This assumption might explain the inhibition observed on addition of alpha-ketoglutarate as shown in Tables II and III.

The coenzyme derivative necessary for the activation of glycine and transamination reactions involving keto acids of the Krebs cycle has been shown to be a pyridoxal derivative[15,16]. It has been demonstrated recently that the incorporation of iron into the tetrapyrrole structure is inhibited in blood obtained from birds which had been fed desoxypyridoxal[17], thus illustrating that pyridoxal plays a role in hemin synthesis. Because of the small amount of hemin formed and the rather large quantity of glycine

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added it seems unlikely that the phenomenon observed can be explained by enzyme saturation. The enhancement in isotope incorporation into hemin brought about by CoA, ATP, acetate and oxaloacetate with labeled acetate is probably due to the formation of citrate[18] which would facilitate the operation of the Krebs cycle.

It is of interest that CoA and succinate stimulated, whereas CoA alone or succinate alone inhibited or had no effect.

It might be pointed out that the effects observed upon addition of CoA could be reproduced using two CoA preparations of different degrees of purity. The same effect was observed upon addition of a crude pork liver CoA preparation (20% pure)[§] or upon addition of a CoA preparation from yeast (70% pure). Equivalent amounts of CoA were equally effective, notwithstanding the source.

The effect of CoA and succinate indicates that these substances are involved in the formation of succinyl-CoA which has been shown to occur in plants[18] and which has been postulated by SHEMIN *et al.*[19,20] as the intermediate involved in hemin synthesis. The effect observed when CoA or succinate was used singly indicates that enhancement or inhibition is a function of the relative concentration of these substances. An enhancement was also observed when magnesium was added. This finding might be explained by the recent observations by MILLERD AND BONNER[18] that magnesium is necessary for the formation of acetyl-CoA and succinyl-CoA.

The results obtained on adding ATP might be interpreted to mean that the system already contained sufficient amounts for hemin formation, or that the added ATP is ineffective. The observation that the addition of fluoride and ATP has no effect indicates that some of the ATP is not decomposed under these conditions. The same interpretation might be offered for the inhibitory action of CoA alone. It could be assumed that the inhibition is the consequence of directing the reaction in other pathways, *e.g.* those involved in fat metabolims. The inhibitory action of substances containing sulfhydryl groups could also be due to the activation of CoA.

Effect of Inhibitors. The effect of inhibitors is presented in Table V. Sodium azide, and malonate showed a strong inhibitory effect, whereas 2,4-dinitrophenol did not influence markedly the incorporation of the alpha carbon atom of glycine into hemin. Sodium fluoride and sodium hydrosulfite had a marked stimulatory action.

TABLE V

EFFECT OF SOME INHIBITORS* ON THE INCORPORATION OF THE α-CARBON ATOM OF GLYCINE INTO HEMIN IN SOY BEAN ROOT NODULE HOMOGENATES

Substances added	*Radioactivity in hemin % of control value*
0.02 *M* Sodium fluoride	180
0.01 *M* Sodium hydrosulfite	150
0.04 *M* Malonate	50
0.01 *M* Sodium azide	30**
0.005 *M* 2,4-Dinitrophenol	90
0.01 *M* Sodium fluoride and 0.01 *M* Magnesium chloride	200

* A ten-fold dilution of the inhibitors did not change the effects observed.
** This measurement was carried out with a probable error of approximately 30%.

§ We wish to thank the Armour Laboratories, Chicago, for a gift of this preparation.

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The action of malonate, sodium hydrosulfite and sodium fluoride merits some comment. The inhibitory action of malonate in soy bean nodule homogenates resembles that observed in animal tissues[21]. SHEMIN AND KUMIN have suggested a scheme of porphyrin synthesis based on observations made with avian blood cells incubated with methylene and carboxyl labeled succinate in the presence and absence of malonate[19]. It is difficult to compare the results of these authors with the results reported here which were obtained using soy bean nodule homogenates incubated in the presence of alpha-^{14}C-labeled glycine. It should be mentioned that malonate inhibited the incorporation of glycine-2-^{14}C into hemin in the presence of added succinate and coenzyme A in all instances observed. This finding is similar to that obtained using bone marrow homogenates[9].

It indicates that malonate blocks the formation of the required succinyl intermediate or its utilization for hemin synthesis or both. Although the mechanism of the inhibitory effect produced by malonate has not been elucidated, the following possibilities might be considered.

(1) Malonate may inhibit reactions other than the conversion of succinate to fumarate[22] namely the formation of succinyl-CoA from succinate or the condensation of succinyl-CoA with a glycine derivative. Malonate may compete with succinate for CoA and the malonyl-CoA formed may also inhibit the condensation of glycine with succinyl-CoA.

(2) The inhibition of the transformation of succinate to fumarate by malonate may result in the accumulation of alpha-ketoglutarate which has been mentioned above.

(3) The inhibition of the Krebs cycle by malonate may decrease the amount of energy available for hemin formation.

The stimulatory effect of sodium fluoride may be the consequence of one or several of the following factors. Sodium fluoride might inhibit the peroxidases present in soy bean nodule homogenates, and consequently prevent the decomposition of the newly formed as well as of the already existing hemoprotein[1]. Sodium fluoride might also inhibit the phosphatases[5,7] present in the preparation, and consequently increase the amount of phosphorylative energy available for hemin formation. The addition of magnesium did not reverse the effect of fluoride. This might indicate that fluoride forms a stable complex with the hemoprotein rather than complexing with dissociable magnesium.

The stimulation observed in the presence of sodium hydrosulfite might be related to the reducing action of this compound on ferrihemoglobin or on peroxidases[1,23]. This reductive action would tend to prevent the decomposition of the hemoprotein since ferrohemoproteins are more stable than ferrihemoproteins.

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SUMMARY

Soy bean nodule homogenates are able to incorporate the carbon atoms of acetate and glycine into hemin. Acids of the Krebs cycle, malonate and sodium azide inhibited the incorporation of the α-carbon atom of glycine into hemin. CoA and substances containing sulfhydryl groups also inhibited the above reaction. Magnesium, acetate, glycine, hydrosulfite and fluoride stimulated. The effects of various combinations of these substances were also investigated. Some implications of the results obtained are discussed.

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RÉSUMÉ

Les homogénats de nodosités de soja incorporent les atomes de carbone de l'acétate et du glycocolle dans l'hémine. Les acides du cycle de Krebs, le malonate et l'azoture de sodium inhibent l'incorporation de l'atome de carbone α du glycocolle dans l'hémine. Le CoA et des substances renfermant des groupements sulfhydryle inhibent également cette réaction. L'acétate, le magnésium, le glycocolle, l'hydrosulfite et le fluorure la stimulent. L'action de diverses combinaisons de ces substances a été également étudiée. Quelques unes des conséquences des résultats obtenus sont discutées.

ZUSAMMENFASSUNG

Homogenate aus Sojabohnenknöllchen sind imstande, die Kohlenstoffatome des Natriumacetats und des Glykokolls in Hämin einzuverleiben. Säuren des Krebs Zyklus, Malonat und Natriumazid hemmen die Einverleibung des α-Kohlenstoffatoms des Glykokolls in Hämin. Coenzym A sowie Verbindungen die Sulfhydrylgruppen aufweisen hemmen diese Reaktion ebenfalls. Magnesium, Acetate, Glykokoll, Hydrosulfit und Fluorid beschleunigen sie. Die Wirkung verschiedener Mischungen dieser Substanzen wurde ebenfalls untersucht und etliche, zu den erhaltenen Ergebnissen in Beziehung stehende Fragen, erörtert.

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