2. Mixtures of the following pairs of amino-acids were separated: glycine-alanine, valine-proline, methionine-leucine. A mixture of serine and threonine did not separate.

3. The isolation of glycine and L-alanine from a protein hydrolysate is described.

4. The order of displacement of fourteen amino-acids was determined by displacing the mixture with dilute hydrochloric acid solution from a column of Dowex 2. This order was substantially the same as the order of pK_a (NH_3^+) for the monocarboxylic

amino-acids, and the result is discussed in relation to the theory of Davies (1949).

5. The use of a strongly basic resin in a simple scheme for the isolation of amino-acids from complex mixtures is discussed.

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The Micro-estimation of Porphyrins by Copper Titration

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Much of the work dealing with porphyrins requires the handling of small amounts of pigments, usually of the order of micrograms. It is thus rarely possible to isolate a sufficient quantity of material of proved homogeneity suitable for carbon, hydrogen, oxygen or nitrogen analysis. Nevertheless, the highly characteristic absorption spectra of porphyrin pigments have enabled workers to make great progress in the field, as structural modifications are reflected strongly in the nature of the spectra.

In the present paper a problem of current interest concerning the porphyrin *a* isolated from the prosthetic group of cytochrome *a* of ox-heart muscle (Rawlinson & Hale, 1949) is taken as an illustration of the use of spectrophotometry in making accurate estimates of pigment concentrations.

The immediate aim was to determine molecular concentrations of porphyrin *a* for the calculation of molar extinction coefficients. The method described here utilizes the ability of free porphyrins to form metalloporphyrins which possess quite different light-absorbing properties. The conditions found most suitable involved the titration of free porphyrin

in glacial acetic acid with a standard solution of cupric acetate in the same solvent. The formation of copper complex is quantitative, and no degradation of pigment is evident during the short period of heating necessary for the reaction. Although the disappearance of the red fluorescence of the free porphyrin, under ultraviolet radiation, can be used to give an approximate measure of the completion of the reaction, the end point is determined with much greater accuracy by measurements of the change of optical density at any wavelength where the two components have marked absorption differences. The reaction involves the replacement of the two hydrogen atoms bound to the nitrogen of each free porphyrin molecule by one cupric ion, so that the least number of metal ions required to produce the maximum change of optical density is equivalent to the number of free porphyrin molecules originally present.

The principles outlined might prove of use for the estimation of other pigments of unknown constitution, where quantitative conversion to another derivative of sufficiently different absorption spectrum is possible.

THEORY OF THE METHOD

If a series of dilutions be prepared containing constant amounts of porphyrin and gradually increasing concentrations of copper acetate, then, after completion of the reaction, the components present will be (i) free porphyrin, (ii) free porphyrin plus copper-porphyrin complex, or (iii) copper-porphyrin plus copper acetate.

The optical densities (D_1 , D_2 and D_3) corresponding to these possibilities, at any selected wavelength, will be given by the equations,

$$D_1 = \{\epsilon_p c + b\} l, \quad (1)$$

$$D_2 = \{\epsilon_p(c-x) + \epsilon_m x + b\} l, \quad (2)$$

$$D_3 = \{\epsilon_m c + \epsilon_o(x-c) + b\} l, \quad (3)$$

where c is the initial molar concentration of porphyrin; x is the standard copper concentration in conditions (ii) and (iii); ϵ_p , ϵ_m and ϵ_o the molar extinction coefficients of free porphyrin, metallo-porphyrin, and copper acetate, respectively; b is any extraneous absorption of light, assumed constant for each wavelength; l is the thickness in cm. of solution absorbing light.

The combination of equations (1) and (2) gives an expression of condition (ii),

$$(D_2 - D_1) = x(\epsilon_m - \epsilon_p) l, \quad (4)$$

so that a plot of $(D_2 - D_1)$ against x will give a straight line passing through the origin with slope depending on $(\epsilon_m - \epsilon_p)$. It is important to select a wavelength giving greatest slope, and this may be done by preliminary inspection of absorption curves of the porphyrin and metal complex at equimolar concentrations (Fig. 1).

Beyond the end point the condition (iii) is expressed by combination of equations (1) and (3).

$$(D_3 - D_1) = \{c(\epsilon_m - \epsilon_p) + \epsilon_o(x-c)\} l, \quad (5)$$

from which it is obvious that any change in optical density will depend on x values, as all other terms are constant. Thus if $(D_3 - D_1)$ values are plotted against x , where it is certain that copper is present in excess, a straight line should result with slope dependent upon the small value, $\epsilon_o(x-c)$.

The end point is given by the point of intersection of the two straight lines defined by equations (4) and (5).

EXPERIMENTAL

Materials

Glacial acetic acid. British Drug Houses Ltd. analytical reagent, was recrystallized and distilled to eliminate metallic impurities.

Standard copper acetate solution (4×10^{-1} mm) was prepared by dissolving CuO (British Drug Houses Ltd. analytical reagent) in warm glacial acetic acid. For titration this solution was diluted to give a range of concentrations suitable for the selected amount of porphyrin.

Coproporphyrin III tetramethyl ester was isolated from cultures of *Corynebacterium diphtheriae*, and purified by the method of Gray & Holt (1947). The results presented in Fig. 3 were obtained with samples each containing 4.22×10^{-4} mmol. in glacial acetic acid.

Protoporphyrin IX was prepared from crystalline protohaemin by the method of Warburg & Negelein (1932) and recrystallized twice by mixing hot saturated solutions of the pigment in pyridine (1 vol.) and glacial acetic acid (10 vol.). The crystals were dried *in vacuo* over H_2SO_4 and then KOH. For the results quoted in Fig. 4 a 4.72×10^{-2} mm solution was prepared by dissolving the porphyrin in 1 vol. pyridine and then diluting to 100 vol. with glacial acetic acid. Samples were used for titration containing 4.72×10^{-4} mmol.

Porphyrin a was isolated from ox-heart muscle and purified by a modification of the method of Rawlinson & Hale (1949). After further purification the pigment was dissolved in $CHCl_3$ and stored at 2° . For titration, suitable samples were evaporated on the water bath and taken into a known volume of glacial acetic acid.

Methods

The sequence of steps involved in the estimation of porphyrin *a* is presented as a typical example of a pigment with unknown extinction coefficient data.

An approximate estimate of concentration is advisable if the minimum number of tubes is to be used in the more accurate method. The selection of suitable wavelengths is also necessary so as to obtain the greatest accuracy.

Fig. 1. Absorption spectra of porphyrin *a* and its copper complex. - - - -, Selected wave-lengths for titration.

(a) **Determination of approximate porphyrin concentration.** By evaporating a known volume of porphyrin *a* in chloroform and transferring into glacial acetic acid, the concentration of pigment was estimated roughly by direct titration with a standard copper acetate solution. The porphyrin solution was maintained at the temperature of the boiling-water bath and irradiated with ultraviolet light. The end point was taken as the disappearance of red fluor-

essence. At concentrations suitable for spectrophotometric work, the results of these fluorescence titrations tended to be too high, but at higher concentrations the method would probably be more accurate. This rough estimate of porphyrin concentration then allowed the preparation of suitable dilutions of standard copper acetate.

(b) *Selection of wavelengths suitable for measurements.* The next step required the selection of wavelengths giving large ($\epsilon_m - \epsilon_p$) values. After preliminary measurements to find a dilution of pigment where the optical density of the most intense absorption band was about 0.4, a sample was treated with a slight excess of standard copper acetate solution, and the original solution diluted to the same extent with glacial acetic acid. The absorption curves of the pigments were plotted, and then compared as shown in Fig. 1. The wavelengths most suitable for titration were readily selected (see broken lines at 604, 598 and 560 m μ .).

Fig. 2. Titration curves for porphyrin *a*.

(c) *Spectrophotometric method for accurate porphyrin estimations.* From the information derived above, 100 ml. of porphyrin *a* in glacial acetic acid was prepared so as to give suitable values of optical density, at the wavelengths selected. Ten ml. samples were pipetted into seven test tubes (6 x 1 in.) and to six of these were added 2 ml. portions of suitably diluted standard copper acetate solutions. The six standard dilutions of copper were selected to give a fairly uniform distribution of values, and arranged so that three points fell on each straight line (Fig. 2). To the seventh tube was added 2 ml. glacial acetic acid. The tubes were covered with glass funnels, heated on the boiling-water bath for 7 min., and then cooled. Each tube was then compared with glacial acetic acid in a Beckman spectrophotometer at 604, 598 and 560 m μ . and the results plotted as shown in Fig. 2. The end point was then measured as the point of intersection of the two 'best' straight lines calculated by the method of least squares.

Various tests on conditions of the method

(a) *Effect of heat on pigments.* Samples of porphyrin *a*, copper-porphyrin *a*, protoporphyrin, and copper-protoporphyrin were heated as described in the method. Samples were removed over a period of 15 min., cooled immediately and then measured in the spectrophotometer at wavelengths of maximum absorption. There was no significant change in optical density values, and so it must be concluded that the pigments were not modified during the heating process.

(b) *Quantitative estimation of coproporphyrin III ester.* This pigment was selected for the first test of the method as it was available in a pure state, and

Fig. 3. Titration curves for coproporphyrin III tetra-methyl ester.

possessed stable peripheral groups. The result of the experiment is shown in Fig. 3, where the 'best' straight lines have been calculated. The point of intersection was obtained from the equations to the lines, and it was found to agree closely with the value expected ($\pm 0.5\%$).

(c) *Quantitative estimation of protoporphyrin IX.* The purpose in making this estimation was to test the method with a porphyrin possessing less stable substituents, in this case the vinyl groups at positions 2 and 4 being the cause of the increased lability. In addition, the porphyrin was used in the unesterified condition and so possessed free carboxyl groups. Another point of importance concerns the possibility of a close structural relationship between porphyrin *a* and protoporphyrin. Any difficulties associated with the estimation of the latter pigment would make it necessary to reserve judgement on the suitability of the method for porphyrin *a*. The results

illustrated in Fig. 4 show that the method gives a satisfactory estimate of concentration ($\pm 2\%$). The great difficulty in completing the removal of solvents from crystalline protoporphyrin was the main cause of error. Unless special care was taken in the removal of solvent, values as low as 80% of theory were obtained. On account of the low solubility of protoporphyrin in glacial acetic acid, it was dissolved first in a small volume of pyridine before dilution. The final solutions of free porphyrin and

Fig. 4. Titration curves for protoporphyrin no. ix.

copper complex were tested for deviations from Beer's law, in case either pigment had commenced to polymerize. There was no evidence for this effect, as the optical density was found to be proportional to concentration in each case.

DISCUSSION

The spectrophotometric methods previously available for the estimation of porphyrins rely upon the data of extinction coefficients calculated from measurements on pure, crystalline compounds. As such information refers to a weight basis, the values are expressed as specific extinction coefficients. The method described in this paper allows of a direct estimate of molar concentration, without the necessity of making any assumptions as to either the molecular weight or extinction coefficients.

In the case of those porphyrins whose composition has not been established completely, values for carbon, hydrogen or oxygen cannot be used for

estimation of molecular weight or molar concentration. The nitrogen content is a more feasible basis, as each porphyrin nucleus contains four nitrogen atoms, and no porphyrin isolated from natural sources has been found to possess additional nitrogen, with the exception of the prosthetic group of cytochrome *c*. Porphyrin *c* contains two cysteine residues (Theorell, 1939). However, even without accepting the assumption of four nitrogen atoms per molecule, the micro-analysis of nitrogen requires much greater amounts of pigment, is not so specific, and is not so convenient for general use. These comments indicate the main reasons for the development of the copper method. As reported earlier by Rawlinson & Hale (1949), the estimation of haemin *a* was possible on the basis of micro-iron analyses. The possibility of using the conversion of free porphyrins into metallo-porphyrins has, therefore, been studied on a

Fig. 5. Copper exchange in zinc porphyrin *a* complex. Zinc porphyrin *a*, —; copper porphyrin *a*, - - -.

quantitative basis. Boas (1933) converted natural porphyrins into iron complexes and estimated these derivatives by their catalytic ability to oxidize benzidine.

Many heavy-metal ions can be made to replace the two hydrogens bound to the pyrrole nitrogen atoms of porphyrins. The speed of the reaction, and the stability of the metal complex depend on the nature of the metal ion, the porphyrin, and the solvent, as well as the temperature. Some idea of the firmness of the metal binding is given by reference to the competition between various ions and protons. Sodium, potassium and arsenic are removed by water. The magnesium and lead complexes are dissociated by dilute acetic acid. Concentrated hydrochloric acid causes the displacement of zinc, silver, tin (Sn^{++}) and iron (Fe^{++}). Concentrated sulphuric acid, on the other hand, is necessary to displace Fe^{+++} , Cu^{++} , Ni^{++} , Mn^{+++} and Co^{+++} , while tin (Sn^{++++}) and aluminium are not removed even under these conditions. It is also possible to displace one metal

by another; for example, the zinc-porphyrin *a* derivative can be converted into the copper complex when dissolved in a dilute solution of copper acetate in glacial acetic acid (Fig. 5).

The iron complex offers certain advantages as the iron or haemochrome contents could have served as a basis of estimation. However, there are some serious disadvantages. In particular, the ease of degradation to bile pigments, the ability to undergo reversible valency changes, and anhydride formation in hot organic solvents constitute the major reasons for avoiding the iron compounds. The direct analysis of heavy metal bound to porphyrin could be used, but would involve more work than the method here described.

There are several reasons which led to the choice of copper for complex formation. Standard solutions of copper acetate are readily prepared and the extremely stable copper-porphyrin forms under mild conditions. As the copper complexes do not fluoresce under ultraviolet radiation, the disappearance of red fluorescence is useful in making quick, preliminary estimates of concentration. In the new method where seven mixtures are compared, the plotting of the two straight lines is facilitated by grouping the results according as to whether or not there is red fluorescence. With some complexes, e.g. sodium, potassium, magnesium, zinc, cadmium and tin (Sn^{++}), this criterion is not available as both components fluoresce. The copper derivatives can be dissociated only by powerful reducing agents, such as sodium amalgam, and unlike the iron complexes do not appear to be capable of forming octahedral complexes by combination with other components of the reaction mixture. In glacial acetic acid the reaction between copper and porphyrin is irreversible.

The method was tested thoroughly by measurements on coproporphyrin ester and protoporphyrin, as well as by studies on the effect of heating and on the relationship between optical density and concentration for the least soluble components, namely, the protoporphyrin derivatives. The use of glacial acetic acid requires some care on account of its corrosive nature, but in spite of this fact it is by far the most satisfactory solvent. The reaction when carried out in pyridine requires longer heating with consequent dangers of loss of solvent and modification of pigments. In particular porphyrin *a*, with its labile formyl group, should be kept in an acidic environment.

Some emphasis could also be laid on the specificity of the method. The elimination of the term *b* in the final equations (4) and (5) means that any component affecting the transmission of incident light need not be considered if the effect remains constant during the conditions of reaction.

Any contaminating substance that forms an irreversible complex with copper would compete with porphyrin and so change the slope of the curve defined as $(\epsilon_m - \epsilon_p)$ until it was all bound to copper (Fig. 6). However, by extrapolating back the straight line beyond this deviation, the intercept on the copper axis would give a true indication of the end point.

Fig. 6. Effect of some interfering factors on the shape of the titration curve: (a) theoretical curve; (b) copper-binding impurities; (c) copper-catalysed decomposition.

Another source of error that requires comment is the possibility of decomposition catalysed by copper. In such a case the titration curve would take the form shown in Fig. 6, but in none of our titrations has this effect been observed.

SUMMARY

1. For the micro-estimation of porphyrin *a*, a new spectrophotometric method, which should prove of more general application, has been developed.
2. The method involves the titration of porphyrin with standard copper solutions by following the changes of optical density at selected wavelengths where the free porphyrin and copper porphyrin complex show greatest differences of absorption.
3. The accuracy of the method has been demonstrated by reference to pure specimens of coproporphyrin III tetramethyl ester and protoporphyrin, under different experimental conditions, and the effects of interfering reactions have been considered.

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The Metabolic Reduction of Progesterone to *allo*Pregnane-3 α :20 α -diol in Human Subjects

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Since the original isolation of *allo*pregnane-3 α :20 α -diol from the urine of pregnant women by Hartmann & Locher (1935) it has been generally assumed that this steroid, like its C-5 stereoisomer pregnane-3 α :20 α -diol, is a metabolic reduction product of progesterone. Hitherto, however, no satisfactory experimental evidence to warrant this assumption has been published.*

The isolation of *allo*pregnane-3 α :20 α -diol from the urine of a post-menopausal woman receiving progesterone treatment has recently been reported from this laboratory (Kyle & Marrian, 1951), but since control urine collected from the same subject before or after the treatment was not examined, it could not be concluded that the isolated steroid had necessarily arisen by reduction of the administered hormone. The possibility that the *allo*pregnane-3 α :20 α -diol had originated from some endogenous source, and otherwise than by the reduction of progesterone, did not seem to be entirely remote in view of the reported findings of Dobriner, Lieberman, Rhoads & Taylor (1948). These workers, in a study of the ketosteroid excretion of a pregnant woman, showed that whereas the urinary pregnan-3 α -ol-20-one increased steadily from the third month until term, the excretion of *allo*pregnan-3 α -ol-20-one excretion remained relatively constant at about 1–2 mg./24 hr. throughout pregnancy. In view of these findings the possibility could not be entirely ignored that the urinary *allo*pregnane steroids, in contrast to those of the pregnane series, might arise from some precursor other than progesterone.

In the present work *allo*pregnane-3 α :20 α -diol has been isolated from the urine of one normal post-menopausal woman and one rheumatoid arthritic

man who were being treated daily with large doses of progesterone by intramuscular injection. In both cases urine specimens were collected over adequate control periods after the cessation of the hormone treatment and were examined by methods as nearly identical as possible with those used for the 'progesterone' urines. In neither instance could any *allo*pregnane-3 α :20 α -diol be isolated from these control urines, and it can therefore be concluded that the material isolated from the 'progesterone' urines had indeed been formed by the reduction of the administered progesterone.

It is of some considerable interest that the relative proportions of pregnane-3 α :20 α -diol and *allo*pregnane-3 α :20 α -diol isolated from the urine during periods of progesterone administration in these experiments (approx. 30:1) agree very closely with the relative proportion of these two steroids isolated from the urine of pregnant women by Kyle & Marrian (1951). This suggests that the *allo*pregnane-3 α :20 α -diol present in the urine during pregnancy arises largely by the metabolic reduction of endogenous progesterone.

EXPERIMENTAL

Methods

Melting points were determined as previously described (Kyle & Marrian, 1951). Al_2O_3 (Peter Spence and Sons) used for chromatography was activated to activity II, as determined by the method of Brockmann & Schodder (1941), by heating to 100° *in vacuo*. In most cases the amount of Al_2O_3 used was 30 times the weight of the fraction to be chromatographed.

All solvents were dried and redistilled before use.

Collection, extraction and fractionation of urine specimens

Urine was collected daily from each case using toluene as preservative, and hydrolysed by boiling under reflux for

* Since the completion of the present work Dorfman & Unger (1950) have reported that they have demonstrated the conversion of progesterone into *allo*pregnane-3 α :20 α -diol in human subjects. Experimental details of this work are not yet available, however.