THE PORPHYRIN REQUIREMENTS OF HAEMOPHILUS INFLUENZAE AND SOME FUNCTIONS OF THE VINYL AND PROPIONIC ACID SIDE CHAINS OF HEME*

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INTRODUCTION

Proteins bearing the prosthetic group Fe protoporphyrin IX (or closely related groups) are now recognized to function in the transport of oxygen, in the catalytic activation of O_2 and H_2O_2, and in the shuttling of electrons from dehydrogenases to the oxygen-activating enzymes. The structure of this prosthetic compound is a complex one. Attention in the past has been focused mainly on the important problem of the attachment of the iron of this compound to some specific group of an apoprotein which endows the complex with its own particular activities. In this paper we shall be concerned primarily with another aspect of the Fe porphyrin ring, namely, its specific side chains and their possible functions.

The structure of Fe protoporphyrin IX (Fig. 1) is well established. It consists of 4 pyrrole rings attached to each other through 4 CH-methene bridges to form a 16-membered resonating ring. In the β β'positions of the pyrrole rings are side chains which characterize the porphyrin type. The naturally occurring protoporphyrin IX has the following order and kinds of side chains around the ring: methyl, vinyl, methyl, vinyl, methyl, propionic acid, propionic acid, methyl.

A survey of all of the structures of the known, naturally occurring Fe porphyrins, including cytochrome C, reveals that all of them, without exception, possess two propionic acid side chains in adjacent positions. Likewise the methyl side chains are a constant feature in all of them. The vinyl side chains show less constancy;here a formyl group may replace one of the vinyl groups, as in *Spirographis* heme; or the vinyl groups may be modified as in cytochrome C.

Several notable contributions are already available as an approach to a study of the functions of the propionic acid and vinyl group side chains. The Lwoffs (1) in their excellent study of the growth requirements of certain trypanosomides found that only protoporphyrin or Fe protoporphyrin could support the growth of these organisms; other porphyrins or their iron derivatives which lacked the vinyl groups could not support growth. In the bacterial organism *Haemophilus influenzae*, none of the

* This is the first of a series of studies on porphyrins and related compounds.

following substances was found by Olsen (2) to support growth: hemocyanin, hematoporphyrin, bilirubin, chlorophyll, pyrrole.

The functions of the side chains have also been studied in connection with the activity of the heme proteins. For example, some clues to the functions of the side chains have been provided by a study of the resynthesis of native globin with different Fe porphyrins by the method of Anson and Mirsky. Hill and Holden (3) were the first to attempt such an experiment, and showed qualitatively that oxygen could be taken up reversibly if Fe mesoporphyrin or Fe hematoporphyrin were the prosthetic group of globin. Later a more systematic study of the coupling of renatured globin with several iron porphyrins was made by Warburg and Negelein (4). They showed that the replacement of two vinyl groups by two ethyl groups, as in Fe rhodoporphyrin, or by two acetyl groups as in Fe diacetyl deuteroporphyrin, did not appreciably affect the oxygen-carrying activity of the resynthesized hemoglobins. Nor did the replacement of one carboxyl for a propionic acid side chain (*i.e.* Fe rhodoporphyrin) affect the activity. The use of Fe pheophorbide *b* as prosthetic group gave a globin complex having no activity; in this compound one of the pyrrole rings is reduced and one of the propionic acid groups is replaced by a phorbin ring which bears no ionized carboxyl group.

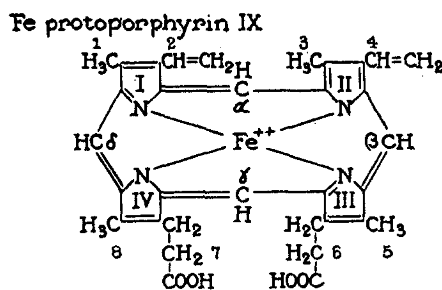

FIG. 1

Theorell, Bergström, and Akeson (5) made a more extensive study of the reconstitution of peroxidase by replacing the normal prosthetic group, Fe protoporphyrin IX, with other Fe porphyrins. The Fe meso- and Fe deutero-compounds in which the vinyl groups are replaced by ethyl, and H, respectively, were found to be quite active. However, Fe hematoporphyrin in which the vinyl groups are replaced by the α hydroxy ethyl groups showed no activity. The hematoporphyrin compound is difficult to purify. Gjessing and Sumner (6) in this connection report that an active peroxidase preparation could be constructed from one of their Fe hematoporphyrin preparations but not from another. Fe rhodoporphyrin was inactive for peroxidase activity, although, as noted above, it was active for hemoglobin function. Fe pyrroporphyrin, in which one propionic acid group is replaced by an H atom, was inactive.

From the studies cited, one may conclude that the vinyl groups are not essential for the functioning of hemoglobin or peroxidase, but that the lack of one acid group appeared to extinguish activity. For the growth of the trypanosomides, however, the vinyl groups appear to be essential. Nothing can be

concluded from the data as to the necessity of the propionic acid side chains for growth of these organisms.

Methods

A. Preparation of the Porphyrin Compounds

The following compounds were prepared in the crystalline state, starting with beef hemoglobin, by methods described in Fischer and Orth (7), and gave theoretical values for N. (For a number of the compounds C, H, and ash[1] analyses were also made): Protoporphyrin, ferric protoporphyrin chloride, ferric protoporphyrin dimethyl ester chloride, protoporphyrin dimethyl ester, mesoporphyrin, mesoporphyrin dimethyl ester, ferric mesoporphyrin dimethyl ester chloride, deuteroporphyrin hydrochloride deuteroporphyrin dimethyl ester, ferric deuteroporphyrin chloride, ferric hematoporphyrin dimethyl ether dimethyl ester. Hematoporphyrin and ferric hematoporphyrin were not obtained in a well crystallized state. Porphyrin (porphin) was prepared according to the method of Fischer and Gleim (8) in very poor yield and identified by its absorption spectrum.

Ferric mesoporphyrin chloride was obtained crystalline and in good yield directly from hemin by Davies' method (9). Coproporphyrin I was isolated from brewers' yeast. Coproporphyrin I tetramethyl ester, originally from Fischer's laboratory, was obtained from Dr. K. Dobriner whom we wish to thank for this sample.

With large volumes of liquid it was found convenient to centrifuge down the Fe^{+++} protoporphyrin chloride crystals in a Sharples centrifuge. This compound was also prepared from human erythrocytes preserved in citrate; the hemin crystals which formed were small and were grossly contaminated with protein material in contrast to those obtained by using beef hemoglobin. The hemin was recrystallized according to the procedure described by Fischer and Orth (7)[2].

Protoporphyrin was prepared by a modification of the method of Fischer and Pützer (10). Six gm. of recrystallized hemin and 400 gm. of 95 per cent formic acid were added to a liter 3 necked flask, with stirrer and reflux attached, and heated on an oil bath. A total of 6.0 gm. of reduced iron powder was added in the following way. Before the formic acid came to a boil 0.3 gm. of iron was added. As boiling began, the iron was added in 6 to 7 portions over a period of 20 to 25 minutes, with an additional 5 minutes' boiling at the end. The flask was removed from the bath and after 10 to 15 minutes the contents were filtered by suction, and the residue on the filter paper washed with a little formic acid. The deep red formic acid solution was poured into 2 liters of distilled water. Then 100 cc. of saturated ammonium acetate solution was added on the side of the beaker without stirring and left overnight at room temperature. The fine, flocculent protoporphyrin was centrifuged and then transferred to a suction filter, washed, dried *in vacuo*, and recrystallized. The yield after recrystallization was 70 to 75 per cent of theory.

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[1] Analyses were done by Dr. A. Elek.

[2] Fischer and Orth (7), **2**, 440.

B. Bacteriological Methods

Six smooth strains and one rough strain (Turner) of *Haemophilus influenzae* have been used in the present studies. Only the bare essentials of the bacteriological method will be mentioned, leaving for a later paper the details and refinements.

The basal medium for the culture of the organisms consisted of 2.0 gm. Difco proteose peptone, 0.6 gm. NaCl, and 0.2 gm. KNO_3, made up to 100 cc., adjusted to pH 7.4 with NaOH, filtered, 10 mg. sodium hydrosulfite added, and the medium then autoclaved. The KNO_3 was added in order to detect the presence of the nitrate-reducing enzyme whose activity is proportional to the growth of the organisms under certain conditions.

The need for supplying porphyrins to the organisms in aqueous solution at neutral pH presented a difficulty owing to the great insolubility of the porphyrins. It was necessary to prepare the solution fresh every day. Most of the porphyrins and iron porphyrins were dissolved in 50 per cent ethanol, being 0.02 N with respect to KOH, to make a concentration of 100 to 500 γ of the porphyrin per cc. This solution was then diluted with sterile aqueous 0.02 N KOH to appropriate volume. Addition of aliquots of this alkaline solution to the well buffered proteose peptone medium changed the acidity by less than 0.1 pH unit. In the case of the esterified porphyrins, alkaline solutions were avoided, dilutions being made from neutral alcoholic solution with sterile distilled water. Controls showed that in no experiments described here was the alcoholic content of the porphyrin solution added to the medium sufficient to cause inhibition by itself.

The experiments were carried out in 5 cc. volumes, containing 4 cc. of the 2 per cent proteose peptone, 0.2 cc. of a solution of coenzyme, 0.1 to 0.5 cc. of the dilutions of porphyrins being tested, and saline to make 4.9 cc. The coenzyme I, prepared by the method of Williamson and Green (11), was of 12 per cent purity as determined spectrophotometrically. The quantity of coenzyme added to the tubes was equivalent to 0.019 γ pure coenzyme and was about five times the amount required for optimum growth. The inoculum consisted of 0.1 cc. of a suspension of organisms prepared by diluting a 6 hour culture of the washed cells to ten times its original volume. The tubes were incubated at 37° for 16 hours. Growth was determined by visual turbidity, later more exactly (but essentially in agreement with the visual method) by spectrophotometric analysis, and also colorimetrically by the reduction of nitrate to nitrite. The *Haemophilus* organism has the ability to reduce nitrate to nitrite, growth being proportional to nitrite produced under certain conditions. The nitrite produced is not further changed nor does it inhibit the growth of the organisms. The nitrite determinations were carried out by the diazotization and coupling method of Shinn (12) as modified by Hoagland and Ward (13), and the intensity of the pink color which resulted was measured at 545 mμ with a spectrophotometer.

EXPERIMENTS

I. Insertion of Iron into the Protoporphyrin Ring to Form Heme

In the biological synthesis of heme two possibilities suggest themselves: Either the porphyrin ring might be imagined to be formed piecemeal around

TABLE I

Growth-Promoting and Inhibitory Properties of the Porphyrins and Iron Porphyrins on Strains of Haemophilus influenzae

	Characteristic side chains at positions			Rough strain Turner					Smooth strain types A, B, C, D, E, F			
	1,3,5,8	2,4	6,7	Support of growth	γ/cc. for just maximum growth	Nitrate reduction	Maximum concentration used for test (γ/cc.)	Inhibition of growth on Fe proto-	Support of growth	Nitrate reduction	γ/cc. for just maximum growth	Inhibition of growth on Fe proto-*
*Proto*porphyrin...	$-CH_3$	$-CH{=}CH_2$	$-CH_2CH_2COOH$	+	0.05–0.10	+		0	+	+	0.1–0.3	0
Fe proto......	"	"	"	+	0.05–0.10	+		0	+	+	0.1–0.3	0
Dimethyl proto-	"	"	$-CH_2CH_2COOCH_3$	0			0.5	0				
Fe dimethyl proto-........	"	"	"	0‡			0.5	0				
Porphyrin (porphin).........	$-H$	$-H$	$-H$	0			35.0					
*Meso*porphyrin....	$-CH_3$	$-CH_2-CH_3$	$-CH_2CH_2COOH$	+§	0.05	0		+§	0	0		+
Fe meso........	"	"	"	+	0.05–0.10	0		0	+	0	0.1–0.3	0
Dimethyl meso-	"	"	$-CH_2CH_2COOCH_3$	0			1.0	0				
Fe dimethyl meso-.........	"	"	"	0			1.0	+‖				
*Hemato*porphyrin.	"	$-CH(OH)-CH_3$	$-CH_2CH_2COOH$	0			12.0	+	0	0		+
Fe hemato-.....	"	"	"	+	0.5–1.0	0		0	+	0	2.0	0
Fe tetramethyl hemato-......	"	$-CH(OCH_3)-CH_3$	$-CH_2CH_2COOCH_3$	0			0.5	0				
*Deutero*porphyrin.	"	$-H$	$-CH_2CH_2COOH$	0			2.0	+	0	0		+
Fe deutero.....	"	"	"	+	0.1–0.3	0		0	+	0	0.1–0.3	0
Dimethyl deutero-......	"	"	$CH_2CH_2COOCH_3$	0			1.0	0				
*Copro*porphyrin I.	1,3,5,7 $-CH_3$		2, 4, 6, 8 $-CH_2CH_2COOH$	0			2.0	+				
Tetramethyl copro-I.......	"		$-CH_2CH_2COOCH_3$	0			12.0	0				
Cytochrome C....				0			12.0					
Catalase-beef (crystalline)....				0			20.0					

* Only strain type E was used for these inhibition tests.

‡ It required a concentration of 50 to 200 times as much Fe dimethyl protoporphyrin as of Fe protoporphyrin to obtain a variable trace of growth. Possibly some hydrolysis of the methyl groups occurred.

§ Mesoporphyrin supported growth of the rough Turner strain at low concentrations but inhibited competitively the growth on Fe protoporphyrin at higher concentrations.

‖ Fe dimethyl mesoporphyrin inhibited only partially even at high concentrations. The inhibition was not of the competitive type.

an iron atom or the porphyrin ring might be first completely formed and the iron inserted afterwards.

When protoporphyrin, *i.e.* heme lacking iron, was added to the medium in place of heme, it was found to be just as effective as heme in supporting growth of all the strains of *H. influenzae* studied (Table I). This confirms the finding of M. Lwoff (1) on one *H. influenzae* strain. In fact, being less susceptible to peroxidative decomposition, protoporphyrin was often more effective than hemin. In order to demonstrate that iron had been inserted into the protoporphyrin ring the Turner strain was first transferred through seven successive passages of media containing protoporphyrin in order to reduce to a minimum the amount of hemin which would be introduced from the original inoculum. Tests were then made on the organisms for the presence of heme. The absorption spectrum of the washed, packed organisms was too diffuse to characterize with certainty the presence of iron porphyrin in the presence of the bands of protoporphyrin; nor were the pyridine hemochromogen bands sufficiently distinct. However, a peroxidase test on the boiled organisms with benzidine and H_2O_2 was distinctly positive, indicating the presence of iron porphyrins. The presence of a relatively strong catalase activity in the organisms grown on protoporphyrin was also demonstrated by measuring the O_2 released in a Warburg manometer. Forty γ dry weight of the organisms was found to decompose three-fourths of the peroxide in 1 cc. of 0.02 M H_2O_2 in 100 seconds at 30°C. During this time no detectable peroxide destruction was observed in the blank of previously boiled organisms. Since catalase contains heme as a prosthetic group this is further evidence that iron was inserted into the complete protoporphyrin ring by *H. influenzae*.

II. Efficiency of Other Porphyrins As Growth Factors for H. influenzae and the Function of the Vinyl Groups

Iron-free porphyrins other than protoporphyrin did not support growth of the six smooth strains of *H. influenzae* used (Table I). (Growth of the rough Turner strain on mesoporphyrin will be discussed below.) These porphyrins differ from protoporphyrin in the substitution of hydroxy ethyl (hemato), or ethyl (meso), or H (deutero) for the vinyl groups at positions 2 and 4.

Since the vinyl groups appear essential for growth, the possibility suggested itself that they might be required for the formation of cytochrome C. However no cytochrome C could be detected in these organisms by several tests. No reduced cytochrome C band was visible in a thick suspension of the organisms. They also failed to oxidize rapidly ascorbic acid or hydroquinone or *p*-phenylene diamine, an oxidation which is characteristic of the cytochrome C-cytochrome oxidase system. Therefore the vinyl porphyrin must have some other function.

Although porphyrins lacking vinyl groups did not support growth, the re-

lated Fe porphyrins did support growth (Table I). Fe deutero- and Fe meso- were as effective as Fe protoporphyrin. Fe hematoporphyrin was about one-tenth as effective. Undenatured cytochrome C of beef heart muscle, and undenatured crystalline beef catalase, *i.e.* iron porphyrins attached to proteins, were not effective in supporting growth, possibly because they were too large to enter the organism. From these results it is apparent that for the growth of the smooth *H. influenzae* the vinyl side chains are unimportant if an iron-containing porphyrin is supplied. When a porphyrin alone is supplied then only the one containing the vinyl groups, namely protoporphyrin, is capable of supporting growth. Since it has been shown that during growth protoporphyrin is converted to iron protoporphyrin it follows that the vinyl groups may be either directly or indirectly concerned with the insertion of iron into the protoporphyrin ring.

Apparently the enzymes formed in *H. influenzae* by replacement of Fe protoporphyrin with other Fe porphyrins are capable of functioning in reactions that are essential for their growth. This does not necessarily mean that it is possible to substitute all the functions of Fe protoporphyrin by other prophyrins. We have found, for example, that the vinyl groups of Fe protoporphyrin have a function which cannot be replaced by non-vinyl-containing porphyrins; that is, they are required for nitrate reduction (Table I). When *H. influenzae,* both smooth and rough strains, were grown in the presence of Fe protoporphyrin or protoporphyrin, the nitrate reduction was found to be proportional to their growth, as judged by turbidity measurements. When growth was supported by other iron porphyrins not the slightest trace of nitrite formation was observed. In both cases tests showed that nitrite, if added to the medium in which the organisms were growing, did not undergo change. It appears then that the vinyl groups play some rôle in nitrate reduction.

III. Competitive Inhibition between Non-Vinyl-Containing Porphyrins and Protoporphyrin in H. influenzae

When porphyrins, which had been shown to be incapable of supporting growth, were added to a medium containing protoporphyrin or iron protoporphyrin, which supported growth, it was found that growth was inhibited. This phenomenon suggested that a competition for heme apoenzymes existed between the vinyl-containing and non-vinyl-containing porphyrins.

It was found that the inhibition was of the competitive type; that is, the ratio between the concentration of the growth-promoting porphyrin and of the porphyrin inhibiting growth was relatively constant. An example of such an experiment is given in Table II. Here the competition between protoporphyrin and hematoporphyrin was studied. Growth was estimated by visual turbidity and also colorimetrically by following the extent of nitrate reduction. The concentration of the growth-promoting protoporphyrin was varied from 0.01 to

0.2 γ per cc. of media. (The insolubilities of the porphyrins limited the range of concentrations at which they could be tested.) From Table II it will be seen that within the range of concentrations used it required approximately 10 molecules of hematoporphyrin to inhibit almost completely the growth-promoting properties of 1 molecule of protoporphyrin.

TABLE II

Competition between the Growth-Promoting Protoporphyrin and the Growth-Inhibiting Hematoporphyrin in H. influenzae Turner

Protoporphyrin γ per cc. media	Hematoporphyrin γ per cc. media	Growth after 16 hrs.	Nitrate reduction after 16 hrs.	Molecular ratio of hemato-proto for almost complete inhibition
0.00		0	0	
0.01		±	±	
0.03		++++	++++	
0.05		++++	++++	
0.10		++++	++++	
0.20		++++	++++	
0.03	0.10	+++	+++	
0.03	0.20	++	+	
0.03	0.40	±	0	←13/1
0.03	0.60	0	0	
0.05	0.10	+++	++	
0.05	0.30	++	±	
0.05	0.50	+	±	←10/1
0.10	0.20	+++	++	
0.10	0.60	++	+	
0.10	1.00	±	0	←10/1
0.20	0.20	+++	+++	
0.20	0.60	++	±	
0.20	1.00	±	0	← 5/1
0.20	1.40	0	0	

Deuteroporphyrin and coproporphyrin were also tested and found to inhibit the action of protoporphyrin to about the same degree as did hematoporphyrin. This is one of the few instances reported in which for nearly complete inhibition the ratio of growth substance to competing molecules is as low as 1:10. The experimental evidence of competitive inhibition supports the assumption that the porphyrins compete with each other for the apoproteins which normally carry iron porphyrin as prosthetic group.

Competition could also be shown to occur between the two iron-containing

porphyrins, Fe protoporphyrin and Fe mesoporphyrin, in regard to nitrate reduction. Both support growth but the nitrate-reducing activity of Fe protoporphyrin is diminished by the presence of Fe mesoporphyrin. Growth as judged by turbidity was enhanced in low concentrations of a mixture of the two compounds and the nitrite production fell off as the Fe mesoporphyrin concentration increased.

IV. Anomalous Behavior of Mesoporphyrin in the Rough Turner Strain

In the six smooth strains of *H. influenzae*, mesoporphyrin not only was ineffective in supporting growth but also acted as an inhibitor to the growth-promoting protoporphyrin. On the other hand, the inhibition of the growth of the rough Turner strain by mesoporphyrin was only observed at concentrations above 0.12 γ/cc. of media; at lower concentrations (*i.e.* 0.01 to 0.10 γ/cc.) mesoporphyrin actually supported growth and the organism could be transferred serially on the lower concentrations of mesoporphyrin as well as on protoporphyrin. The positive benzidine peroxidase test on the boiled organisms grown on mesoporphyrin indicated that iron had been inserted into the mesoporphyrin ring. The nitrate-reducing property, however, was lacking when growth occurred on mesoporphyrin as was to be expected in view of the necessity of vinyl groups for this reaction. The ability of the rough Turner strain to grow on mesoporphyrin at low concentrations was not due to its property of "roughness." When two strains of "smooth" *H. influenzae* were made "rough" they were not capable of growing on mesoporphyrin at any concentrations used. Since Fe mesoporphyrin regularly supports growth in all strains, it appears that the Turner strain is capable of inserting iron into mesoporphyrin, and it is the only one of these organisms which can do so.[3]

The phenomenon observed can be explained on the basis of two assumptions; one is that the rate of insertion of iron into mesoporphyrin is slow compared with the rate at which iron is incorporated into protoporphyrin. The other assumption is that iron is first inserted into the porphyrin ring and the completed Fe porphyrin then adsorbed to the apoenzyme, rather than that the porphyrin ring is adsorbed to the apoenzyme and the iron inserted afterwards. When mesoporphyrin is provided at low concentrations to the Turner organism the mesoporphyrin would be converted relatively slowly to Fe mesoporphyrin and adsorbed to the apoenzymes to function in the support of growth. When

[3] Using a sample of crystalline hematoporphyrin kindly supplied us by Dr. A. Pappenheimer, Jr., we have observed a slight tendency to support growth of the Turner organism. A "one plus" growth at a concentration of 0.05 γ/cc. of hematoporphyrin was occasionally observed. However, this occurred only when the inoculum was larger than usual and it was not possible to transfer the organism serially with this compound.

mesoporphyrin is supplied at high concentrations, so much mesoporphyrin would enter the cell that it would compete for the apoenzymes with the small amounts of Fe mesoporphyrin that were being formed and thus bring about inhibition of growth. It can be shown that mesoporphyrin at high concentrations is inhibitory to these organisms only in so far as it competes for the heme apoenzymes, since Fe protoporphyrin has been found to overcome the inhibitory action of high mesoporphyrin concentrations. Very high concentrations of protoporphyrin (up to 5 γ/cc.) were not found to inhibit growth, perhaps because the rate of iron insertion into protoporphyrin may be so rapid that the protoporphyrin concentration in the neighborhood of the apoenzymes will be low compared to the Fe protoporphyrin concentration.

V. Growth and Inhibition of H. influenzae As Related to the Propionic Acid Side Chains of the Porphyrins and Fe Porphyrins

When protoporphyrin or the Fe porphyrins of proto-, meso-, deutero-, and hematoporphyrin which normally supported growth were methylated at the two propionic acid side chains to form the propionic acid methyl esters, they no longer supported growth (Table I). It is evident that the free propionic acid groups are required for growth. In the case of growth of the Turner strain on low mesoporphyrin concentrations, methylation likewise extinguished the growth-promoting properties of this compound.

When the porphyrins deutero-, meso-, hemato-, and copro-, which normally inhibited growth, were methylated at the propionic acid side chains they no longer possessed inhibitory properties. It is evident then that the free propionic acid groups are required for this function.

These findings lead to the interpretation that the propionic acid side chains are required for attachment to the specific apoenzymes. When the propionic acid groups are esterified they cannot attach to the proteins and therefore can not function either to support growth, or to inhibit growth in the presence of Fe protoporphyrin. There is some evidence that the coordination of the central iron atom to some one group of the apoprotein surface, which endows it with its own particular function (for example, as in hemoglobin where the iron is postulated to be attached to the imidazol histidine nitrogen of the globin (14)), is not sufficiently strong by itself to bind the heme. Our experiments suggest that the propionic acid side chains serve to orient the heme in proper position on the apoprotein at the time of attachment, and to anchor the heme more firmly to the protein.

In protoporphyrin IX, the propionic acid groups are in positions 6 and 7 on the pyrrole rings. In coproporphyrin I there are 4 propionic acid groups at positions 2, 4, 6, and 8. Since coproporphyrin I is as inhibitory as is deuteroporphyrin IX for example, the distances separating the acid groups on the porphyrin do not appear to be critical for protein atttachment. The experiment

of Warburg and Negelein (4) also supports this view, since rhodoporphyrin in which a propionic acid group is replaced by a —COOH group was found to attach to globin and function as a carrier of oxygen.

DISCUSSION

A survey of the known Fe porphyrins occurring in plants and animals indicates a fundamental uniformity as regards their side chain structure. Some of the reasons for this uniformity of the porphyrins in nature may be suggested, if it may be assumed that the experimental results on the porphyrin chemistry of *Haemophilus* can be applied to other cells.

All of the naturally occurring Fe porphyrins contain two propionic acid groups. These have been shown to be essential since blocking them by methylation makes these compounds incapable of supporting growth. It is postulated that the two acid groups are required for orienting and firmly attaching the Fe porphyrins to two basic groups of the protein carriers (apoenzymes) so that the weak but all important iron coordination link with the protein may be stabilized.

With the exception of cytochrome C, all the naturally occurring Fe porphyrins contain either one or two vinyl groups. (If cytochrome C is presumed to be derived from protoporphyrin then this compound might also be included.) In the smooth strains of *H. influenzae* it was observed that of the iron-free porphyrins only protoporphyrin, *i.e.* containing vinyl groups, could function to support growth, those lacking vinyl groups failing to support growth. Fe porphyrins could also function as prosthetic groups to support growth even when they lacked vinyl groups. The vinyl groups appeared essential for the insertion of iron into the porphyrin ring.[4] In contrast to *H. influenzae* most cells synthesize their own iron porphyrins. If one can assume that the vinyl groups have a similar function in other cells as in *H. influenzae* one may conclude that these cells can insert iron into porphyrins only if these porphyrins contain vinyl groups; *i.e.*, protoporphyrin.

To complete the picture of the porphyrin side chains it is necessary to consider the function of the remaining groups, namely, the methyl side chains. The fully substituted pyrrole rings in protoporphyrin make it appear reasonable to postulate that the methyl side chains are present to stabilize the rings in order to prevent any undesirable side reactions from occurring within the cell. No direct evidence is yet available, however, to support this supposition.

[4] In the rough Turner organism mesoporphyrin in low concentrations was also effective in supporting growth, so that at least in this organism Fe insertion is not limited to vinyl groups but to a two carbon chain at positions 2 and 4 of the porphyrin ring. Mesoporphyrin, however, does not occur physiologically. Therefore one may consider that it is primarily the vinyl groups which are physiologically active in other organisms in connection with iron insertion.

SUMMARY AND CONCLUSIONS

1. Iron protoporphyrin IX was required for the growth of *H. influenzae*. It could be replaced by protoporphyrin IX. When grown on protoporphyrin evidence was obtained for the presence of Fe porphyrin in the organism. It was concluded that the organism could insert iron into the protoporphyrin ring.

2. In the smooth strains, other porphyrins containing no iron such as deutero-, hemato-, meso-, and coproporphyrins could not replace protoporphyrin for growth. Since protoporphyrin has two vinyl groups which other porphyrins lack, it was concluded that the two vinyl groups were essential for growth.

3. When porphyrins lacking vinyl groups were converted chemically into iron porphyrins and then supplied to the organisms it was found that these iron porphyrins supported growth. It was concluded that the "smooth" organisms were able to insert iron only into the porphyrin containing the vinyl groups; *i.e.*, protoporphyrin. One function of the vinyl groups then was to permit iron to be inserted biologically into the porphyrin ring.

4. An anomalous behavior in the rough Turner strain was observed and discussed. This organism was able to insert iron into mesoporphyrin at low concentrations but was inhibited by this compound at higher concentrations. In all other reactions with the porphyrins this rough strain behaved in the same was as did the smooth strains.

5. All strains which were grown on iron porphyrins lacking vinyl groups could not reduce nitrate to nitrite. When grown on protoporphyrin or Fe protoporphyrin reduction of nitrate occurred. It was concluded that the nitrate-reducing mechanism required the presence of the vinyl groups either for its formation or function.

6. The porphyrins lacking iron and lacking vinyl groups inhibited the growth of *H. influenzae* on Fe protoporphyrin. The inhibition between a porphyrin and Fe protoporphyrin was a competitive one. It was suggested that the porphyrin inhibited the growth-promoting properties of Fe protoporphyrin by attaching on to a particular apoprotein, thus preventing the formation of a heme catalyst. Likewise, competition between two growth-promoting Fe porphyrins for apoenzymes could be shown to occur.

7. Protoporphyrin and Fe protoporphyrin supported growth. When their propionic acid side chains were esterified they no longer supported growth. It was suggested that the esterified carboxyl groups could not attach to the specific apoproteins to form the heme enzymes and so could not act to support growth. For the same reason the inhibitory action of porphyrins lacking vinyl groups could be prevented by esterifying their propionic acid groups.

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