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The Effect of Haemorrhage on the Incorporation of [α - 14 C]Glycine into Stercobilin

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It was shown by Tarchanoff (1874) that injected haemoglobin gave rise to bile pigments. This observation led eventually to an approximate correlation of the rate of haemoglobin breakdown with the daily amounts of bile pigment produced (Eppinger & Ranzi, 1920; Hawkins, Shribhishaj, Robschert-Robbins & Whipple, 1931; Miller, Robschert-Robbins & Whipple, 1945). From the nature and arrangement of the β -substituents of biliverdin, from which the other naturally occurring bile pigments are derived, it is evident that, of the free porphyrins, only protoporphyrin IX could theoretically give rise directly to biliverdin. Evidence for such a conversion has been obtained by using isotopically labelled protoporphyrin (London, Yamasaki & Sabella, 1951); however, when a rabbit's liver was perfused with a solution of protoporphyrin only 2% of the porphyrin was recovered, while the remainder did not give rise to bile pigments (Watson, Pass & Schwartz, 1941; Salzburg & Watson, 1941). Since it is known that bile pigments are formed in the liver, it may be inferred that they arise directly only from haem (London, 1950) or from haem proteins.

Isotope studies have shown that bile pigment is derived not only from red cells at the end of their life span, but also from a second source, responsible for the labelling of faecal stercobilin during the first

few days after feeding isotopically labelled glycine. The origin of this second fraction of bile pigments, indistinguishable chemically from the catabolic fraction produced from the breakdown of red cells at the end of their life span, is unknown but the increase in percentage of this fraction from about 10% in the normal person to about 80 or 90% of the bile pigments in patients with congenital porphyria, pernicious anaemia and sickle-cell anaemia, conditions associated with increased haemopoiesis, has suggested that this fraction may arise in association with haemopoiesis. Since this suggestion has been confirmed by our findings described in the present paper, the early labelled fraction may conveniently be called the haemopoietic fraction. It has been suggested that the haemopoietic fraction may arise in the bone marrow from premature breakdown of haemoglobin from intact red cells (London, Shemin, West & Rittenberg, 1949; Gray, Neuberger & Sneath, 1950), or from haem in excess of globin (Neuberger, Muir & Gray, 1950). On the other hand, direct synthesis from pyrrolic compounds without the formation of haemoglobin as an intermediate has been considered (London, West, Shemin & Rittenberg, 1950), and some support for this was obtained by Berlin, Neuberger & Scott (1956), who showed that a proportion of δ -aminolaevulinic acid admin-

istered to man is rapidly converted into stercobilin by a pathway which may not include haemoglobin. If the formation of the haemopoietic fraction of the bile pigment is related to haemopoiesis, there should be proportionality between the haemopoietic activity in the marrow and the labelling of stercobilin during the first few days after feeding isotopic glycine. If, on the other hand, this fraction arises, for example, from the turnover of haem-containing enzymes in the liver or in the body as a whole (Theorell, Beznak, Bonnicksen, Paul & Åkeson, 1951), then its rate of production should not be affected by changes in the rate of blood formation. Haemopoiesis may be stimulated by bleeding, and it seemed that this problem might be studied by examining the incorporation of [α - ^{14}C]glycine into the faecal stercobilin before and after venesection. The patient who was the subject for this study was suffering from a form of porphyria characterized by mild intermittent photosensitivity (porphyria cutanea tarda) in contrast with the continuous severe sensitivity associated with congenital porphyria. There is evidence that the former type of porphyria is not related to a defect specifically in the erythropoietic system (Watson, Lowry, Schmid, Hawkinson & Schwartz, 1951), and it was shown by Lowry, Hawkinson & Watson (1952) that the formation of bile pigment in porphyria cutanea tarda is normal, 85–90% occurring in the catabolic fraction. A preliminary report of the present work has been published (Gray & Scott, 1958).

EXPERIMENTAL

Clinical material

The subject was a man of 53 years with very mild porphyria cutanea tarda. He was in excellent general health. Blisters on the backs of the hands, and on the forehead and cheeks, which had developed on exposure to sunshine a year before, were almost completely healed. No lesions were present on the covered parts of the body and when sunshine was avoided the lesions healed with minor scarring. There was no excessive pigmentation or hirsutism, nor abdominal or neurological symptoms suggestive of acute porphyria. There was no anaemia, and no abnormality of mean corpuscular haemoglobin or of the differential white-cell count except for a slight eosinophilia of 8%. The blood film was normal. The detailed results of other haematological examinations are presented below. Consumption of alcohol had not been excessive and on general examination there was no abnormality except for a blood pressure of 200/100 mm. Hg (systolic/diastolic). There was no history of jaundice, no evidence of abnormal liver function, and at the time of investigation the urine did not contain an excess of porphyrins; porphobilinogen had never been detected in the urine. The faeces contained a small but significant excess of coproporphyrin. During the 34 days of the investigation the mean quantities of

faecal coproporphyrin and protoporphyrin excreted were 1.8 ± 1.1 mg. (0.84–4.81) and 0.5 ± 0.23 mg. (0.32–1.08)/day respectively.

Methods

Isolation of faecal stercobilin. The faeces were ground in a mortar with about a quarter of their bulk of acetic acid and then with an equal volume of ether. After standing, a deeply pigmented layer separated and was decanted through filter paper. After repeated extraction of the residue with further quantities of ether, acetic acid being added at about every fourth extraction, the united extracts were distilled on the water bath and the resulting dark brown solution, containing much acetic acid, was added to ten times its volume of 0.27 N-HCl. After it had stood overnight the dark-brown fatty material was filtered off through a large Büchner funnel and saturated potassium acetate solution added to the filtrate until it was no longer acid to Congo red. This solution was repeatedly extracted with ether until the ether extract showed only weak porphyrin absorption bands with a hand spectroscope. The ethereal extract was analysed for porphyrins and the aqueous phase was extracted with chloroform. The stercobilin hydrochloride was then isolated as previously described (Gray *et al.* 1950).

Estimation of faecal porphyrins. The united ethereal extracts were extracted with 0.15 N-HCl until the acid extract showed no significant absorption bands in the hand spectroscope. The volume was noted and after suitable dilution the coproporphyrin was estimated spectrophotometrically at $401 \text{ m}\mu$ ($E_{1\%}^{1\text{cm}}$ 6500). The remaining ethereal solution was exhaustively extracted with 0.7 N-HCl and the protoporphyrin determined spectrophotometrically at $409 \text{ m}\mu$ ($E_{1\%}^{1\text{cm}}$ 5000). The optical densities were not corrected for interfering substances.

Blood volume. This was determined with ^{51}Cr by a method modified from that of Mollison & Veall (1955).

Blood haemoglobin. This was determined by the method of Haldane (1900), by using the British Standards Institution standard.

Measurement of radioactivity. All samples of stercobilin were kindly gas-counted by Dr A. S. McFarlane with the method of Bradley, Holloway & McFarlane (1954); the standard error was less than 3%.

Faecal urobilinogen. This was measured by the method of Schwartz, Sborov & Watson (1944) as modified by Gray (1957).

Plan of experiment

The time relationship between the various phases of the experiment is shown in Fig. 1, together with relevant haematological data. [α - ^{14}C]Glycine ($25 \mu\text{C}$, 4.957 mg.) was dissolved in 50 ml. of water and drunk by the patient, the vessel being washed out with water ($6 \times 20 \text{ ml.}$). The faeces were subsequently collected during the succeeding 17 days at intervals of 2, 2, 3, 6 and 4 days. On the 15th day of the experiment, two 540 ml. quantities of blood were removed by two venesections separated by about 3 hr. Haematological examinations were carried out at convenient intervals throughout the investigation. On the 17th day of the experiment the reticulocyte count had risen from 0.2 to 0.6%, showing that haemopoiesis had been stimulated, and a second dose of $25 \mu\text{C}$ of [α - ^{14}C]glycine was given, precisely as before. The reticulocyte count continued to increase up to the twenty-third day. Faecal collections were continued

and worked up at the same intervals as in the preliminary experiment. Stercobilin hydrochloride was isolated from each batch of faeces as soon as collection was complete. Stercobilin was also isolated from faeces collected at convenient intervals over the period 130–157 days after the first administration of glycine, that is 113–140 days after the second administration. It was not possible to collect samples of faeces over the period 110–130 days, when the red cells formed during the first administration of labelled glycine were being broken down.

RESULTS AND DISCUSSION

It is convenient to consider together the quantities and radioactivities of the stercobilin obtained at corresponding intervals in phases I and II, the 17-day periods before and after the second dose of labelled glycine.

The rates of excretion of stercobilin during the two 17-day periods are shown in Table 1. Fig. 1 shows the haematological data obtained during the experiments. The concentration of haemoglobin fell from 17.5 to 14.5 g./100 ml. after the bleeding and was 14.2 g./100 ml. at the time of the first collection of faeces, and had risen to 16.3 g./100 ml. 3 days before the end of the experiment. As is usual in values for excretion of bile pigment determined in this way (see, for example, Gray & Neuberger, 1952) there was considerable variation in the rates of excretion even during phase I when the total haemoglobin was not changing. It may be seen from Table 1 and Fig. 1, however, that after the removal of blood there was a substantial increase in reticulocyte count and an apparent decrease of over 40% in the average rate of excretion of stercobilin. This apparent decrease is considered to be too great, for reasons which are discussed below.

Fig. 1. Plan of experiment and relevant haematological data. On both occasions the [α - 14 C]glycine given contained 25 μ C in 4.957 mg. The volume of blood removed was 1.08 l.; the total volume of blood was 5.5 l. throughout phases I and II, both of which lasted for 17 days. O, Concentration of haemoglobin (left-hand scale of ordinates); ●, reticulocyte count (right-hand scale of ordinates).

The observed specific radioactivities of the stercobilin isolated from the faeces during each interval are shown in Fig. 2. The radioactivities in phase II are considerably higher than in phase I, but the values for the specific activities during

Table 1. *Excretion of stercobilin before and after removal of blood*

The time intervals are measured from the day when isotopic glycine was first given. After 17 days a second dose of glycine was given; the faeces were then collected at intervals corresponding to those in the first period. Blood (1.08 l.) was removed on the 15th day. The method for estimating stercobilin gives values which include stercobilinogen.

Collection period (days)	No. of days	Wt. of faeces (g.)	Stercobilin excreted (mg./day)
Phase I			
1–2	2	140	111
3–4	2	406	156
5–7	3	250	87
8–13	6	440	141
14–17	4	705	120
		Mean	125
Phase II			
18–19	2	350	79
20–21	2	220	59
22–24	3	474	92
25–30	6	823	55
31–34	4	769	87
		Mean	72

Fig. 2. Mean specific activities of stercobilin after feedings of [α - 14 C]glycine before and after removal of blood. ●, During phase I; ■, during phase II; □, phase II, corrected for residual radioactivity from phase I; ○, corrected both for residual radioactivity and for changes in the total quantity of circulating haemoglobin during phase II. For further details of corrections, see text and Fig. 1.

phase II require correction because a proportion of the stercobilin would still have been labelled as a result of the [α - ^{14}C]glycine given at the start of phase I. When the specific activity of the stercobilin during phase I is plotted on a logarithmic scale against time, an approximately linear relationship is obtained, corresponding with a half-life of the faecal haemopoietic stercobilin of about 6 days. It was therefore assumed that the approximately exponential decrease of radioactivity of the haemopoietic fraction during phase I was maintained with respect to the first dose of glycine throughout phase II, and the small extrapolated values were subtracted from the specific activities of the haemopoietic fraction in phase II. This has permitted the correction of the observed specific activities of the stercobilin isolated after the second feeding of glycine for the persistence of labelling resulting from the first administration. A second correction is necessary to permit a valid comparison of the specific activities of the stercobilin samples because, after the bleeding, the circulating haemoglobin was decreased and caused a decrease in the quantity of unlabelled catabolic stercobilin derived from red cells at the end of their life span. This would increase the specific activity of the combined fractions whether any increased incorporation into the early fraction took place or not. The values, corrected for residual radioactivity, were accordingly multiplied by the ratio of the mean haemoglobin concentration in the appropriate interval in phase II to the haemoglobin concentration in phase I; the resulting values give approximately the specific activities which would have been observed had there been no change in the total haemoglobin. These corrections, particularly the second, lower the values in phase II by about 20 %, but the values are still substantially higher throughout than those in the first period.

Table 2 shows the ratios of the corrected specific activities of stercobilin isolated at corresponding intervals in phases I and II. The ratio was always

Table 2. *Incorporation of [α - ^{14}C]glycine into faecal stercobilin before and after haemorrhage*

The time intervals relate to corresponding intervals after giving isotopic glycine before (phase I) and after (phase II) haemorrhage. The corrected values of the specific activities have been used in calculating the ratios; for further details see text.

Days after glycine feeding	Specific activities of faecal stercobilin (ratio: phase II/phase I)
1-2	1.48
3-4	2.07
5-7	2.89
8-13	1.00
14-17	1.35

greater than unity and reached a value of 2.89 between the 5th and 8th days after the glycine feeding.

It is possible to calculate the output of ^{14}C -labelled stercobilin from the results of the estimations shown in Table 1. Such a calculation is, however, rendered invalid because of the variation in the daily weight of faeces (see Table 1) and of the unpredictable destruction of a part of the bile pigment in the gut to compounds not reacting with Ehrlich's aldehyde reagent under the conditions of the estimation; moreover, this destruction may be affected by diet. A further consequence of such destruction is that relatively more may be lost when less is present.

It is therefore more valid to assume a daily production of catabolic bile pigment approximately equivalent to 1/120 of the total haemoglobin in the body; since there is no reason to suppose that labelled material will be destroyed at a different rate in the gut from unlabelled material, the total amounts of stercobilin ^{14}C produced can be determined by numerical integration of the two curves of specific activity plotted against time. The total radioactivities calculated in this way amount to 62.4 and 104.4 $\mu\mu\text{C}$ before and after the bleeding respectively; there was thus an increase of about 60 % in the incorporation of glycine into the haemopoietic stercobilin.

Blood was removed towards the end of the first period and the second dose of labelled glycine was given at a time when the response of the bone marrow to the blood loss was established. This was judged by reticulocyte counts of the circulating blood and it was reckoned that the peak of activity in the bone marrow would precede the maximum reticulocyte count by three or four days. Reference to Fig. 1 shows that the glycine was probably given a few days too early to achieve a maximum effect. Moreover, the methods of correction used, to permit a direct comparison of the specific activities of the stercobilin in the two periods, are only approximate. In particular, correction for decreased dilution by catabolic pigment in the second period has been based on the approximation that all the stercobilin arises from haemoglobin breakdown whereas in fact only 85-90 % is normally so derived. Nevertheless, it is considered that the increases in specific activity are considerably greater than could arise from errors in the corrections. It is therefore concluded that the haemopoietic stercobilin is at least in part correlated with the biosynthesis of haemoglobin. We have not sufficient data to decide whether other sources of haemopoietic stercobilin exist or not.

Table 3 shows the specific activity of the faecal stercobilin hydrochloride isolated between the 129th and 157th day of the experiment, i.e. at a

Table 3. *Radioactivity of faecal stercobilin when labelled erythrocytes were being broken down*

The time intervals are measured from the day when isotopic glycine was first given.

Collection period (days)	Wt. of faeces (g.)	Specific activity of stercobilin hydrochloride ($\mu\mu\text{C}/\text{mg.}$)
130-131	540	12.0
132-135	550	14.4
136-145	1160	10.9
146-149	510	13.5
150-153	350	9.3
154-157	500	9.0

time when the red cells labelled after the second glycine feeding were breaking down. Unfortunately it was not possible to collect faeces at the time when red cells labelled from the first glycine experiment were being broken down.

In patients with congenital porphyria, the total daily excretion of stercobilin is greatly increased. Further, the studies with isotopic glycine (Gray *et al.* 1950; London *et al.* 1949) have shown that 80-90% of the isotope is excreted in the haemopoietic fraction; this has been interpreted to indicate that the increased excretion of bile pigment is due to an increase in the actual quantity of the haemopoietic fraction. It is of interest, therefore, that at a time at which the red cells labelled after the second feeding of [α - ^{14}C]glycine would be expected to be breaking down maximally, the specific activity of the catabolic stercobilin was showing a broad peak at about $14 \mu\mu\text{C}/\text{mg.}$, a value which is slightly higher than the corrected maximum specific activity corresponding with the much narrower peak of the early fraction ($13.5 \mu\mu\text{C}/\text{mg.}$) measured during phase II (cf. Gray *et al.* 1950). Although these figures cannot be corrected for the persistence of labelling from the breakdown of red cells labelled during the first feeding of [α - ^{14}C]glycine they show clearly that the labelling of the stercobilin at this time was as great as that observed during the first few days, and is not made extremely low by dilution with large amounts of haemopoietic stercobilin. Porphyria cutanea tarda thus differs from the congenital form of the disease in this respect, in confirmation of the earlier findings of Lowry *et al.* (1952).

SUMMARY

1. The incorporation of [α - ^{14}C]glycine into the early-labelled ('haemopoietic') stercobilin has been studied in man before and after haemopoiesis had been stimulated by bleeding.

2. The radioactivity of stercobilin formed after haemorrhage was consistently greater than the radioactivity of that formed in the corresponding

period before haemorrhage, showing that the haemopoietic stercobilin is related to the rate of production of red cells.

3. The subject studied was suffering from a mild form of porphyria cutanea tarda. A comparison of the specific activities of stercobilin excreted between days 1 and 34, and 129 and 157 days after the first feeding of glycine, shows that there was no increase in the amount of haemopoietic stercobilin relative to late-labelled stercobilin such as occurs in congenital porphyria, in confirmation of the findings of other workers.

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