The Effects of Vitamin B₁₂ Deficiency and of Copper Deficiency on the Concentration of Free Protoporphyrin in the Erythrocytes of Sheep

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Estimations of the concentrations of free protoporphyrin within the erythrocytes may assist in the understanding of the nature of the metabolic disturbances responsible for certain anaemic states. Normal concentrations of free protoporphyrin are found when haemoglobin synthesis is retarded by a dietary deficiency of protein (Cartwright & Wintrobe, 1948*b*); the relatively low concentrations that accompany the anaemia of pyridoxine deficiency (Cartwright & Wintrobe, 1948*a*) suggest an impairment of protoporphyrin production; and the abnormally high concentrations that supervene on infection, haemorrhage, iron deficiency, etc. (Cartwright, Lauritsen, Jones, Merrill & Wintrobe, 1946; Cartwright, Huguley, Ashenbrucker, Fay & Wintrobe, 1948) indicate that the synthesis of haemoglobin is then limited by factors other than those associated with the production of protoporphyrin.

Watson (1950) has reported that in human subjects suffering from Addisonian pernicious anaemia the concentrations of both coproporphyrin and free protoporphyrin in the red cells are within the normal range, and that during remission after treatment with vitamin B₁₂ the concentration of coproporphyrin increases as the number of reticulocytes increases, whilst the concentration of free protoporphyrin rises more slowly to reach a maximum subsequent to the reticulocyte peak.

A study of the concentration of free protoporphyrin in the red cells of vitamin B₁₂-deficient sheep has shown (see below) that the concentration increases with the severity of the condition, and gradually returns to normal after therapy with vitamin B₁₂. Unequivocal evidence has also been obtained of abnormally high concentrations of free protoporphyrin in the red cells of sheep rendered anaemic by the virtual iron deficiency which supervenes on a dietary deficiency of copper (cf. Marston, 1952).

Bénard, Gajdos & Gajdos-Török (1951) have claimed that the concentration of free protoporphyrin in the red cells of normal rabbits increases after parenteral injection of either vitamin B₁₂ or pteroylglutamic acid. As the concentrations of free protoporphyrin reported by these authors

were, before treatment, extraordinarily low and, after treatment, within the normal range found by other workers (Schwartz & Wikoff, 1952), the experiments were repeated in the course of the present study. The results did not confirm the claims that the concentration of free protoporphyrin in the red cells of normal rabbits is influenced by supplementation with either vitamin B₁₂ or pteroylglutamic acid in excess of the normal requirements. The possibility that the experimental animals used by Bénard *et al.* were vitamin B₁₂-deficient before treatment is remote, as this vitamin is unlikely to be a limiting factor in an animal that is coprophagous, e.g. the rabbit, which is pre-eminently so (Eden, 1940, 1945).

METHODS

The vitamin B₁₂-deficient sheep were experimental animals included in long-term investigations of various aspects of vitamin B₁₂ deficiency, the results of which will be published elsewhere. The animals were confined either in pens or in metabolism cages and were fed on rations containing less than 0.03 µg. of Co/g. dry wt. (Marston & Smith, 1952) until their stores of vitamin B₁₂ had become depleted to a level which no longer fulfilled the requirements of normal metabolism, at which stage appetite fails. The final criterion of uncomplicated vitamin B₁₂ deficiency under these conditions was the capacity of parenteral injection of vitamin B₁₂ to maintain, indefinitely, normal health in sheep on these rations and to induce rapid recovery in those which invariably developed the deficiency syndrome when confined to cobalt-deficient rations. The administration of appropriate amounts of cobalt *per os*, either as a drench or as an addition to the deficient fodder, induces the synthesis of sufficient vitamin B₁₂ by micro-organisms in the paunch to meet the animal's requirements, and the effects are identical with those which supervene on parenteral injection of vitamin B₁₂ (Marston, 1952).

The copper-deficient sheep and the normal (copper-treated) control sheep were drawn from groups of experimental animals that had grazed together as a flock on copper-deficient pastures throughout the previous 30 months. The control animals (group A) had been kept in a normal state of health by dosing them with the equivalent of 10 mg. Cu/day administered as copper sulphate thrice weekly. The copper-deficient animals (group C) had not received a copper supplement and had developed the advanced syndrome of copper deficiency (Marston, Lee & McDonald, 1948*a, b*).

Male Chinchilla rabbits aged approx. 14 weeks and approx. 1.4 kg. in weight were used when repeating the experiments of Bénard *et al.* (1951). These were fed on a ration comprised of lucerne and compressed nuts, containing bran (41%), pollard (41%), crushed oats (4%), meat meal (4%), wheat germ (2%), brown peas (6%), salt (1%) and a supplement of vitamins A and D.

Analytical

The oxygen-carrying capacity of blood was estimated by an alkaline-haematin method (cf. Marston & Lee, 1948).

Plasma iron was estimated by a modification of the methods of Barkan & Walker (1940) and Kitzes, Elvehjem & Schuette (1944), i.e. by incubation of the plasma with HCl as recommended by Barkan & Walker, followed, with slight modification, by the colorimetric estimation of iron as described by Kitzes *et al.*

Reagents

Ethyl acetate. Alcohol was removed from commercial ethyl acetate by heating under reflux for about 2 hr. with one-tenth of its volume of acetic anhydride; the product was fractionally distilled. Ethyl acetate may be recovered from residues by crude fractional distillation, followed by neutralization of the distillate with saturated Na_2CO_3 solution, dehydration first with Na_2SO_4 and then with K_2CO_3 , and finally fractional distillation. The use of CaCl_2 as a drying agent, recommended by Schwartz, Zieve & Watson (1951), should be avoided, as CaCl_2 is freely soluble in ethyl acetate and decomposes when heated with it (Le Canu, 1885).

Ethyl ether. High-grade commercial ether was shaken with FeSO_4 solution (60 g. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 6 ml. of conc. H_2SO_4 , 110 ml. of H_2O), dried over CaCl_2 and distilled.

Chloroform. Commercial chloroform was shaken, in the cold, with about half of its volume of conc. H_2SO_4 , then washed successively with water, dilute Na_2CO_3 solution and water, and, after drying over CaCl_2 in an atmosphere of CO_2 or N_2 , fractionally distilled as required.

Hydrochloric acid solutions. These were 25% (w/v) and 10% (w/v).

Estimation of porphyrins

As analytical procedures in current use for the estimation of the porphyrins depend essentially on differences in the partitions of individual porphyrins between aqueous solutions and various organic solvents, more than usual care must be taken to ensure that the solvents are pure. The method outlined below, based on that of Schwartz & Wikoff (1952), but differing from it in an important detail, was used in an attempt to estimate both copro- and free protoporphyrin in the same sample of blood. For the estimation of free protoporphyrin only, 2 ml. samples of blood (or sufficient to give 0.5–1.0 ml. of red blood cells) and smaller volumes of extracting fluids are satisfactory. Reproducible and comparable estimates of the free protoporphyrin content of red blood cells may be obtained by following the procedure suggested, but no claim is made that such estimates represent the absolute concentration of free protoporphyrin.

The blood, 15 ml., measured into a 50 ml. round-bottomed centrifuge tube, was spun, the plasma removed, and the red cells were triturated with a 4:1 (v/v) mixture of ethyl acetate and glacial acetic acid. Between each of the five or

six extractions the mixture was centrifuged and the supernatants were decanted through a Whatman no. 4 filter paper, previously wetted with 10 ml. of the extracting fluid. After the first decantation a second 10 ml. of the extracting fluid was passed through the filter paper; in all, 220 ml. of extracting fluid was used. The extract was washed with 3% (w/v) sodium acetate solution and three times with successive 44 ml. portions, and the porphyrins were extracted from the ethyl acetate solution into 10% HCl by five successive extractions, each of 10 ml. The filtered (Whatman no. 1) HCl extract was neutralized to Congo-red paper with saturated sodium acetate solution and extracted into ether by four extractions—10 ml. followed by three successive 5 ml. portions. The use of ethyl acetate as an extractant at this stage, which is recommended by Schwartz & Wikoff (1952), was, in the hands of the author, unsatisfactory; from pure solutions very poor recoveries of added proto- and especially of copro-porphyrin were obtained on re-extracting the porphyrins from ethyl acetate into small volumes of the appropriate concentrations of HCl.

The ether extract was washed with water (2 ml.), and the coproporphyrin, together with some protoporphyrin, extracted into 0.1 N-HCl, by nine successive extractions each of 0.5 ml. The 0.1 N-HCl extract was freed from protoporphyrin by extraction with half of its volume of chloroform, clarified by centrifuging, made up to volume, approx. 7 ml., and the concentration of coproporphyrin determined from the optical density of the solution at a wavelength of 401–402 μ , in a Beckman model DU photoelectric quartz spectrophotometer. The optical densities of the extracts so obtained suggested the presence of coproporphyrin; further purification, however, showed that little, if any, was present (see below).

The fraction of protoporphyrin extracted by chloroform was discarded; that remaining in the ether phase was extracted into 25% HCl by seven successive extractions—two of 0.5 ml. and five of 0.3 ml. portions—made up to volume, approx. 6 ml., and the concentration of protoporphyrin determined from the optical density of the solution at a wavelength of 410–412 μ , in the Beckman instrument.

Centrifugings were carried out at temperatures below 5° in covered tubes. All manipulations were performed under electric light in a room in which the blinds were drawn, as porphyrin solutions tend to decompose when exposed to ultraviolet light. For all extractions the period of shaking was 1 min., for washings $\frac{1}{2}$ min., and at least 10 min. was allowed for the phases to part before separating. If several analyses were attempted, it was usually not feasible to complete them in one day. The procedure was then followed until extraction into 10% HCl had been effected and the analysis was completed on the following day.

The number of extractions and the volumes specified were determined by extraction from pure solvents of known amounts of the pure porphyrins which had been added to them. The amounts were chosen to give, in the HCl extracts in which absorptions were measured, for coproporphyrin, 5–50 $\mu\text{g.}/100$ ml., and for protoporphyrin, 10–100 $\mu\text{g.}/100$ ml. With the procedure outlined, recoveries of protoporphyrin added to pure solutions are of the order of 97–100%, provided that the ether phase is not extracted with 0.1 N-HCl; the recovery of protoporphyrin after extraction of coproporphyrin with 0.1 N-HCl is of the order of 85%. The recovery of coproporphyrin added to pure solutions is close to 100%.

Standard solutions of coproporphyrin were prepared from the dimethyl ester of coproporphyrin III by hydrolysis with HCl (Jope & O'Brien, 1945).

Standard solutions of protoporphyrin were prepared by dissolving pure protoporphyrin—prepared by the method of Fischer & Pützer (1926), and purified as described by Grinstein & Watson (1943)—in a small volume of 10% HCl containing one-third of its volume of acetic acid, and diluting to the required volume with 10% HCl.

Porphyrin standards for determination of spectrophotometric absorption were diluted with ether-saturated HCl of the appropriate concentration (Schwartz *et al.* 1951).

Washing of ether extract. During washing of the ether extract with water, coproporphyrin passes much more readily than protoporphyrin into the aqueous phase from pure solutions to which they have been added; about 7% of each is removed by two and ten 2 ml. washings respectively.

Extraction of protoporphyrin from ether by 0.1 N-HCl. In the procedure adopted, nine extractions each with 0.5 ml. of 0.1 N-HCl remove from the ether phase approx. 15% of the protoporphyrin added to pure solutions. Conversion of protoporphyrin into haematoporphyrin may be responsible for a portion of this loss.

Chloroform extraction of the 0.1 N-HCl extract. In the estimation of coproporphyrin added to pure solutions, extraction of the 0.1 N-HCl extract with half of its volume of chloroform results in the loss of approx. 2.5 and 5% of coproporphyrin after one and two extractions respectively. In the presence of relatively large amounts of protoporphyrin, however, and in extracts of blood, protoporphyrin is always predominant; one extraction with chloroform, while removing all but a small trace of protoporphyrin, results in an apparently complete recovery of coproporphyrin.

Extraction of protoporphyrin from ether by 25% HCl. In more recent work 12.5% (w/v) HCl has been used to extract protoporphyrin from the ether phase and it gave results identical with those obtained by extraction with 25% HCl, with the advantage that the 12.5% HCl dissolves less of the ether phase.

Extracts of red blood cells. Vigorous trituration of the red cells is necessary in order to extract the porphyrins.

When the extracts from blood cells are submitted to the procedures outlined above, a number of substances, chiefly haemin and certain unidentified substances that fluoresce blue-green, and which absorb strongly at 400–410 m μ ., are carried through to the final extracts.

In an attempt to determine the extent of this interference, the ether extracts from a number of blood samples were subjected to a series of washings with water, and the optical densities of the aqueous solutions determined. A low and approximately constant 'blank' reading (optical density 0.013) was obtained only after eight to ten washings. The optical densities of the washings were due to interfering impurities and not to porphyrins, because no red fluorescence was detectable when they were submitted to electrophoresis on paper (cf. section on coproporphyrin under Results). It was considered therefore that the amount of protoporphyrin extracted into 25% HCl (omitting previous extraction with 0.1 N-HCl) after the ether phase had been subjected to ten 2 ml. washings with water would give a close approximation to the actual amount of protoporphyrin present in a free state in the blood cells, although in pure solutions of similar concentrations this number of washings would result in a loss of approx. 7% of the protoporphyrin.

The apparent concentrations of protoporphyrin obtained by following exactly the method of estimation outlined were found to be identical with those obtained when the extraction with 0.1 N-HCl was omitted and the ether phase subjected to ten washings with water, each of 2 ml., and are therefore a compromise between the amount of protoporphyrin which was extracted by the 0.1 N-HCl, and discarded, and the amount of interfering impurities which were carried into the 25% HCl extract. Application of the correction suggested by Rimington & Sveinsson (1950), however, indicated the presence of little, if any, impurity.

It is obvious that the 0.1 N-HCl extracts, even after extraction with chloroform, would contain a considerable quantity of substances which absorb in the ultraviolet, and as washing with water would remove a large proportion of the coproporphyrin, if present, as well as the impurities, the 0.1 N-HCl extracts were further concentrated and examined by electrophoresis on paper to determine unequivocally if coproporphyrin were present and, if so, to indicate the order of its concentration.

Separation of porphyrins by electrophoresis on paper. The paper employed, Whatman no. 4, was washed well with 2% (w/v) aqueous NH₃, rinsed thoroughly with distilled water, and dried before the porphyrin solution was applied. The transporting medium used for these separations was 0.1 N-NaOH, and the apparatus and technique were similar to those described by Foster (1952).

Chloroform-treated 0.1 N-HCl extracts of red cells, or of porphyrins which had been added to pure solutions to test the procedure, were prepared for electrophoresis as follows: a number of extracts (see below) were pooled, neutralized to Congo-red paper with saturated sodium acetate solution, extracted into ethyl acetate (two extractions of 4 and 3 ml. respectively) and the ethyl acetate extract was evaporated to dryness, in the dark, at room temperature, under reduced pressure in a desiccator containing P₂O₅, NaOH and paraffin wax. The residue was dissolved in 0.01–0.02 ml. of 0.1 N-NaOH and applied to the paper.

The coproporphyrin ion migrates towards the positive electrode when a potential difference is applied, and may thus be separated from protoporphyrin, which, under the conditions specified, either does not move or moves much more slowly.

RESULTS

Free protoporphyrin in the red blood cells of normal and of vitamin B₁₂-deficient sheep

Analyses of the bloods of thirty-four animals, some single observations and some taken serially over a period of time from the same individuals—in all 110 determinations—indicate that the concentration of free protoporphyrin in the red blood cells of normal sheep is within the range 31–89 μ g./100 ml., with an average value of approx. 50 μ g./100 ml. of red blood cells.

A series of estimations of the concentrations of free protoporphyrin in the red blood cells of sheep in various stages of vitamin B₁₂ deficiency, and during the recovery period, was undertaken. As the syndrome of vitamin B₁₂ deficiency developed and the animals became anaemic, the concentration of free protoporphyrin in the erythrocytes invariably

increased to levels that were from two to four times what might be considered the normal concentration for the particular animal. After appropriate treatment with vitamin B₁₂ (or with cobalt *per os*) these high levels fell slowly, taking from 6 to 14 weeks to regain the normal concentration. In general, the concentration of free protoporphyrin in the erythrocytes of vitamin B₁₂-deficient sheep was related reciprocally to the concentration of haemoglobin; the curves shown in Figs. 1 and 2 are typical of the results obtained.

When the deficiency syndrome was allowed to proceed to its fatal termination, the concentration of free protoporphyrin in the erythrocytes rose, before death, to approximately ten times the normal figure.

The abnormally high concentrations of free protoporphyrin were accompanied by normal to high concentrations of iron in the plasma; the average value for twenty-one normal sheep ex-

amined was close to 1.5 µg. of Fe/ml. of plasma (range 0.85–2.7), the average value for the vitamin B₁₂-deficient animals was 1.8 µg. of Fe/ml. of plasma (range 1.1–2.7).

Watson, Grinstein & Hawkinson (1944) have reported that the concentration of free protoporphyrin in the erythrocytes of human subjects suffering from various types of blood dyscrasias increases on incubation under sterile conditions. Although it seemed unlikely that the relatively high concentrations of free protoporphyrin found in the red blood cells examined in this series were due to breakdown of haemoglobin, samples of whole blood from vitamin B₁₂-deficient sheep were taken aseptically and incubated at a temperature of 37° for periods of from 24 to 48 hr., a total of twenty-four samples being treated in this way; *in no instance* did the concentration of free protoporphyrin in the erythrocytes increase during incubation.

Fig. 1. Effect of treatment with vitamin B₁₂ (or cobalt *per os*) on the concentration of free protoporphyrin and on the oxygen-carrying capacity of the red blood cells (R.B.C.) of vitamin B₁₂-deficient sheep. At the beginning of the periods indicated, the animals, aged 15 months, had been on cobalt-deficient rations for 40 weeks; all had developed marked symptoms of vitamin B₁₂ deficiency. Treatments: either 100 µg. of vitamin B₁₂/day (intramuscular) or 1 mg. of Co/day *per os* administered as a drench three times a week as indicated. (EP=erythrocyte protoporphyrin.)

Fig. 2. Effect of treatment with vitamin B₁₂, cobalt or pteroylglutamic acid (PGA) on the concentration of free protoporphyrin and on the oxygen-carrying capacity of the red blood cells (R.B.C.) of vitamin B₁₂-deficient sheep. (a) At the beginning of the period indicated, no. 001, aged 14 months, and no. 5009, aged 8 years, had been on cobalt-deficient rations for 40 and 26 weeks respectively. Treatments: no. 001, 50 µg. of vitamin B₁₂/day (intramuscular) from day 225; no. 5009, 500 µg. of vitamin B₁₂/day *per os* from day 173. (b) At the beginning of the period, no. 1108, aged 11 months, had been on cobalt-deficient rations for 21 weeks. Treatment: 20 mg. of pteroylglutamic acid/day (intraperitoneal) for 1 week, 100 µg. of vitamin B₁₂/day (intramuscular) for 1 week, followed by 50 µg. of vitamin B₁₂/day (intramuscular) from day 138. The effect of treatment with pteroylglutamic acid will be discussed elsewhere.

Table 1. *Constituents of the bloods of treated and copper-deficient sheep grazed on copper-deficient pastures*

	Haemoglobin (vol. of O ₂ /100 ml. of blood)	Copper (µg./ml. of blood)	Protoporphyrin (µg./100 ml. of red cells)	Iron (µg./ml. of plasma)
Mean ± s.e.	12.3 ± 0.41	0.91 ± 0.027	37 ± 3.8	0.95 ± 0.043
No. of animals*	(6)	(6)	(6)	(6)
Range	10.8–14.3	0.82–1.01	24–45	0.85–1.12
		Group C (copper-deficient)		
Mean ± s.e.	7.5 ± 0.50	0.15 ± 0.017	171 ± 22.6	0.295 ± 0.008
No. of animals†	(7)	(7)	(7)	(6)
Range	5.7–9.6	0.05–0.28	107–310	0.22–0.46

* Single analyses.

† Duplicate analyses.

Free protoporphyrin in the red blood cells of copper-deficient sheep

This set of observations was obtained from blood samples withdrawn from sheep in an advanced state of copper deficiency (group C) and from their normal controls (group A), which received adequate copper. The concentrations of free protoporphyrin in the erythrocytes of the control animals (group A)

were eight within the normal range or lower than those usually found in normal sheep; the concentrations of iron in the plasma were normal. The concentrations of free protoporphyrin in the erythrocytes of the anaemic, copper-deficient animals (group C) were high, the mean value being nearly five times that of the normal controls; the concentrations of iron in the plasma were low, the mean value being less than one-third of that of the control group (Table 1).

Free protoporphyrin in the red blood cells of rabbits

On repeating the experiments of Bénard *et al.* (1951), it was found that in the normal rabbit parenteral injection of either vitamin B₁₂ or pteroylglutamic acid does not increase significantly the concentration of free protoporphyrin in the red blood cells (Table 2).

Coproporphyrin in the red blood cells of sheep

Repeated attempts to estimate coproporphyrin in the red blood cells of either normal or vitamin B₁₂-deficient sheep failed to detect its presence.

The absorption at 401 m μ . of chloroform-treated 0.1N-HCl extracts obtained by the method outlined above indicated concentrations of coproporphyrin of the order of 30 μ g./100 ml. of red blood cells. This absorption, however, was, at least in part, due to substances other than coproporphyrin, as these solutions when exposed to ultraviolet light emitted a green fluorescence: no red fluorescence was detectable by eye. Because it was possible that the red fluorescence of coproporphyrin was being quenched by the green fluorescence arising from impurities, resort was made to detect the presence of coproporphyrin in these extracts by electrophoresis on paper.

A solution of coproporphyrin recovered by pooling the extracts from two assays, to each of

which had been added 0.01 μ g. of coproporphyrin and 3 μ g. of protoporphyrin as pure substances before the extraction procedure, was plainly visible by its red fluorescence under ultraviolet light after it had been spotted on to the paper. On electrophoresis the coproporphyrin separated cleanly from this spot, leaving a small trace of residual protoporphyrin that had escaped extraction into chloroform.

Coproporphyrin (0.01 μ g.), added directly to the red cells from each of two lots of 15 ml. of blood and subjected to the whole of the extraction and electrophoretic procedures, moved similarly under the applied potential and became visible when viewed under ultraviolet light, though the intensity of the fluorescence was less than that from the same amount of coproporphyrin extracted from pure solution (Fig. 3).

Coproporphyrin could not be detected by this means in extracts made from the red cells of either normal or vitamin B₁₂-deficient sheep. On several occasions, the material from three analyses, representing the extracts from 45 ml. of blood, was pooled and applied to the paper as one spot. Initially, when viewed under ultraviolet light, these spots fluoresced green with a brown discoloration at the centre. After electrophoresis for about an hour, the contaminating, green fluorescent material had moved towards the negative electrode, leaving a red

Table 2. *Effect of injection of vitamin B₁₂ or of pteroylglutamic acid on the concentration of free protoporphyrin in the red blood cells of the normal rabbit*

Date	Haemoglobin (vol. O ₂ /100 ml. of blood)	Protoporphyrin (μ g./100 ml. of red cells)	Haemoglobin (vol. O ₂ /100 ml. of blood)	Protoporphyrin (μ g./100 ml. of red cells)
	Rabbit 1		Rabbit 2	
6. xi. 52	12.7	47	13.8	88
11. xi. 52	—	48	15.8	67
13. xi. 52	13.1	40	13.9	77
Mean	12.9	45	14.5	77
17. xi. 52	20 μ g. vitamin B ₁₂ intramuscularly			
18. xi. 52				
20. xi. 52				
24. xi. 52				
Mean	13.5	53	12.9	78
S.D. of a mean of protoporphyrin concentration = 5.2				
	Rabbit 3		Rabbit 4	
	Haemoglobin (vol. O ₂ /100 ml. of blood)	Protoporphyrin (μ g./100 ml. of red cells)	Haemoglobin (vol. O ₂ /100 ml. of blood)	Protoporphyrin (μ g./100 ml. of red cells)
6. xi. 52	14.1	81	12.4	73
11. xi. 52	14.2	37	13.9	48
13. xi. 52	14.5	51	13.4	49
Mean	14.3	56	13.2	57
17. xi. 52	15 mg. PGA subcutaneously			
18. xi. 52				
20. xi. 52				
24. xi. 52				
Mean	14.4	58	15.0	75
S.D. of a mean of protoporphyrin concentration = 12.1				

fluorescing material at the initial spot, together with the non-fluorescing brown material. The coproporphyrin, when and only when this had been added to the extract, began at this stage to move towards the positive electrode, being detectable under ultraviolet light as a red fluorescing streak not yet separated from the origin. As electrophoresis proceeded, the coproporphyrin moved away from the initial spot and passed slowly, with some tailing, towards the positive electrode; the other red fluorescing material streaked out slightly but on no occasion was observed under these conditions to break away from the initial spot even after electrophoresis for 7 hr. The brown, non-fluorescent material moved towards the anode but more slowly than the coproporphyrin. At least the greater portion of the red fluorescence remaining near the starting line was probably due to the very small amounts of protoporphyrin which had escaped extraction into chloroform: it is possible, however, that other porphyrins were also present. The differences in the rate of movement of coproporphyrin during electrophoresis may be explained by differential wetting of the paper by the transporting medium. Relatively large amounts of impurities from blood extracts were applied on to the dry paper, together with the porphyrins, and in some instances a considerable period of time elapsed before all of the spots were completely wet.

That no appreciable amount of protoporphyrin was removed from the ether extracts of red blood cells by the exhaustive washings with water, which were found necessary if extraction with 0.1 N-HCl was omitted, was proved by pooling the washings of the extracts of a number of blood samples and, after concentrating either by evaporation or extraction into ethyl acetate, subjecting to electrophoresis on paper. No red fluorescing spots were detectable after this procedure.

It is clear that the methods in current use for the estimation of copro- and of free proto-porphyrin in red blood cells may lead to erroneous results, at least in sheep's blood. Electrophoresis on paper provides a rapid and effective means of separating porphyrins from the interfering impurities found in blood extracts, and might be adapted to give a very good approximation of the amount of coproporphyrin present in red blood cells.

Comparison of the behaviour of extracts of red blood cells, with and without the addition of pure coproporphyrin prior to extraction and electrophoresis on paper, led to the conclusion that the concentration of coproporphyrin in the blood of normal sheep is certainly less than 0.2 $\mu\text{g.}/100\text{ ml.}$ of red blood cells and in the vitamin B₁₂-deficient sheep certainly less than 0.3 $\mu\text{g.}/100\text{ ml.}$ of red blood cells.

Fig. 3. Electrophoresis of porphyrins. Tracings of spots, revealed by red fluorescence when viewed under ultraviolet light, obtained after electrophoresis of porphyrin solutions on paper. The conditions prevailing for the runs illustrated were: at beginning, 700–750 v, 10–12 mA; at finish, 550–600 v, 15 mA; duration 3–3½ hr. Series A: pure solutions. (i) 0.1 $\mu\text{g.}$ of protoporphyrin; remained on starting line. (ii) 0.02 $\mu\text{g.}$ of coproporphyrin; moved towards positive electrode under influence of applied voltage. (iii) 0.02 $\mu\text{g.}$ of copro- + 0.02 $\mu\text{g.}$ of protoporphyrin; protoporphyrin remained on the starting line, coproporphyrin separated from the protoporphyrin. (iv) 0.02 $\mu\text{g.}$ of coproporphyrin added, at end of extraction procedure, to the combined extracts of two 'blank' assays. (v) The combined final extracts of two mixtures of 0.01 $\mu\text{g.}$ of copro- + 3 $\mu\text{g.}$ of proto-porphyrin which had been submitted to the whole of the assay procedure. Series B: extracts from blood. (i) 0.01 $\mu\text{g.}$ of coproporphyrin; pure solution not submitted to extraction procedure. (ii) Final extract from 30 ml. of blood which had been submitted to assay procedure; no coproporphyrin detectable. (iii) Final extract from 30 ml. of blood + coproporphyrin: 0.02 $\mu\text{g.}$ of coproporphyrin added at end of extraction procedure; coproporphyrin easily detected. (iv) Final extract from 30 ml. of blood + coproporphyrin: 0.01 $\mu\text{g.}$ of coproporphyrin added to each of two assays of red blood cells after removal of plasma and just before the first addition of extracting fluid; coproporphyrin detectable on wet paper. (v) Final extract from 30 ml. of blood + coproporphyrin: 0.04 $\mu\text{g.}$ of coproporphyrin added as in (iv); coproporphyrin easily detected. The hatched circles on the negative side of the starting line represent the green fluorescent spots which at times disappeared before completion of the run.

DISCUSSION

The results described in this paper suggest that, in the sheep, vitamin B₁₂ is not implicated in any of the steps (cf. Lemberg, 1954) of protoporphyrin synthesis. In the erythrocytes of all the thirty-two experimental, vitamin B₁₂-deficient sheep examined, the concentration of free protoporphyrin was very considerably in excess of that of normal sheep—according to the severity of the deficiency from 1.6 to 10 times the average normal concentration found in this series. In every instance the concentration of free protoporphyrin increased as the deficiency syndrome progressed; and in every instance the abnormally high concentration of protoporphyrin receded, more or less slowly, to the normal level when the physiological requirements of vitamin B₁₂ were reinstated by appropriate treatment. In general, this decrease reflected the increase in the concentration of haemoglobin in the blood (Figs. 1 and 2). Iron was not limiting in these circumstances, for in the plasma the concentration was either normal or higher than normal. A claim (Bénard *et al.* 1951) that the concentration of free protoporphyrin in the red cells of normal rabbits is increased by injection of vitamin B₁₂ or of pteroylglutamic acid was critically examined and not substantiated (Table 2).

The red cells of sheep that had developed a virtual iron-deficiency anaemia as a result of a dietary deficiency of copper were found also to contain abnormally high concentrations of free protoporphyrin, but in these circumstances the condition is associated with low concentrations of iron in the plasma (Table 1). These latter observations are in agreement with those of other workers (Cartwright *et al.* 1946, 1948), who have found that iron-deficiency anaemias are almost invariably associated with high concentrations of free protoporphyrin in the erythrocytes.

An attempt was made to demonstrate the presence of coproporphyrin in the red blood cells of normal and of vitamin B₁₂-deficient sheep, for it became clear early in these studies that the analytical methods which are in current use and which depend ultimately on the absorptions at 400–401 m μ . by solutions into which the coproporphyrin has been concentrated by a series of partitions between aqueous solutions and organic solvents are capable of yielding misleading information, at least for sheep's blood. Electrophoresis on paper of extracts prepared as described above from the erythrocytes of normal and of vitamin B₁₂-deficient sheep indicated that little if any of the absorption was due to coproporphyrin. Comparison of the results obtained by electrophoresis of the extracts of blood cells and of the extracts of blood cells to which pure coproporphyrin had been added at the

first stage of the analytical procedure proved that in the red cells of normal sheep the concentration of coproporphyrin is certainly less than 0.2 μ g./100 ml. of red blood cells, and in the red cells of vitamin B₁₂-deficient sheep < 0.3 μ g./100 ml. of red blood cells, and suggested that the actual concentrations might be very considerably lower.

The effect of vitamin B₁₂ deficiency on the free protoporphyrin status of the red blood cells of sheep certainly differs from that in the human subject. Red blood cells from human subjects suffering from Addisonian pernicious anaemia were examined by the methods outlined above. The findings, which will be reported in detail elsewhere, confirm those of Watson (1950): coproporphyrin was detected in the red cells, and the concentration of free protoporphyrin increased during remission of the dyscrasia subsequent to treatment with vitamin B₁₂.

It is probable that protoporphyrin is not a direct intermediate in haem formation (Dresel & Falk, 1956); during its production, however, an excess of protoporphyrin seems always to appear as a by-product, and thus the increase in concentration of free protoporphyrin which occurs in the red cells of patients suffering from Addisonian pernicious anaemia after therapy with vitamin B₁₂ may be merely the result of the general stimulation of haemopoiesis rather than a specific effect on the production of protoporphyrin.

SUMMARY

1. In normal sheep the concentration of free protoporphyrin in the red blood cells falls within the range 31–89 μ g./100 ml. of red blood cells; the average of 110 determinations was close to 50 μ g./100 ml. of red blood cells.

2. In vitamin B₁₂-deficient sheep the concentration of free protoporphyrin in the red blood cells is invariably much higher than normal: it increases more or less steadily as the anaemia develops and may, in the terminal stages of the deficiency syndrome, reach concentrations up to 500 μ g./100 ml. of red blood cells. During re-establishment of a normal vitamin B₁₂ status, by appropriate treatment, these abnormally high concentrations gradually return to normal as the regeneration of haemoglobin proceeds. Concurrently with these high concentrations of free protoporphyrin in the erythrocytes, the concentrations of iron in the plasma are normal or higher than normal.

3. The claim of Bénard *et al.* (1951) that the injection of either vitamin B₁₂ or pteroylglutamic acid into the normal rabbit will bring about an increase in the concentration of free protoporphyrin in the red blood cells could not be confirmed.

4. In copper-deficient sheep the concentration of free protoporphyrin in the red blood cells is

much higher than normal: in the series of animals studied, concentrations ranging from 107 to 310 $\mu\text{g./100 ml.}$ of red blood cells with an average value of 170 $\mu\text{g./100 ml.}$ of red blood cells were observed. Concurrently with high concentrations of free protoporphyrin in the erythrocytes the concentrations of iron in the plasma are abnormally low.

5. Coproporphyrin was not detected in the red blood cells of either normal or vitamin B₁₂-deficient sheep. Electrophoresis on paper indicated that if coproporphyrin is present, the concentration is less than 0.2–0.3 $\mu\text{g./100 ml.}$ of red blood cells.

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South African Pilchard Oil

5. THE ISOLATION AND STRUCTURE OF AN EICOSAPENTAENOIC ACID FROM SOUTH AFRICAN PILCHARD OIL*

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In recent years structural diagnosis of the highly unsaturated fatty acids of natural origin has been facilitated by the increased use of spectral data, and by improvements in the methods of oxidative degradation due to Klenk & Bongard (1952). However, there is still considerable disagreement on

the suitability of some of the experimental methods that have been used in the isolation of individual acids. Thus Farmer & van den Heuvel (1938) have criticized the use of conventional vacuum-distillation techniques, and several authors have criticized the polybromide method on the grounds that structural alteration of such labile molecules can, and probably does, occur. Kolb & Brown (1955)

* Part 4: Silk & Hahn (1954b).

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