3. The virtual absence of linolenic acid in the ox and sheep, as compared with its presence in quantity in the horse, the rabbit, and the pasture on which these animals feed, would indicate a difference in the nature of fat metabolism of these animals.

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Bile Pigment Precursors in Normal Human Erythrocytes

By C. GARDIKAS, J. E. KENCH AND J. F. WILKINSON
Department of Haematology, Manchester Royal Infirmary

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In studies on the *in vitro* breakdown of haemoglobin Lemberg, Lockwood & Legge (1941) have obtained evidence of the occurrence of a chromoprotein intermediate, to which they have given the name choleglobin. The presence of this substance in the reaction mixture of ascorbic acid and oxyhaemoglobin is revealed by the appearance of a new absorption spectrum, and treatment with acetic acid will then liberate the bile pigments, biliverdin and bilipurpurin.

Reduced choleglobin has a well defined absorption band in the red at 628–630 m μ ., reacts with carbon monoxide, and can be oxidized to the ferric compound, the maximum absorption of which occurs at

670 m μ . The pigment will therefore be green whether oxidized or reduced. Because many of its physical characteristics are similar to those of haemoglobin, Lemberg and his co-workers have been unable to effect separation of choleglobin from the great excess of its precursor. There is a paucity of direct knowledge of the pigment, and it is not known if it can undergo reversible oxygenation. Chemical evidence indicates that choleglobin arises by oxidative removal of the methene group in the prosthetic group of haemoglobin. The haem molecule is thereby opened, but otherwise unchanged, and the iron and globin linkages remain undisturbed. This chemical change is sufficient to endow the new protein

with two properties not possessed by haemoglobin, viz. light absorption at 628–630 $m\mu$., and lability in 66 % acetic acid. Lemberg *et al.* (1941) observed that the choleglobin band at 628–630 $m\mu$. is dispersed by acetic acid treatment, and simultaneously bile pigments can be recovered from the mixture. The yield of bile pigments was associated with the intensity of the 628 $m\mu$. band, with an average in all experiments of 11 %.

Following these *in vitro* experiments, Lemberg and his co-workers (Lemberg *et al.* 1941; Lemberg & Legge, 1942) subjected normal erythrocytes to the acetic acid treatment used for disruption of choleglobin, and were able to demonstrate the formation of bile pigments. This constituted evidence for the presence of an intracorporeal bile-pigment precursor, labile, like choleglobin, in 66 % acetic acid. The relationship between choleglobin and erythrocyte bile pigment precursor is not yet clear, but by assuming that the two compounds give similar yields of bile pigment, Lemberg *et al.* (1941) were able to calculate that bile pigment precursor constitutes approximately 1 % of the total oxyhaemoglobin in sheep's erythrocytes; 1–5 μg . of biliverdin was isolated per ml. of normal human erythrocytes (Lemberg & Legge, 1942).

For the detection of intracorporeal bile pigment precursor the pigment was broken down in 66 % acetic acid to biliverdin which was extracted from the mixture with ether, transferred to 5 % (w/v) hydrochloric acid and determined spectrophotometrically. During the acetic acid treatment further oxidation of biliverdin to bilipurpurin was observed, but this complication was prevented by addition of 10 mg. of ascorbic acid as an anti-oxidant.

As choleglobin is rapidly formed *in vitro* by coupled oxidation of ascorbic acid and oxyhaemoglobin (Table 1), it appeared to the present authors that addition of ascorbic acid introduces an error. The formation of choleglobin by the action of intracorporeal ascorbic acid and glutathione was circumvented by performing the whole Lemberg procedure anaerobically. As pointed out recently by Lemberg (1949), bilipurpurin is formed even anaerobically, and the present paper describes how this pigment can be determined in admixture with biliverdin.

In previous experiments (Gardikas, Kench & Wilkinson, 1948) no bile pigments could be detected photometrically in twelve specimens of normal blood. Subsequent studies have shown that 2.47 μg . of bile pigments can be obtained per ml. of erythrocytes, as measured by oxidation of biliverdin to bilipurpurin, the zinc complex salt of which by its intense red fluorescence in ultraviolet light can be detected in a solution containing 0.08 μg ./ml.

It appeared important to compare the bile pigment precursor content of erythrocytes, as computed

from this bile pigment formation, with that of pure crystalline human haemoglobin. It was hoped thereby to assess the true physiological changes which had occurred in the erythrocyte haemoglobin. The proportion of bile pigment precursor in the crystalline product was found to be slightly greater than that in the circulating erythrocyte. Possible implications of these observations are discussed later.

METHODS

Materials

Biliverdin was prepared in pure form from bilirubin (Lemberg, 1934). Purification was by crystallization of the methyl ester, m.p. 207°.

Crystalline human oxyhaemoglobin was prepared according to the method of Drabkin (1945).

Ether. The ether used was rendered peroxide-free by reduction with $Fe(NH_4)_2(SO_4)_2$, $Ca(OH)_2$, and reduced Fe. The absence of peroxide was checked frequently with acidified KI solution and starch suspension. Sodium acetate was Fe-free and other reagents A.R.

Determination of bile pigment precursor

Bile pigment precursor in the erythrocytes was disrupted in acetic acid and the biliverdin transferred to ether. The ether phase was washed with Na acetate to remove acetic acid and afterwards shaken with water to eliminate traces of acetate. Finally, biliverdin was extracted from the ether phase with 5 % (w/v) HCl, and bilipurpurin passed into 20 % (w/v) HCl.

The train of events was as follows: (a) red blood corpuscles (10 ml.), washed with saline, were lysed with distilled water (50 ml.); (b) glacial acetic acid (100 ml.) was added and allowed to react for 15 min.; (c) peroxide-free ether (300 ml.) was added and the mixture gently shaken, Fe and globin were retained in the acid layer, which was re-extracted and then discarded; (d) the pooled ether extracts were washed successively with 100 ml. of 18 % (w/v) Na acetate solution, 50 ml. of 1.8 % (w/v) Na acetate, and finally with 50 ml. of distilled water, the washings being withdrawn on each occasion; (e) the purified ether phase was fractionated with 5 % (w/v) HCl for biliverdin, and with 20 % (w/v) HCl for bilipurpurin.

The manipulations (a)–(d) were accomplished anaerobically in two vessels *A* and *B* arranged as shown in Fig. 1. *B* is a small vacuum desiccator, *A* a 500 ml. Pyrex round-bottomed flask. All connexions were of Portex plastic, which is not attacked by the reagents, while rubber stoppers and sealing grease were out of contact with the solvents. Both vessels were connected by two-way stopcocks to a Hyvac pump or a cylinder of pure N_2 (kindly supplied by Osrarn G.E.C. Ltd., to contain >0.001 % O_2). Thus the apparatus could readily be evacuated, and then filled with N_2 , the pressure changes being read on the Hg manometer connected to *B*.

At the outset of a determination, 10 ml. of washed erythrocytes were introduced into the well of *B*, which was evacuated thoroughly, and filled with N_2 three times, until all O_2 had been removed from the blood. At the same time, 100 ml. of glacial acetic acid introduced into *A* through the inlet tube *E* was similarly made O_2 -free and saturated with

N₂. Then with a partial vacuum in *B*, and *A* connected to the N₂ supply, the acetic acid was forced over into *B*, being dispersed at the point of entry by a narrowing at the end of the tube. The blood was mixed with the acid, and allowed to remain in contact for 15 min. Then 300 ml. peroxide-free ether were put under reduced pressure in *A*, and introduced into *B* to extract the bile pigments produced. The lower (acid) phase was expelled through the outlet tube *F* by increasing the N₂ pressure. These manipulations were repeated with saturated (18% w/v) and dilute (1.8% w/v) Na acetate solutions, and finally with distilled water. The purified ether phase, containing haematin and bile pigments, was then fractionated repeatedly with 5, 10 and 20% (w/v) HCl in air.

Fig. 1. Apparatus used for the extraction of bile pigments in absence of air. *A*, Pyrex round-bottomed flask (500 ml.); *B*, small vacuum desiccator; *C*, *C*, connexions to Hyvac pump; *D*, *D*, connexions to N₂ supply; *E*, inlet; *F*, outlet; *G*, connexion to mercury manometer.

Determination of bile pigments

Each HCl solution was in turn extracted with CHCl₃, neutralized to congo red with Na acetate solution and re-extracted with CHCl₃ to remove all the pigments. The CHCl₃ solutions were pooled for measurement of fluorescence.

Preparation of standard

To 1 ml. of CHCl₃ solution of biliverdin (containing 1 µg. of crystalline pigment) was added 0.5 ml. of 5% (w/v) Zn acetate in methanol, followed immediately by 0.03 ml. of a methanol solution of 0.006% (w/v) I₂ and 0.008% (w/v) KI. The maximum ultraviolet fluorescence of the Zn salt of bilipurpurin was reached within 10 sec., and the solution was then diluted rapidly to 10 ml. with methanol. This gave the standard fluorescence. There was some fading, and the standard had to be freshly prepared for each set of readings. The intensity could readily be matched in thin non-fluorescent test tubes.

Critical attention is required in certain respects: (1) all apparatus and solvents must be quite dry; (2) the quantity and concentration of Zn acetate and I₂ must be kept within narrow limits to produce optimum conversion without further oxidation of bilipurpurin to choletelin.

Determination of biliverdin and bilipurpurin in a mixture

The CHCl₃ extract of the mixture (1 ml.) was treated with 0.5 ml. of 5% (w/v) Zn acetate solution in methanol. Bilipurpurin reacts to give a red fluorescence, and the solution

was diluted with methanol to match the standard. By measuring the yield of bilipurpurin in terms of crystalline biliverdin the unsolved technical difficulties of preparing pure bilipurpurin have been circumvented.

Total bile pigments were determined in a further 1 ml. of CHCl₃ solution by addition of Zn acetate and I₂, and matched as before.

Biliverdin was calculated by difference.

Traces of protoporphyrin sometimes appeared in the CHCl₃ solution but seldom caused trouble, since the fluorescence of this pigment was rapidly quenched on addition of Zn acetate.

RESULTS

Table 1 presents the data collected concerning the effect of addition of ascorbic acid on the bile pigment yield from solutions of crystalline human oxyhaemoglobin. Experiments were made in air at 15°.

Table 1. Effect of addition of ascorbic acid on the bile pigment yield from solutions of crystalline human oxyhaemoglobin

Crystalline oxyhaemoglobin present (mg.)	Ascorbic acid added (mg.)	Yield of biliverdin (µg.)	Yield of bilipurpurin (µg.)	Total yield of bile pigments (µg.)
350	0.0	3.0	3.9	6.9
	10.0	12.1	8.9	21.0
	50.0	8.4	5.2	13.6
3000	0.0	8.8	0.8	9.6
	10.0	19.2	4.3	23.5
	40.0	45.2	13.5	58.7
	100.0	73.2	19.8	93.0

Crystalline oxyhaemoglobin was dissolved in 50 ml. of distilled water, and ascorbic acid added immediately before acetic acid treatment. It is clear that addition of 10 mg. of ascorbic acid as an anti-oxidant will vitiate the results for bile pigment yield and bile pigment precursor concentration in the original material.

The erythrocyte bile pigment values of twelve specimens of normal blood were determined anaerobically as described in the experimental section. (Each specimen was first examined for the presence of free bile pigments by the anaerobic method, with omission of acetic acid treatment. In no instance could free bile pigment be detected.) The mean of this series of values was 2.47 µg. of bile pigment per ml. of erythrocytes (range 1.04–4.26 µg./ml.). By calculation, using the factor of Lemberg *et al.* (1941), the corresponding bile pigment precursor represents 0.17% of the corpuscular haemoglobin.

Four separate preparations of crystalline human haemoglobin were likewise examined anaerobically for bile pigments (Table 2). From these figures it can be calculated that bile pigment precursor constitutes 0.21% by weight of crystalline human haemoglobin.

Table 2. *Bile pigments from crystalline human oxyhaemoglobin*

Specimen	Weight of crystalline oxyhaemoglobin (g.)	Total yield of bile pigments (μ g.)
1	3.7	32.1
2	3.7	24.0
3	3.8	32.6
4	4.3	45.9

DISCUSSION

It is evident from this investigation that the bile pigment precursor content of human erythrocytes is not greater than that associated with the corresponding amount of crystalline oxyhaemoglobin. Such a result could arise if either of two quite different conditions obtain. The minute yields of bile pigment might be caused artificially by the drastic chemical processes involved. Alternatively, bile pigment precursor may pass along with oxyhaemoglobin through all the steps of purification into the crystalline product, maintaining the original quantitative relations with the mother pigment which obtained in the original erythrocytes. This latter hypothesis appears unlikely, but no means are available at present of deciding which is the true interpretation. The suggestion that early changes in the breakdown of haemoglobin to bile pigments are initiated during circulation receives little support

from the present experiments. It is possible that a more fruitful search for intermediates of pigment metabolism could be made in situations where the reticulo-endothelial system is overloaded with haemoglobin catabolic products, as in the spleen of patients suffering from haemolytic jaundice or polycythaemia.

SUMMARY

1. An anaerobic method for the study of bile pigment precursor in human erythrocytes and in crystalline human haemoglobin is described.

2. The bile pigment precursor content of human erythrocytes was found to be 0.17 % of the contained haemoglobin. Bile pigment precursor also constituted 0.21 % of crystalline human oxyhaemoglobin.

3. A fluorimetric method for measurement of bile pigments has been developed. Its special advantages are: (a) it has a high sensitivity, adequate for the small quantities of pigments encountered in this problem; (b) it is specific, with minimal interference from other pigments; (c) it is not dependent on the preparation of a pure bilipurpurin standard.

4. The experiments provide little support for the hypothesis that early degradative changes of oxyhaemoglobin are initiated in the circulating erythrocyte.

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The Action of Enzymes on Chlorophenylalanines

By H. BLASCHKO AND JEAN STIVEN

The Department of Pharmacology, University of Oxford

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The enzymic oxidation and decarboxylation of tyrosine and its homologues has recently been studied (Blaschko, Holton & Sloane-Stanley, 1949; Blaschko, 1949; Sloane-Stanley, 1949). It was found that *o*-hydroxyphenylalanine and *m*-hydroxyphenylalanine were decarboxylated by extracts of mammalian tissues, which contain the enzyme 3:4-dihydroxyphenylalanine (DOPA) decarboxylase, an enzyme known not to act on tyrosine. On the

other hand, acetone-dried preparations of *Streptococcus faecalis* R, containing the enzyme tyrosine decarboxylase, acted not only on tyrosine, but also on *m*-hydroxyphenylalanine; they did not decarboxylate *o*-hydroxyphenylalanine. The amino-acid oxidases showed a lesser degree of specificity: the L-amino-acid oxidase of cobra venom and the D-amino-acid oxidase of pig kidney oxidized all three hydroxyphenylalanines.