Although the end-point of the titration curve (Fig. 2) is not very sharp, it offers no explanation for this difference. It seems likely that the ammonia effect has to be explained by the difference in the electrode used. In our comparative studies on the determination of -SH groups in haemoglobins we therefore used a median ammonia concentration (0.1M) for both untreated and treated platinum-wire electrodes.

An important finding is the effect of the pre-treatment of the platinum electrode with ammoniacal mercuric chloride solution before use. The final results obtained with such a system for studying native human haemoglobin are in agreement with the data obtained by Ingram (1957), except that only one end-point was found in our experiments. The differences between the results in titrations with the untreated platinum electrode and with the mercury-coated electrode must be explained rather by the different experimental conditions than by a different reactivity of some of the sulphhydryl groups in the haemoglobin, as examination of the current-voltage curves has shown an increased sensitivity of the mercury-coated electrode. Moreover, the results of repeated titrations with a mercury-coated platinum electrode show a gradual decrease in the amount of silver bound. We therefore believe that the number of reactive -SH groups of different haemoglobins, and perhaps of other proteins, can be studied in the native state and after denaturation very clearly by the procedure using a mercury-coated platinum-wire electrode.

SUMMARY

1. A detailed description of the argentometric-titration procedure with a rotating mercury-coated platinum electrode for the estimation of the number of reactive sulphhydryl groups in normal adult human haemoglobin is given.

2. A comparison of this method with the technique using an untreated platinum electrode revealed that with the latter procedure lower amounts of titratable -SH groups were found to be present in native adult human haemoglobin. It is suggested that this decrease is caused by the different experimental conditions.

3. The titration curves obtained by amperometric titration of native normal adult human haemoglobin with silver nitrate at the mercury-treated platinum electrode show only one sharp end-point.

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The Determination of Phylloerythrin in Blood

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Diseases of livestock in which there is an acute sensitivity of exposed parts to sunlight occur in many parts of the world. The need to identify the photodynamic agents responsible has been stressed (Clare, 1952). Photosensitivity directly due to components of the diet has been shown to occur in some instances, such as in poisoning by plants of the genus *Hypericum* (Pace, 1942), by *Fagopyrum esculentum* (Wender, Gortner & Inman, 1943), and after administration of phenothiazine to calves (Clare, 1947) and lambs (Gordon & Green, 1951).

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More commonly, where liver damage is also found, the pigment phylloerythrin may be responsible (Clare, 1952). Phylloerythrin has been shown conclusively to be the photodynamic agent in geeldikkop (Rimington & Quin, 1934), in facial eczema (Clare, 1944), in congenital photosensitivity in Southdown sheep (Clare, 1945), in bile-duct ligation in sheep (Rimington & Quin, 1934) and in poisoning of sheep by *Lippia rehmanni*, *Panicum miliaceum*, *Myoporum laetum* and copper (Clare, 1952).

Phylloerythrin, a normal component of the bile and faeces of herbivores, is formed by microbial degradation of chlorophyll and related pigments in

their passage through the gut (Quin, Rimington & Roets, 1935), from which it is partially absorbed and re-excreted into the bile. In certain types of liver dysfunction, phylloerythrin accumulates in the peripheral circulation, causing intense photosensitization.

In photosensitivity diseases where the photodynamic agent is unknown it is desirable to examine affected animals for phylloerythrinaemia, but no quantitative method for the estimation of phylloerythrin in blood or urine has previously been described.

To provide information on levels of blood phylloerythrin in photosensitive animals, and as part of an investigation of sporadic photosensitization in sheep and cattle, a method has been developed for the estimation of phylloerythrin in small blood samples. This method depends on the measurement of the light absorption of phylloerythrin in ether at one or other of the peaks in the banded absorption spectrum in the visible and the near ultraviolet.

The procedure described in this paper is based on Fischer's (1926) method of porphyrin extraction from blood serum, modified slightly to obtain a more granular protein precipitate. The number of extractions, volumes and concentrations specified was selected to minimize interference by protoporphyrin without undue loss of phylloerythrin, and were checked with normal sheep plasma to which had been added known amounts of phylloerythrin and protoporphyrin.

EXPERIMENTAL

Spectrophotometry. All measurements were made on a Beckman model DU photoelectric quartz spectrophotometer checked frequently for wavelength calibration against the more intense lines of a mercury arc between 2225 and 5791 Å. For phylloerythrin estimations the slit width was set at 0.03 mm. (band width 7 Å at 4200 Å). The microcells (Pyrocell Manufacturing Co., U.S.A.) had a minimum volume of 100 mm.³ and a light path of 1 cm.

Reagents. All reagents except ethyl ether were A.R. grade. Ether was laboratory-reagent grade purified by shaking with acid FeSO₄ solution and redistilling.

Pure phylloerythrin was prepared from dried ox bile by the method of Rimington & Quin (1934). The ether extract was washed with 2% (w/v) HCl during repeated transferences between ether and 10% (w/v) HCl. The 10% HCl solution was extracted with chloroform, which was washed with water, dried and evaporated in air. The dried material was taken up in a small volume of pyridine and the cycle repeated several times. After two such cycles no changes were observed in the number and positions of the absorption maxima or in their relative intensity. The final chloroform extract was slowly evaporated, yielding a crystalline material that was used without further purification. The visible absorption maxima in ether (519.5, 560.0, 584.0, 638.0 mμ) are in reasonable agreement with earlier data for phylloerythrin in this solvent [e.g. Hellström

(1931): 512.3–526.6, 558.0, 579.8, 595.0, 635.2 mμ; Fischer (1933): 521, 561, 584, 593, 637 mμ; Keys & Brugsch (1938): 521, 559, 588, 637 mμ; Clare (1944): 522, 561, 585–597, 636 mμ]. These maxima and the ratios of their intensities (1.0, 1.52, 1.03, 0.220 respectively) agree closely with values obtained for phylloerythrin in dioxan by Stern & Wenderlein (1935) (517.5, 557.5, 581.5, 634 mμ; intensities 1.0, 1.52, 1.00, 0.218) and Lemberg & Falk (1951) (519, 559, 583.5, 637.5 mμ; intensities 1.0, 1.47, 0.98, 0.224).

Protoporphyrin was prepared from haemoglobin by the method of Keys & Brugsch (1938) and purified by repeated distribution between ethyl ether and aq. HCl solutions. Absorption maxima in ether-acetic acid were at 5030, 5365, 5780 and 6320 Å, in good agreement with the values of 5020, 5370, 5760 and 6325 Å given by Lemberg & Legge (1949a). The concentration of protoporphyrin in the final ether solution was determined by the method of Grinstein & Wintrobe (1948).

Procedure. Serum or plasma (10 ml.) was shaken with ethyl ether (20 ml.), followed by the addition of ether-acetic acid (1:1; 30 ml.). The flask was stoppered, shaken and left for 30 min. The ether layer was decanted into a separating funnel and the residue washed with ether (3 × 10 ml.), which was allowed to stand in contact for several minutes before decanting. The combined extracts were shaken with 2% (w/v, 0.55 M) HCl (3 × 20 ml.). The combined HCl washings were extracted with ether (2 × 5 ml.), the ether layers being transferred by test pipette to the original ether extract, which was then shaken with 20% (w/v, 6 M) HCl (3 × 4 ml.). The combined 20% HCl extracts were centrifuged to ensure separation of any ether emulsion and washed with ethyl ether (1–2 ml.) to remove traces of ether-soluble pigments. Ether (2 ml.) was added before shaking with alkaline sodium acetate (25 ml. of 0.9 M-sodium acetate; 1.5 M in NaOH), followed by further extraction with ether (3 × 3 ml.). This amount of alkaline sodium acetate left the aqueous phase slightly acid and thereby reduced further the risk of contamination by porphyrins of lower acid number. The ether extracts were mixed in a stoppered graduated cylinder and the volume was noted.

The optical density of the ether solution was measured with stoppered 2 cm. cells at 4120, 4140, 4170, 4230, 4290 and 4700 Å, against water. The phylloerythrin concentration in the original plasma was obtained from the relation

$$c \text{ (mg./100 ml.)} = 0.020V (E_{4140} - E_{4700}),$$

where V is the final volume of ether. The factor 0.020 includes a correction for the recovery of only 87% of phylloerythrin (see below).

The reliability of the determination was checked:

(i) by comparing $(E_{4170} - E_{4230}) - (E_{4230} - E_{4290})$ with $(E_{4140} - E_{4700})$. The ratio, R , of these quantities should be close to 0.23;

(ii) by confirming that E_{4140} was greater than E_{4120} and E_{4170} .

For more dilute solutions approximate values can be obtained by extracting the final ether solution into 20% HCl (1 ml.) and driving any phylloerythrin back into a small volume of ether with alkaline sodium acetate (2 ml.).

Higher concentrations can be measured with shorter absorption cells, dilution of the ether extract or absorption measurements in the visible. Beer's Law is obeyed at the four visible maxima for concentrations of phylloerythrin in

ether up to at least 3.5 mg./100 ml., and the ratios of the optical densities at the maxima provide a purity check. The phylloerythrin in the original plasma is given by

$$c \text{ (mg./100 ml.)} = 0.23 \sqrt{E_{5800}}.$$

RESULTS

Absorption spectrum of phylloerythrin

Fig. 1 shows the absorption spectrum of phylloerythrin in ethyl ether saturated with aq. acetic acid under the conditions described above for the estimation of phylloerythrin. Observed maxima, minima and extinction coefficients are given in Table 1.

The shape of the absorption curve and the positions of the maxima were unchanged when the ether extract was washed with water and with 2% HCl.

The inflexion at 4260 Å, which is absent from the spectral-absorption curves for most porphyrins, including protoporphyrin, mesoporphyrin and coproporphyrin, provides a convenient check on the purity of solutions prepared for phylloerythrin estimation. Over the successive and equal wavelength intervals 4170–4230, 4230–4290 Å, there is a considerable difference in the optical-density increments (Fig. 2). This difference

$$[d = (E_{4170} - E_{4230}) - (E_{4230} - E_{4290})]$$

is a constant fraction of the optical densities at any selected wavelengths, e.g. 4140 and 4700 Å. This follows directly from Beer's Law ($E = \epsilon ct$), which holds for all wavelengths. Thus

$$d = [(\epsilon_{4170} - \epsilon_{4230}) - (\epsilon_{4230} - \epsilon_{4290})] ct,$$

$$\text{and } E_{4140} - E_{4700} = (\epsilon_{4140} - \epsilon_{4700}) ct.$$

Hence

$$d = \frac{(\epsilon_{4170} - \epsilon_{4230}) - (\epsilon_{4230} - \epsilon_{4290})}{(\epsilon_{4140} - \epsilon_{4700})} (E_{4140} - E_{4700}) \\ = R (E_{4140} - E_{4700}),$$

where R is a constant.

For phylloerythrin in ether R has a value of 0.23. The inflexion is absent from solutions of phylloerythrin in aqueous hydrochloric acid. Although the inflexion is also absent from solutions of protoporphyrin in ether the absorption spectrum of this pigment shows sufficient curvature between 4170 and 4290 Å to give a value of $R = 0.10$.

Sensitivity

The method as described enables plasma phylloerythrin levels to be determined over the range 0.05–0.5 mg./100 ml. The use of visible absorption-spectrum measurements for high plasma phylloerythrin concentrations extends this range to

Fig. 1. Absorption spectrum of phylloerythrin in ethyl ether saturated with aq. acetic acid.

Table 1. *Phylloerythrin in ether-acetic acid*

Maxima (Å)	Minima (Å)	$E_{1\text{cm.}}^{1\%}$
4140	—	2800
4260	—	1560
(inflexion)	—	—
—	4760	50
—	(very flat)	—
5195	—	159
—	5420	82
5600	—	242
—	5735	99
5840	—	163
—	6215	13.3
6380	—	35

Fig. 2. Use of inflexion in absorption spectrum of phylloerythrin in ether saturated with aq. acetic acid as purity check. The ratio $d:h$ (0.23) is independent of concentration.

3.5 mg./100 ml. By the use of microcells and application of the *R* test in the modified method for very low levels of phylloerythrin a limit of detection of the order of 0.005 mg./100 ml. is given.

Specificity

The wavelength of the Soret maximum provides a useful qualitative check on the pigment obtained in the method described. Thus phylloerythrin in ether has $\lambda_{\text{max.}} = 4140 \text{ \AA}$; protoporphyrin, $\lambda_{\text{max.}} = 4040 \text{ \AA}$. Owing to the sharpness of the Soret band and the rapid rate of change of optical density with wavelength near the maximum, the position of the maximum is relatively little affected by the presence in the solution of compounds such as bilirubin, which show a more general light-absorption. For this reason an independent check on the purity of the ether extracts is necessary and this is provided, quantitatively, by calculation of *R*.

The hydrochloric acid number of protoporphyrin [2.0, Keys & Brugsch (1938)] is close enough to that of phylloerythrin [8.5, Keys & Brugsch (1938)] for significant amounts to be carried through. In human red-blood cells normal protoporphyrin levels are less than 0.05 mg./100 ml. (Cartwright, Lauritsen, Jones, Merrill & Wintrobe, 1946); in sheep the values are 0.03–0.09 (Allen, 1956). Kench, Gillam & Lane (1942) found whole-blood protoporphyrin concentrations up to 0.4 mg./100 ml. in human chronic lead-poisoning cases and reported that in this condition generally protoporphyrin concentrations were higher in plasma than in red cells. Values up to 1 mg./100 ml. of red blood cells have been reported (Dr E. C. Vigliani, quoted by Lemberg & Legge, 1949*b*). Recovery experiments with protoporphyrin added to plasma showed that a plasma protoporphyrin concentration of 1 mg./100 ml. gave an apparent phylloerythrin value of 0.04 mg./100 ml. This was shown to be spurious when *R* was found to be 0.10 and not 0.23 as required for phylloerythrin. Concentrations of protoporphyrin high enough to cause significant interference are unlikely to be encountered in practice.

Deutero-, meso-, haemato- and copro-porphyrins, which are known or believed to occur in normal or pathological metabolism, have much lower hydrochloric acid numbers (Keys & Brugsch, 1938), and present no difficulty in obtaining quantitative separation from phylloerythrin. Uroporphyrin is insoluble in ether acidified with acetic acid.

Bilirubin, biliverdin and carotene (which is a normal constituent of bovine plasma) are quantitatively rejected in the extraction process described. Haemolysis of the sample does not affect the determination.

The possible effects of chlorophyll-degradation products other than phylloerythrin have not been

investigated experimentally. However, Rothe-mund (1935), in his examination of ox bile for porphyrins derived from chlorophyll, found only phylloerythrin and smaller quantities of pyrroporphyrin, and no phylloporphyrin or rhodoporphyrin. Pyrroporphyrin, with an acid number of 1.5, is unlikely to interfere in the estimation of phylloerythrin by the method described.

Recoveries. The extraction process entails some loss of phylloerythrin because of the necessity to remove most of the protoporphyrin, the greatest loss occurring during the washing with 2% hydrochloric acid. The extent of the overall loss was determined by recovery experiments. Recoveries of phylloerythrin in ether added to plasma, within the range 0.06–1.37 mg./100 ml., followed by evaporation of the ether, amounted to $87 \pm 6\%$ (range 80–96). On the basis of these results, multiplication by 1.15 is included in the factor used to convert optical densities into plasma phylloerythrin concentrations.

Below about 0.05 mg./100 ml. differences are not sufficiently great for accurate calculation of *R* and hence there is no reliable check on the apparent phylloerythrin values obtained. In a series of experiments with plasma to which had been added 0.007–0.032 mg. of phylloerythrin/100 ml., recoveries were 0.012–0.054 mg./100 ml., including a (spurious) plasma blank of 0.004 mg./100 ml. In a typical experiment an ether extract with an apparent phylloerythrin content of 0.015 mg./100 ml. was concentrated into 0.5 ml. of ether. Measurement by microcell (slit width 0.06 mm.) gave the original phylloerythrin level as 0.007 mg./100 ml., *R* = 0.19.

DISCUSSION

In their work on geeldikkop, Rimington & Quin (1934) isolated phylloerythrin from large volumes of blood from affected sheep but did not determine its concentration. Clare (1944), using existing methods of porphyrin extraction and various modifications, followed by fluorimetric estimation, was unable to obtain even roughly quantitative recoveries of phylloerythrin added to blood, and in some of his experimental sheep photosensitization after injection of phylloerythrin occurred when peripheral-blood phylloerythrin had fallen below levels he could detect. Clare concluded that if, in affected sheep, the pigment was found in the blood sufficient was present to cause photosensitivity.

The present method enables phylloerythrin in low concentrations in blood to be identified and estimated. The use of an internal spectroscopic check which depends on the shape of the phylloerythrin-absorption curve considerably increases the reliability of the determination. The sharpness

of the phylloerythrin absorption bands and the dependence of *R* on small wavelength differences require accurate calibration of the spectrophotometer and the use of narrow slit widths.

The highest plasma phylloerythrin level so far found with the method described has been 0.3 mg./100 ml., in a sheep poisoned by *Panicum miliaceum*, although Clare (1944) estimates that levels up to at least 1.6 mg./100 ml. occur in severe cases of facial eczema in sheep.

The method appears to be applicable to other biological materials, especially urine. Phylloerythrin has been detected in the urine of humans on vegetable diets (Kemer, 1924; Fischer & Hilmer, 1925), indicating that absorption of chlorophyll-degradation products from the gut can take place in humans. Whether phylloerythrinaemia occurs in human-liver dysfunctions and porphyrias is not at present known, but the method described makes feasible the examination of blood from affected persons. In addition to its application to photosensitivities of unknown origin it also enables information to be obtained on the concentrations of phylloerythrin involved in photosensitization in sheep and cattle.

SUMMARY

1. Details are given of a method for estimating phylloerythrin in blood, consisting in extraction into ether-acetic acid solution, distribution between ether and hydrochloric acid solutions, and spectrophotometric measurement.

2. The inflexion at 4260 Å in the absorption spectrum of phylloerythrin provides a quantitative check on the purity of the material being measured.

3. The method is applicable over the range 0.05–3.5 mg. of phylloerythrin/100 ml. of plasma, but a modification giving approximate values down to 0.005 mg./100 ml. is described. Proto-porphyrin and other porphyrins do not cause significant interference.

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APPENDIX

The Form in which Phylloerythrin Occurs in Bile and Blood

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Although phylloerythrin is extremely insoluble in water it occurs as a normal constituent of sheep and ox bile, from which it is not thrown down on centrifuging. Similarly, although it can be quantitatively extracted into ether from bile acidified with acetic acid, phylloerythrin is not readily extracted

from fresh bile with ether alone. A similar observation was made with plasma from a congenitally photosensitive Southdown sheep. Dialysis of bile in collodion tubes led to the deposition of most of the phylloerythrin on the walls, with very little present in the diffusate. The effect of pH on