The Biosynthesis of Prodigiosin, the Tripyrrylmethene Pigment from *Bacillus prodigiosus* (*Serratia marcescens*)

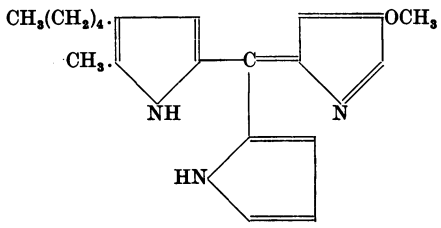
By R. HUBBARD and C. RIMINGTON
Department of Chemical Pathology, University College Hospital Medical School, London

(*Received* 20 *September* 1949)

Following the pioneer work of Schoenheimer, relatively simple organic compounds have, in many instances, been identified as precursors of much larger and more complicated molecules of biological importance. Efforts to bridge the gap between these small organic building stones and the complex final product have, however, met with less success. By investigating the biosynthesis of prodigiosin, the only known naturally occurring tripyrrylmethene, we hoped not only to elucidate this process for its own sake, but also to study it as a possible intermediate or side reaction in the biosynthesis of porphyrins (Rimington, 1949). A preliminary communication on this subject has already appeared (Hubbard & Rimington, 1949).

In an effort to explain the occurrence in nature of porphyrins belonging to both the I and III series of isomers, Turner (1940) postulated a tripyrrylmethene as a possible intermediate in porphyrin biosynthesis. His suggestion derived support from the earlier work of Corwin & Andrews (1936), who identified a tripyrrylmethane intermediate in the pyrrol aldehyde synthesis of dipyrrylmethenes and offered an explanation, thereby, of the unexpected occurrence of more than one dipyrrylmethene in the products of the reaction.

Prodigiosin



(I)

Since a tripyrrylmethene structure (I) was assigned to prodigiosin by Wrede & Rothhaas (1934), it seemed appropriate to study its biosynthesis and to compare the results with those obtained for haemin biosynthesis by other workers (for summary see Shemin, 1948; Muir & Neuberger, 1948).

EXPERIMENTAL

Extraction of prodigiosin

Prodigiosin was prepared from cultures of *Bacillus prodigiosus* (*Serratia marcescens*), National Collection of Type Cultures no. 4612. The cultures were grown on a medium containing Difco-proteose (1%), Lab-Lemco (1%), agar (2%), and NaCl (0·5%) in tap water. The medium (usually 1 l. distributed in ten Roux bottles) was inoculated, after sterilization, by spreading on it a thin film of a suspension of the organism in a meat-extract broth. To prepare the suspension, a red colony was picked off a stock agar slope and cultured in meat-extract broth overnight at room temperature. The inoculated Roux bottles were kept at room temperature in the dark. Pigmentation was visible after 24–48 hr., but cultures were not harvested until 4–7 days after inoculation. The pigment was extracted by one of the two following methods.

Alkaline extraction. For this procedure, Wrede & Hettche's (1929) method was slightly modified. The cells were washed off the medium with 0·9% (w/v) NaCl and solid NaOH added with constant agitation up to a concentration of 10% (w/v). After 2 hr., absolute ethanol and light petroleum (b.p. 40–60°) were added in amounts roughly equal to the volume of the solution. The mixture was shaken thoroughly, and the lower layer drawn off. Both layers were then repeatedly re-extracted with light petroleum until no more pigment could be removed. The light petroleum extracts were pooled for further purification. The cell residue was so degraded by the alkali that it had to be discarded. This method, was therefore used only when no isotope analysis was planned.

Acid extraction. The culture was washed off the agar with acetone containing 10% (v/v) of 3N-HCl (in later experiments 2% (v/v) of glacial acetic acid) and was repeatedly extracted by mechanical shaking for 30–45 min. followed by centrifugation. The extracts were pooled and the cell residue was washed three times with acetone to remove all traces of acid and once with ether and then dried in a vacuum desiccator. For isotope analysis, the cells were at first extracted continuously with acetone (Soxhlet) to remove all traces of pigment and fat. After it was found that extracted and unextracted cells gave identical results, such further extraction was omitted. The total acidic acetone extract was diluted with an equal volume of distilled water and made alkaline by addition of 10% (w/v) NaOH until the colour changed from deep red to yellow-orange; it was then extracted with several portions of light petroleum. When no more pigment could be extracted, the aqueous acetone layer was discarded and the light petroleum extracts pooled.

Purification of prodigiosin

The method of purification adopted depended on whether the material was to be used for ^{15}N analysis, for isotopic C analysis or for crystallization. Crystallization was found to be far too wasteful of material to be practicable for runs containing isotopes. Satisfactory and fairly quantitative purification procedures were, however, elaborated and applied as follows:

Procedure for ^{15}N analysis. The pigment in light petroleum was extracted with 85% (v/v) ethanol containing 1% glacial acetic acid until no more could be removed. The deep-red ethanolic solution was then diluted with an equal volume of water, treated with aqueous NaOH until the colour changed to orange, and extracted repeatedly with $CHCl_3$. The combined $CHCl_3$ extracts were concentrated to a small volume under reduced pressure and slight warming, and the optical density at the maximum (540 mμ.) as well as the N (micro-Kjeldahl) content of the sample were determined. The optical density was measured with the Beckman spectrophotometer. In later experiments, N determinations were omitted.

For isotope analysis, the purified samples of pigment were evaporated to dryness in Kjeldahl digestion flasks and digested overnight with 50% (v/v) H_2SO_4 containing 1% (w/v) SeO_2 with addition of catalytic amounts of $CuSO_4$ and K_2SO_4. The samples were then distilled and analysed for ^{15}N. Analyses for ^{15}N were always performed on duplicate samples.

Procedure for ^{13}C or ^{14}C analysis. It was found that although the above purification procedure removed all nitrogenous contaminants, enough fatty material remained to make C analyses unreliable. The pigment was therefore further purified by warming under reflux for 1 hr. at about 60° in 85% (v/v) ethanol containing 10% (w/v) NaOH. The alkaline ethanolic solution was then diluted with an equal volume of water and extracted with purified light petroleum. The transfer between slightly acidic 85% ethanol and light petroleum, after dilution and addition of alkali, was repeated until the light petroleum, after extraction of the pigment, showed no trace of a fatty residue on evaporation. The pigment was then transferred once more to light petroleum, the solution concentrated to 1–2 ml. and evaporated to dryness in a small weighing vessel. The vessel with the pigment was weighed and the pigment transferred to a small volumetric flask with a few drops of glacial acetic acid. The vessel was then dried in a vacuum desiccator containing pellets of KOH and reweighed. The pigment was diluted to a given volume with $CHCl_3$ and the optical density at the maximum (540 mμ.) measured. The pigment was considered sufficiently pure if the extinction coefficient on a weight basis fell within 15% of the value calculated on the basis of N content. It was then transferred to a small tube, dried overnight in a vacuum desiccator and analysed for isotopic C. The yield was approximately 10 mg. from 1 l. of culture medium (ten Roux bottles).

Procedure for crystallization. For easy crystallization, it was necessary to remove most of the fatty contaminants. This was accomplished either by the alkaline extraction procedure (p. 220) or by the acid extraction followed by the same purification steps as for carbon analysis. The pure dry sample of pigment was dissolved in about 1 ml. or less of hot absolute ethanol and was treated while hot with a few drops of 5% (v/v) aqueous $HClO_4$ to the point of slight turbidity. The solution was then allowed to cool to room temperature and stored overnight at $-10°$. The purple crystals of the perchlorate came out readily and another crop could be obtained from the mother liquor after further concentration and addition of some drops of 1% (v/v) aqueous $HClO_4$. For recrystallization, the crystals were dissolved in about 0·5 ml. of hot absolute ethanol and the hot solution treated with a few drops of 1% (v/v) aqueous $HClO_4$.

Miscellaneous procedures

Estimation of glycine. Glycine in the medium was determined by the method of Alexander, Landwehr & Seligman (1945).

Preparations of labelled compounds. DL-Leucine labelled with ^{15}N was prepared by the method of Schoenheimer & Ratner (1939). Glycine, labelled with ^{13}C on the carboxyl and with ^{14}C on the methylene group respectively were kindly made available by Drs Neuberger, Bentley and Arnstein, of the National Institute for Medical Research, Hampstead, N.W. The isotopic acetates were supplied by the National Chemical Laboratory, Teddington and the NH_4NO_3 by the Tracer elements Subcommittee of the Medical Research Council. The chloride was prepared from the nitrate. Analar reagents were used throughout the experiments and light petroleum was specially purified by repeated washing with conc. H_2SO_4 followed by washing with distilled water and redistillation. All taps in contact with the prodigiosin solutions were greased with a silicone grease (Dow-Corning Co., U.S.A.)

RESULTS

Spectral properties of prodigiosin

Since there are no detailed data available on the spectral absorption of prodigiosin, and since the only previous attempt to determine its extinction coefficient quantitatively (Ehrismann & Noethling, 1936) failed for technical reasons, we shall report our investigations on the striking spectral behaviour of prodigiosin in some detail.

Prodigiosin in solution can exist in one of two distinct but readily interconvertible forms or as a mixture of these two forms, depending on the hydrogen ion concentration of the medium. The two extreme spectra are illustrated in Fig. 1 which shows the difference in the position of the absorption maxima as well as in the width and intensity of the absorption bands of the two forms. In acid solution, the pigment is red, due to a sharp, high, narrow main band with a maximum at 535–540 mμ. and a slight shoulder on the low wavelength limb of the curve at about 510 mμ. This hump is always present and is independent of the purity of the sample. In alkaline solution, the pigment is orange-yellow due to a broader, less intense, roughly symmetrical band centered at about 470 mμ. The secondary hump at 530 mμ. (Fig. 1) is due to a trace of the 535 mμ. chromogen. Transition forms between these two extremes have been studied and there appears to be a definite isobestic point at about 495 mμ. showing that the two forms are probably the only participants

in this reversible reaction. As the ordinate in Fig. 1 is the same for both curves, it can be seen that the optical densities at the maxima of the two chromogens are in the ratio of about 2·5 : 1.

Since the pigment is soluble only in non-aqueous media, the pK associated with this colour change has not been accurately determined. It is, however, certainly on the alkaline side of neutrality, probably between pH 8·5 and 9·5. In partitions between aqueous ethanol and light petroleum, the behaviour of prodigiosin depends on the acidity of the ethanol phase. With acid aqueous ethanol and light petroleum, the 540 mμ. chromogen is hypophasic down to a concentration of 30% ethanol, beyond which point the aqueous solution is too turbid for estimations of optical density. The 470 mμ. chromogen, on the other hand, is always epiphasic to alkaline ethanol. This distinction forms the basis of the method of purification by repeated partitions described above (p. 221).

The pure 470 mμ. band can only be obtained in fairly alkaline solvents, pyridine giving a mixture of the two bands. The pure 540 mμ. band is easily obtained in all non-alkaline organic solvents and

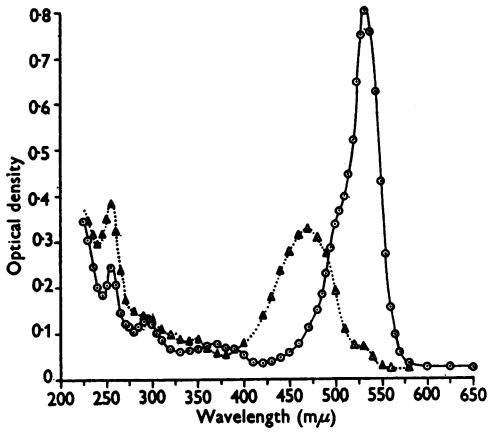


Fig. 1. Spectral properties of prodigiosin: ⊙, in 85% ethanol; △, in 85% ethanol + NH_4OH.

seems to be the more stable form as the 470 mμ. chromogen passes into the 540 mμ. chromogen spontaneously on prolonged standing. This transition is not appreciably accelerated by bubbling a stream of carbon dioxide through the solution but can be accomplished instantaneously by adding a drop of acid and somewhat more slowly by vigorous mechanical agitation, but not by bubbling oxygen through the liquid. A 540 mμ. band is also characteristic of the zinc complex of prodigiosin as well as of prodigiosin perchlorate.

The extinction coefficient of the 540 mμ. chromogen has been measured in chloroform containing 1% glacial acetic acid on the basis of nitrogen content as well as dry weight. As the figures on the basis of nitrogen have been consistently about 15% higher, they have been considered more reliable and they yield a value for ϵ (mol. extinction coefficient) of 110,000 ± 5000. The ϵ value of the 470 mμ. chromogen is therefore about 44,000.

It is difficult to interpret these spectrophotometric data chemically, since almost nothing is known concerning the spectral behaviour of tripyrrylmethenes. Dipyrrylmethenes are known to exist in two spectral forms with absorption properties similar to those described above (Granick & Gilder, 1947). However, it is safe to assume from the pK value quoted above that the reversible spectral shift is due to the formation of a sodium salt on one of the pyrrole nitrogens.

Comparison of the spectra of prodigiosin and of another tripyrrylmethene

As the structural formula of Wrede & Rothhaas (1934) for prodigiosin has not been confirmed by synthesis, it seemed worth while to study the spectral properties of one of the two tripyrrylmethenes

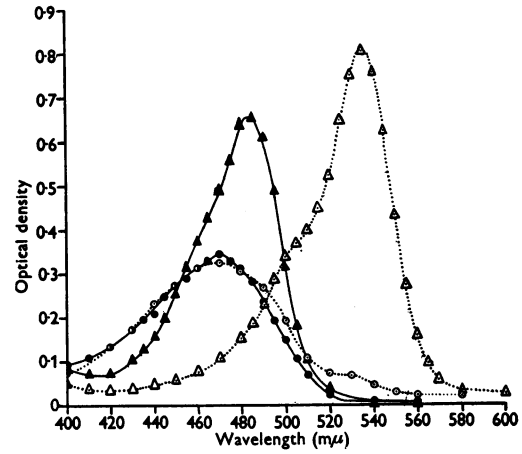


Fig. 2. Spectral comparison of prodigiosin and Fischer & Gangl's(1940) tripyrrylmethene; ▲—▲, tripyrrylmethene in acid ethanol; ●—●, tripyrrylmethene in alkaline ethanol; △ . . . . △, prodigiosin in acid ethanol; ⊙ . . . . ⊙, prodigiosin in alkaline ethanol.

synthesized by Fischer & Gangl (1940). We were fortunate to obtain a small sample of the perchlorate of Fischer & Gangl's 4-bromo-2:3′:3″-tricarbethoxy-3:2′:4′:2″:4″-pentamethyltripyrrylmethene, through the generosity of Dr Siedel, and were able to measure its spectrum in acid and alkaline ethanolic solution and compare this with the spectrum of prodigiosin. Fig. 2 presents the comparison, using the same ordinate and abscissa for all four curves. The striking similarity, if not complete identity, of the

two alkaline spectra is immediately apparent. There is also a qualitative similarity in the change of the spectra on addition of acid. In both cases the band becomes narrower and more intense and acquires a slight hump on the short wavelength limb of the absorption curve. However, whereas in the case of prodigiosin, this change is accompanied by a shift of about 65 mμ. toward longer wavelengths, there occurs only a slight shift of about 15 mμ. with the synthetic tripyrrylmethene. This difference is very likely attributable to the different ring substituents in the two compounds. Although the interpretation of these similarities should not be pushed too far, they lend definite support to the tripyrrylmethene formula proposed for prodigiosin.

The biosynthesis of prodigiosin

Nitrogen precursors. Since it has been shown (Shemin, 1948; Muir & Neuberger, 1948) that glycine is a specific precursor for all four pyrrole nitrogens of the porphyrin ring, we investigated its utilization for the biosynthesis of prodigiosin, and compared the utilization found with that of a few other nitrogenous substances. Table 1 summarizes the results. It will be seen clearly that glycine is a specific precursor for the pyrrole nitrogen of prodigiosin, whereas all the other compounds tested are utilized for prodigiosin synthesis to the same extent or less than for the synthesis of cellular protein. The efficiency of glycine utilization is 74%, i.e. of the 6% excess ^{15}N fed, about 4·5% excess appears as prodigiosin ^{15}N. This highly efficient utilization makes it seem likely that glycine is the only specific nitrogen precursor for all three pyrrole rings of prodigiosin.

Carbon precursors. In this series of experiments, the utilization of the two carbon atoms of glycine and of acetate was tested. The results are summarized in Table 2. They show that both carbon atoms of acetate are utilized at a higher level for prodigiosin synthesis than for the synthesis of the non-fatty cellular constituents. The two carbon atoms seem to be roughly equipotent as prodigiosin precursors. With glycine as a source of carbon atoms, however, only the α-carbon is utilized and this with a high degree of efficiency; the carboxyl carbon is not used at all. It is not possible to compare the acetate and glycine carbon results quantitatively since our baseline of utilization for non-fatty cellular constituents is not comparable for glycine, a protein precursor, and acetate, a fat precursor. It should, however, be stressed that acetate is definitely specifically used here as in the case of porphyrin biosynthesis.

The utilization of glycine. Table 3 gives in summary form a comparison of the utilization of the nitrogen and α-carbon atoms of glycine for prodigiosin biosynthesis. As mentioned above, the carboxyl carbon atom of glycine is not used. The results in Table 3 show that the nitrogen and α-carbon atoms are used to the same extent and with the same efficiency for prodigiosin biosynthesis and that, therefore, the nitrogen and α-carbon atoms seem to be incorporated together. Decarboxylation must occur at some stage in the formation either of the pyrrole ring or of

Table 1. *Utilization of* ^{15}N *by* Bacillus prodigiosus

Isotope supplied (Constant amount of N added)		Isotope (^{15}N) utilized		
Compound	^{15}N content (atom % excess)	Cell protein (atom % excess)	Prodigiosin (atom % excess)	Prodigiosin-^{15}N / Cell protein-^{15}N
Glycine	32	1·64	4·43*	2·70
Glycine	32	1·83	4·74*	2·59
NH_4NO_3	31·5	1·46	0·868	0·59
NH_4Cl	31·5	1·63	1·60	0·98
DL-Leucine	31·2	1·08	0·442	0·41

* Glycine content of medium reduces ^{15}N supplied as glycine to 6·0 atom percentage excess. Therefore the efficiency of incorporation of glycine $^{15}N = 74\%$.

Table 2. *Utilization of isotopic carbon* (*C) *by* Bacillus prodigiosus

Isotope supplied			Isotope utilized		
Compound	Amount added (g.)	Isotope content (atom % excess or counts/min./mg. of C)	Cell protein *C (atom % excess or counts/min./mg. of C)	Prodigiosin *C (atom % excess or counts/min./mg. of C)	Prodigiosin *C / Cell protein *C
$CH_3.{}^{13}COONa$	2·0	8	0·52	1·26	2·52
$CH_3.{}^{13}COONa$	0·5	8	0·22	0·67	3·04
$^{13}CH_3.COONa$	1·25	8	0·69	1·25	1·83
$NH_2.CH_2.{}^{13}COOH$	0·40	27	0·27	0	0
$NH_2.{}^{14}CH_2.COOH$	0·39	17,000 (counts/min./mg. of C)	865 (counts/min./mg. of C)	2080 (counts/min./mg. of C)	2·40

Table 3. *Utilization of* $NH_2{}^{14}CH_2.COOH$ *and of* $^{15}NH_2.CH_2.COOH$ *by* Bacillus prodigiosus

(Basal medium contains 1·6 g. of normal glycine.)

Isotope supplied				Isotope utilized					
^{14}C-Glycine		^{15}N-Glycine		Cell protein		Prodigiosin			
Added (g.)	^{14}C content (counts/ min./ mg. of C)	Added (g.)	^{15}N content (atom % excess)	^{14}C content (counts/ min./ mg. of C)	^{15}N content (atom % excess)	^{14}C content (counts/ min./ mg. of C)	^{15}N content (atom % excess)	$\frac{^{14}C \text{ Prodigiosin}}{\text{Cell protein}}$	$\frac{^{15}N \text{ Prodigiosin}}{\text{Cell protein}}$
0·39	17,000	0·04	32	865 (efficiency 26·4 %)	0·189 (efficiency 30·0 %)	2080 (efficiency 63·6 %)	0·444 (efficiency 70·4 %)	2·40	2·35

the tripyrrylmethene structure, but it is noteworthy that deamination reactions leading to the incorporation of residues derivable from glycine do not occur in the formation of prodigiosin, although they do so in the biosynthesis of haemin (Radin, Rittenberg & Shemin, 1949; Muir & Neuberger, 1949).

DISCUSSION

In recent years, studies of the biosynthesis of large molecules have revealed that glycine and acetate are among the most versatile small molecules which take part in synthetic processes (for review see Bentley, 1949). The discovery of yet another large molecule which is built from these two precursors therefore raises the question whether one can assume some parallelism in the mechanism of these various biosynthetic processes; in other words, whether there exist certain fundamental and general processes of chemical biosynthesis in which these simple molecules play a prominent role, and which may be adapted and elaborated to provide molecules which when completed bear little formal structural similarity to one another. As an example of such a line of thought, already familiar in biochemistry, may be mentioned the potentialities attributed to isoprene units in the synthesis, by polymerization, etc., of the mono-, di- and tri-terpenes and even other types of equally complex isocyclic structures. It is, however, also possible that the discovery of so many biosynthetic processes involving glycine and acetate may be merely fortuitous. Be this as it may, the realization that prodigiosin and the porphyrins are synthesized from the same precursors inevitably raises the question whether in porphyrin synthesis there is first formed a common pyrrole precursor which by further reaction gives rise to the cyclic structure or whether pyrrole and porphyrin rings are formed in a single event (Rimington, 1949). It is noteworthy in this connexion that there are some quantitative differences between porphyrin and prodigiosin synthesis which, however, can probably be explained by the differences in the structure of the two molecules. Thus Radin *et al.* (1949) have shown that in porphyrin biosynthesis six methyl carbons of acetate are used for every carboxyl carbon, whereas in our experiments, the two carbon atoms of acetate seem to be used to about the same extent. This difference may find its explanation in the fact that acetate is almost certainly utilized in the formation of the ring substituents which are quite different and considerably more complex in the case of the porphyrins than in prodigiosin. Similarly, Muir & Neuberger (1949) have shown that the α-carbon atom of glycine is utilized to more than twice the extent that the nitrogen atom is in porphyrin biosynthesis, whereas we have shown above that there is a strict 1:1 relationship between the utilization of the nitrogen and α-carbon atoms of glycine. This difference is almost certainly due to the fact that, as Muir & Neuberger have postulated, there is a rough 1:1 relation in the nitrogen and α-carbon content of the pyrrol rings, but they, and also London, Shemin, West and Rittenberg (1949), have shown that all the methyne bridges of the porphyrin molecule are derived from the α-carbon atom of glycine. Since the over-all architecture of the prodigiosin molecule is quite different from that of the porphyrins, this may account for the discrepancy in the carbon : nitrogen ratios.

On the basis of evidence presented above, it does not seem possible to decide definitely whether there exists a common precursor for all porphyrins as well as prodigiosin or whether each is synthesized individually *ab initio*, but the data strongly suggest some interrelation between the biosyntheses of the various pyrrole compounds. Although our experiments offer no proof of the formation of a tripyrryl intermediate, they do not exclude this possibility and we feel that the striking similarities between prodigiosin and porphyrin biosynthesis must be given due consideration in the elaboration of any final scheme of porphyrin biogenesis.

SUMMARY

1. Methods have been evolved for the isolation of prodigiosin and of bacterial cell residues suitable for isotopic analysis.

2. The absorption spectrum of prodigiosin in various solvents has been studied quantitatively, and the existence of two spectral forms depending upon the pH of the solvent demonstrated. In acid chloroform, prodigiosin has a band at 535–540 mμ. with $\epsilon = 110{,}000 \pm 5000$.

3. A comparison has been made of the spectral properties of prodigiosin and of a tripyrrylmethene synthesized by Fischer & Gangl (1940). This comparison supports the tripyrrylmethene structure assigned to prodigiosin by Wrede & Rothhaas (1934).

4. Both the nitrogen and α-carbon atoms of glycine are utilized specifically and to the same extent with a high degree of efficiency in the biosynthesis of prodigiosin. The carboxyl carbon atom of glycine is not utilized. All three nitrogen atoms of prodigiosin must be derived from glycine.

5. Both carbon atoms of acetate are utilized specifically and to about the same extent in the biosynthesis of prodigiosin.

6. The nitrogen of DL-leucine and of ammonium salts is incorporated into prodigiosin to the same extent as into the bacterial protein.

7. The experiments recorded show a striking similarity between the modes of synthesis of prodigiosin and porphyrin pigments but offer no conclusive evidence relative to the participation of a tripyrrylmethene intermediate in the formation of the porphyrin ring.

One of us (R. H.) wishes to thank the Division of Research Grants and Fellowships of the United States Public Health Service for a Predoctorate Research Fellowship. The Beckman spectrophotometer was purchased out of a grant from the London University Research Grants Fund.

The isotopes employed were kindly made available by the Tracer elements Subcommittee of the Medical Research Council and we are greatly indebted to Drs R. Bentley and H. R. V. Arnstein for performing the isotope analyses.

We wish to thank Prof. F. Wrede for generously sending us a specimen of his crystalline prodigiosin perchlorate for comparison with our material, and Dr B. K. Blount of the Research Branch, Zonal Office of the Economic Adviser, B.A.O.R. 30, for putting us in touch with Dr W. Siedel who kindly supplied us with crystalline specimens of the perchlorate salts of prodigiosin and Fischer & Gangl's synthetic tripyrrylmethene.

We are indebted for technical assistance to Miss K. Hale and Miss P. A. Miles and to Mr W. F. Napier, the Chief Technician of the Bacteriological Department of this School.

REFERENCES

Alexander, B., Landwehr, G. & Seligman, A. M. (1945). *J. biol. Chem.* **160**, 51.

Bentley, R. (1949). *Rep. Progr. Chem.* **45**, 239.

Corwin, H. A. & Andrews, J. S. (1936). *J. Amer. chem. Soc.* **58**, 1086.

Ehrismann, O. & Noethling, W. (1936). *Biochem. Z.* **284**, 376.

Fischer, H. & Gangl, K. (1940). *Hoppe-Seyl. Z.* **267**, 201.

Granick, S. & Gilder, H. (1947). *Advanc. Enzymol.* **7**, 305.

Hubbard, R. & Rimington, C. (1949). *Biochem. J.* **44**, l.

London, I. M., Shemin, D., West, R. & Rittenberg, D. (1949). Personal communication.

Muir, H. M. & Neuberger, A. (1948). *Biochem. J.* **43**, lx.

Muir, H. M. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.

Radin. N. S., Rittenberg, D. & Shemin, D. (1949). *Fed. Proc.* **8**, 240.

Rimington, C. (1949). *Haemoglobin*, p. 241. London: Butterworth's Scientific Publications.

Schoenheimer, R. & Ratner, S. (1939). *J. biol. Chem.* **127**, 301.

Shemin, D. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 185.

Turner, W. J. (1940). *J. Lab. clin. Med.* **26**, 323.

Wrede, F. & Hettche, O. (1929). *Ber. dtsch. chem. Ges.* **62**, 2678.

Wrede, F. & Rothhaas, A. (1934). *Hoppe-Seyl. Z.* **226**, 95.