5. The components characterizing the three groups have a common property, not shared by any other of the serum proteins, of binding haemoglobin under the conditions used for the electrophoresis. They migrate in the α_2-globulin position when filter-paper electrophoresis is used.

6. The occurrence of the characteristic proteins may be related to age and to hereditary factors.

7. Possible reasons for the high resolving power of the new method and its limitations are discussed.

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The Absorption Spectra of Porphyrin *a* and Derivatives

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In recent years, there has been a revival of interest in the problem of the structure of the prosthetic group of cytochrome *a* and cytochrome oxidase. Negelein (1933) and Roche & Benevent (1936) made the earliest attempts to isolate haemin *a* from heart muscle, but improvements in the preparative method by Rawlinson & Hale (1949) allowed of greater success. Warburg & Gewitz (1951) have claimed the isolation of crystalline 'cytohaemin', but publish no experimental details. Attempts to isolate haemin *a* from cytochrome oxidase and cytochrome *a* obtained in 'solution' by the use of sodium cholate have been reported by Dannenberg & Kiese (1952), and Falk & Rimington (1951) have studied the preparative methods for porphyrin *a*. Lemberg & Falk (1951) compared the spectroscopic properties of the porphyrin *a* prepared by Rawlinson & Hale with other carbonyl-porphyrins, and Lemberg (1953) has recently published more extensive results on porphyrin *a* and other porphyrins of heart muscle.

We have now shown that specimens of porphyrin *a* possessing identical spectra can be obtained under different experimental conditions. By applying a new method of porphyrin estimation (Oliver & Rawlinson, 1951), it has been possible to express the extinction data on a millimolar basis. The effects of solvents, oxime and copper-complex formation on the absorption spectrum of porphyrin *a* have also been examined.

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EXPERIMENTAL

Preparation of porphyrin a

Method A. The method is based on that of Rawlinson & Hale (1949).

(1) Six ox hearts freed of fat and ligaments were minced finely and extracted three times with 4% NaCl (total vol., 36 l.) at 0°. After draining, the pulp was treated twice with 2 vol. of cold ethanol; the pulp was pressed free from solvent each time. Extractions were then made with 2 vol. portions of acetone at 0°, until no more yellow pigments appeared in the extracts (usually three times). Excess of acetone was removed in a mechanical press.

(2) The dehydrated tissue was stirred with 2 vol. of chilled, acidified acetone (containing 2·5% v/v of 10N-HCl) and left standing at 0° for 1 hr.

(3) The dark-brown acetone solution was separated, filtered and added to an equal volume of peroxide-free ether. Addition of water (2 vol.) caused transfer of the pigments into the ether phase. Acetone was removed from the ether solution by washing with 2N-HCl. The ether solution contained both haemin *a* and protohaemin, as judged by the haemochrome test. The ether solution was filtered through a column of alumina (British Drug Houses Ltd., for chromatographic analysis) and the mixed haemins were obtained as a dark band at the top of the column.

(4) After thorough washing with fresh ether, the haemins were eluted with glacial acetic acid at 60–70°. The removal of iron by ferrous acetate and glacial acetic acid (Warburg & Negelein, 1932) gave a mixture of protoporphyrin and porphyrin *a*.

(5) After dilution with water, the mixed porphyrins were transferred to ether and washed free of acetic acid. Rapid extraction with 25% (w/v) HCl removed the porphyrin. By careful addition of solid Na_2CO_3 it was found that

porphyrin *a* alone could be displaced into a layer of fresh ether. At this stage the aqueous phase contained only negligible amounts of porphyrin *a* and practically all the protoporphyrin. Traces of this pigment detected in the ether solution were extracted by shaking two or three times with 5% (w/v) HCl.

(6) The ether was washed free of acid and then removed at room temperature under reduced pressure; the green residue was dried thoroughly over KOH. The product was washed with light petroleum four or five times and then dried in air. After this treatment the pigment could be shaken in ether–HCl systems without the formation of emulsions so often experienced in earlier stages of the purification. This indicated that much lipid material had been removed. The porphyrin was stored in chloroform solution at 0° and remained unchanged for at least 6 months.

Method B. This method of preparation gave porphyrin *a* that was spectroscopically indistinguishable from the previous preparation, the logarithmic density plots of the absorption curves being identical (Fig. 1).

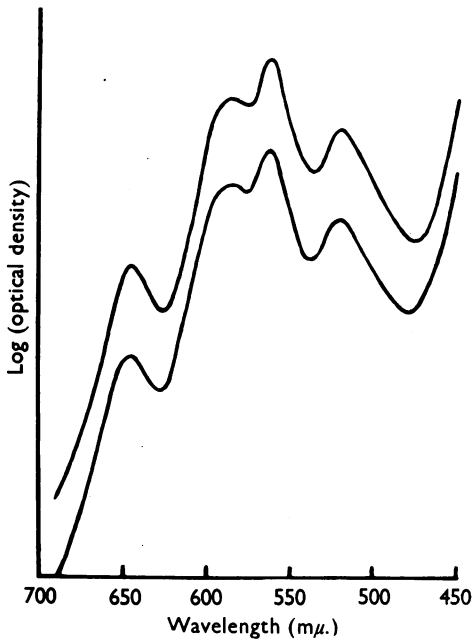

Fig. 1. Characteristic absorption spectra of porphyrin *a* prepared by different methods. Chloroform solution. Bottom curve, method A; top curve, method B.

Stages (1) and (2) were carried out as in method A.

(3) Crushed ice was added to bring the acid–acetone extract to 20% (v/v) with respect to water, and the solution filtered in the cold to remove lipid matter thus precipitated. The total haemins were then precipitated as the barium salts, the optimum conditions being found by trial. For each litre of acetone extract, 670 ml. of a 10% (w/v) barium acetate solution was prepared. The extract was then stirred into the barium acetate solution. The haemins were completely precipitated as the barium salts, and after standing overnight at 0° the supernatant was decanted. The dark-green precipitate was centrifuged off, washed once with ethanol and then with water to remove excess of barium acetate.

(4) The barium salts were decomposed in the following way: The precipitate was stirred into a sludge with water and 10 N-HCl was added dropwise with stirring until the colour changed from green to brown, indicating conversion of the barium salts into acid haematins. Water was added and the mixture centrifuged. The brown residue was then washed with 0·03 N-HCl until the supernatant was free from barium. Haemochrome tests of this residue indicated the presence of haemin *a* and protohaemin.

(5) The precipitate was dissolved in pyridine and diluted with 2·3 vol. of 0·2 N-HCl. Shaking this solution with ether transferred most of the haemin *a*, together with small amounts of protohaemin, to the organic phase. The ether solution was washed with 2 N-HCl to remove pyridine, and then with water. The haemin, after adsorption on alumina and elution with warm glacial acetic acid (stage 4 of method A), was converted into the free porphyrin by the method of Warburg & Negelein (1932).

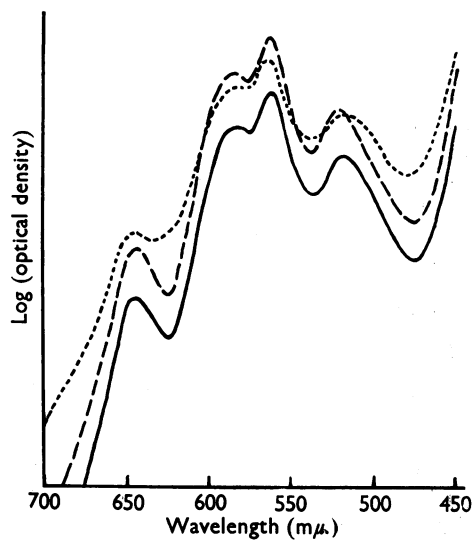

Fig. 2. Characteristic absorption spectra of fractions obtained during preparation of porphyrin *a* by method B. Chloroform solution. —, Fraction I (0–5% (w/v) HCl fraction); – – –, fraction II (5–12% (w/v) HCl fraction); - - - -, fraction III (unextracted by 12% (w/v) HCl).

(6) The porphyrin was transferred into ether solution in the usual way, and then shaken with 2% (w/v) HCl to remove a small amount of protoporphyrin. Further fractionation was then carried out against 5% (w/v) and 12% (w/v) HCl. Shaking with 5% HCl was continued until no further porphyrin fluorescence was detected in the aqueous phase. This fraction (I) contained a porphyrin of the *a* type and traces of protoporphyrin remaining after the 2% (w/v) HCl extraction. Fraction II was obtained by repeating the process with 12% (w/v) HCl, and the porphyrin remaining in ether constituted fraction III.

Spectrophotometric examination of the three fractions was carried out in chloroform solution (Fig. 2). Fraction I had a high content of protoporphyrin, fraction II was identical with the porphyrin *a* of the previous method, and fraction III was obviously contaminated with some unknown pigment. From this evidence it was considered that the preparation of a 5–12% (w/v) HCl fraction as described above represents another method of obtaining porphyrin *a*.

Although the two methods of preparation involve largely the same principles, they differ in some important details. In method B, protohaemin is removed from the preparation and conversion into the porphyrin gives a preparation of porphyrin *a*, almost free of protoporphyrin, which requires relatively little treatment with hydrochloric acid to complete the separation.

In method A, the mixed porphyrins must be separated by the acid fractionation procedures, and concentrated acid is used. Falk & Rimington (1951) have claimed that treatment with hydrochloric acid alters the absorption spectrum of porphyrin *a*, so the time taken over the acid procedure had been kept to a minimum.

In each case we attempted to obtain a preparation which was completely free of protoporphyrin, had band positions and relative band intensities similar to the preparation of Rawlinson & Hale (1949), and consisted of a single porphyrin, as far as could be ascertained from spectroscopic and spectrophotometric measurements of the neutral and acid spectra.

Estimation of porphyrin concentration

The copper-titration method of Oliver & Rawlinson (1951) determines porphyrin. It does not detect non-porphyrin impurities that do not react with copper, and it was shown (Oliver & Rawlinson, 1951) that preparations of porphyrin *a* contained no copper-binding impurities that contribute to the optical-density change at the wavelengths used for measurements.

Copper–porphyrin a complex

This was prepared from a solution of porphyrin *a* of known concentration by treatment in glacial acetic acid solution with a slight excess of copper acetate, in a boiling-water bath. The pigment was transferred to ether solution and washed with distilled water to remove acetic acid and any excess of copper salt. It was stored in dry chloroform at 0°.

Oxime derivatives

Oximes of both the free porphyrin and the copper complex were prepared by the addition of a few mg. of hydroxylamine hydrochloride to a pyridine solution of the pigment containing anhydrous Na_2CO_3. The reaction was judged to be complete when no further shift in band positions could be detected in the Hartridge reversion spectroscope.

Measurement of absorption spectra

All solvents used in spectrophotometry of the pigments were carefully purified by distillation in glass, and, for glacial acetic acid, by crystallization before distillation. Where possible, solvents were dried. A Beckman spectrophotometer (Model DU) was used, at slit widths not exceeding 0·1 mm. All solutions were compared in 1 cm. cells with a blank of the pure solvent, over the wavelength range 700–350 mμ.

In all cases, 5 ml. portions of the stock solution of porphyrin *a* in chloroform (concentration 0·03 mM) were rapidly evaporated to dryness in test tubes placed in a boiling-water bath, and redissolved at room temp. by gently shaking with 5 ml. of the solvent to be used. Two 5 ml. portions of the stock solutions were transferred to an equal volume of pyridine, anhydrous Na_2CO_3 was added to each and a few mg. of hydroxylamine hydrochloride were then added to one of them. Gentle warming of the solution resulted in the rapid formation of the oxime derivative, and the solution was cooled to room temperature before measurement.

With the metalloporphyrin, solutions in different solvents were prepared from a stock solution in chloroform (concentration 0·02 mM) by the same methods as those used for the free porphyrin. The oxime derivative was prepared from a pyridine solution of the metal complex as described above. For wavelengths below 450 mμ. the porphyrin solutions were diluted 11 times and the metalloporphyrin solutions were diluted 6 times, in order to keep optical-density values below about 0·7.

When organic solvents were used, precautions were taken to avoid errors due to evaporation and experimental checks made to ensure that these were adequate.

RESULTS

These are presented as figures showing the absorption spectra of porphyrin *a* and copper–porphyrin *a* in various solvents, and also of their oxime compounds. The absorption spectrum of porphyrin *a* in *p*-dioxan and chloroform is shown in Fig. 3. Fig. 4 shows the spectrum of porphyrin *a* and its oxime in pyridine solution. Fig. 5 shows the spectrum of copper–porphyrin *a* in chloroform and acetic acid, and Fig. 6 the spectrum of the metalloporphyrin and its oxime in pyridine solution. The

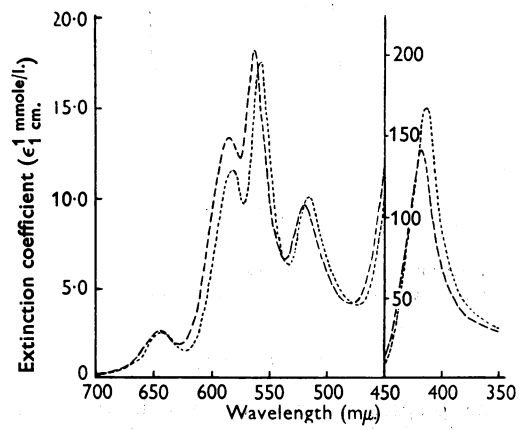

Fig. 3. Absorption spectra of porphyrin *a* in neutral solvents. - - - - -, In *p*-dioxan; – – –, in chloroform.

curves are defined in terms of millimolar extinction coefficients (E, cm.^{2}/millimole). Table 1 shows the millimolar extinctions of porphyrin *a* and derivatives at the wavelengths of maximum and of minimum absorption.

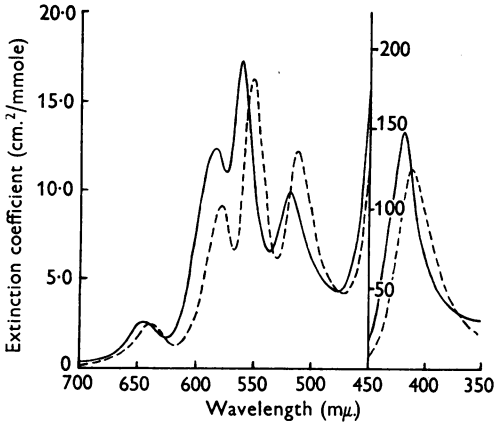

Fig. 4. Absorption spectra of porphyrin *a* and its oxime. Solvent, pyridine. —, Porphyrin *a*; – – –, oxime.

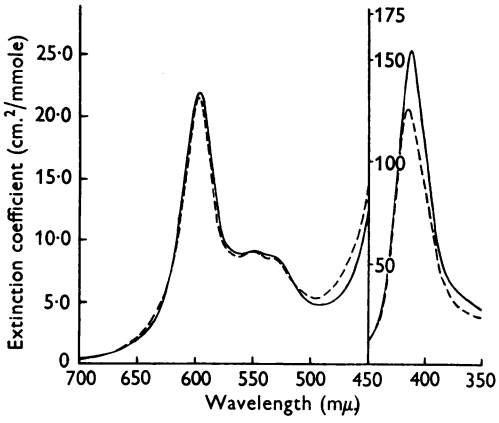

Fig. 5. Absorption spectra of copper–porphyrin *a* in different solvents. —, In acetic acid; – – –, in chloroform.

DISCUSSION

In the preparation of pure haemin *a* and its porphyrin, it is necessary to select conditions carefully if modification is to be avoided. Whatever procedures are adopted, the time of treatment must be short and the temperature kept as low as possible. In view of the lability of the aldehyde group (Rawlinson & Hale, 1949) alkaline conditions must be avoided, particularly in crude extracts. Strongly acid conditions have been shown to bring about a slow change (Falk & Rimington, 1951) and we have therefore avoided the use of strongly acidic conditions. Although still not ideal, these conditions represent a possible compromise. The hydrophobic character of haemin *a* simplifies its separation from other haemin compounds but makes complete purification more difficult. The lipid material probably inhibits crystallization, and in our experience its removal enhances the stability of haemin *a* to alkali.

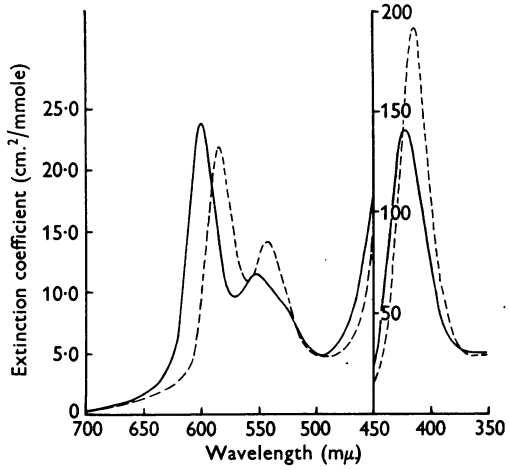

Fig. 6. Absorption spectra of copper–porphyrin *a* and its oxime. Solvent, pyridine. —, Copper–porphyrin *a*; – – –, oxime.

In developing the methods described for preparing porphyrin *a* we have kept a continual check on the possibility of modification of pigment. After the removal of protoporphyrin in the final purification it was found in method B that further fractionation was necessary to obtain a pigment identical with the porphyrin from method A. In Fig. 2 the use of the 'characteristic' absorption spectra reveals the spectroscopic differences between porphyrin *a* (fraction II) and the other fractions obtained.

Fraction II, which we term porphyrin *a*, possesses a spectral pattern similar to the preparations obtained by Rawlinson & Hale (1949) and Falk & Rimington (1951), namely oxo-rhodo type (i.e. intensities of absorption bands descend in the order III, II, IV, I). On the other hand, the porphyrin isolated by Dannenberg & Kiese (1952) possessed a rhodo-type spectrum, namely the order is III, IV, II, I. These authors have mistakenly compared their porphyrin with the oxime derivative of Rawlinson & Hale.

The preparations of porphyrin *a* used in this work show an absorption-band ratio III/IV of 1·9 (1·88–1·94) in three different solvents (from Table 1). This value is considerably higher than

Table 1. *Absorption band maxima and minima of porphyrin* a *and some derivatives in various solvents*

Wavelengths are in mμ. The figures in brackets are millimolar extinction coefficients.

Substance and solvent		I	II	III	IV	Soret
Porphyrin *a*						
(i) Chloroform	Max.	645 (2·65)	585 (13·4)	562 (18·2)	520 (9·6)	418 (141)
	Min.	626 (1·93)	576 (12·5)	536 (6·6)	478 (4·1)	
(ii) Dioxan	Max.	644 (2·54)	582 (11·6)	558 (17·4)	516 (10·1)	414 (167)
	Min.	622 (1·60)	572 (9·7)	534 (6·3)	472 (4·0)	
(iii) Acetic acid	Max.	646 (2·81)	586 (12·8)	562 (15·9)	520 (8·2)	414 (161)
	Min.	634 (2·58)	576 (12·3)	534 (6·9)	480 (4·1)	
(iv) Pyridine	Max.	645 (2·58)	583 (12·4)	561 (17·3)	519 (9·9)	419 (148)
	Min.	624 (1·77)	574 (11·1)	535 (6·5)	476 (4·3)	
Porphyrin *a* oxime						
(i) Pyridine	Max.	639 (2·48)	578 (9·1)	552 (15·9)	512 (12·2)	412 (125)
	Min.	616 (1·30)	567 (6·7)	530 (6·2)	472 (4·2)	
Copper–porphyrin *a*						
(i) Chloroform	Max.	598 (21·8)	550 (9·2)	534 (8·7)	—	415 (125)
	Min.	560 (8·72)		496 (5·40)		
(ii) Acetic acid	Max.	596 (22·1)	550 (9·2)	534 (8·8)	—	413 (155)
	Min.	558 (9·00)		492 (4·8)		
(iii) Pyridine	Max.	600 (23·8)	552 (11·4)	—	—	422 (140)
	Min.	570 (9·7)		496 (4·9)		
Copper–porphyrin *a* oxime						
(i) Pyridine	Max.	584 (21·8)	544 (14·2)	—	—	414 (190)
	Min.	560 (11·0)		492 (4·8)		

those of Falk & Rimington (1951) or Rawlinson & Hale (1949) but lower than those reported by Lemberg (1953), who obtained values between 2·03 and 2·11. Lemberg also isolated from heart muscle a crystalline cryptoporphyrin *a* which is found in close association with porphyrin *a* and is obtained in the 8% (w/v) HCl fraction in the Willstätter separation.

The method of preparing porphyrin *a* reported in this paper makes it highly probable that our product was contaminated with cryptoporphyrin *a*. The extinction data given by Lemberg (1953) for bands III and IV of pure porphyrin *a* and crystalline cryptoporphyrin *a* allow the calculation of the band ratio III/IV to be expected with mixtures of porphyrin *a* and cryptoporphyrin *a*. These calculations show that only 2% of cryptoporphyrin *a* present in a sample of porphyrin *a* would suffice to lower the III/IV ratio from 2·09 to 1·91.

Assuming that cryptoporphyrin *a* is the most likely impurity in our preparation, 98% purity can be claimed for the porphyrin *a*. Further, we do not claim an accuracy greater than 2% for the copper-titration method used for the estimation of porphyrin concentration, and the degree of purity given above is therefore reasonable.

In this work we have avoided the use of the more volatile solvents such as ether, in order to minimize evaporation during measurement. In addition, we wished to compare the spectra of porphyrin *a* in solvents with different characteristics. Fig. 3 shows the absorption spectra of porphyrin *a* in chloroform and dioxan. The general shift of visible bands to longer wavelength in the solvent of higher refractive index (chloroform) is consistent with that expected (Granick & Gilder, 1947). In pyridine and acetic acid, the absorption spectra of porphyrin *a* show no appreciable difference in the band positions in these two solvents, but the absorption bands are sharper in pyridine.

The curves for porphyrin *a* and its oxime derivatives in pyridine (see Fig. 4) confirm previous work. There is a general shift of absorption bands to shorter wavelengths, and the ratio between band II and band IV changes so as to give a typical rhodo-type spectrum for the oxime.

The spectra of copper–porphyrin *a* in glacial acetic acid and in chloroform (see Fig. 5) are similar to those of haemochrome *a*. Although most other covalent metalloporphyrin complexes possess very definite α- and β-bands in the visible region, the copper complex of porphyrin *a* shows only weak relative intensity in the β-region, i.e. its spectrum resembles that found by other workers for pyridine-haemochrome *a*. In pyridine, (see Fig. 6) copper–porphyrin *a* shows a typical β-band instead of two feeble bands in the same region in glacial acetic acid and in chloroform. On conversion into the oxime, the copper complex has the more usual type of two-banded spectrum, showing

the effect of the free aldehyde group. The Soret band of the oxime of the copper complex, in addition to being shifted to shorter wavelengths, is much more intense than that of the copper–porphyrin *a* itself.

The similarity of the absorption spectra of copper–porphyrin *a* and pyridine-haemochrome *a*, and their difference from the usual two-banded systems of most covalent metal–porphyrin derivatives, leads us to consider cytochrome *a* and cytochrome oxidase. It appears to be widely accepted that these components possess the same or closely related prosthetic groups, and Lemberg (1953) has produced good evidence that haemin *a* is in fact the prosthetic group common to both moieties. In general, the two-banded type of spectrum is found when the metal bonds are covalent, e.g. in oxyhaemoglobin. The ionic complexes, such as reduced haemoglobin, usually show a more diffuse absorption spectrum. Reduced cytochrome *a* has a visible absorption band at a wavelength (605 mμ.) longer than that of copper–porphyrin *a* and pyridine-haemochrome *a* (598 and 587 mμ. respectively). The absence of a strong β-band is common to all three pigments, and is attributable to the presence of a free aldehyde group, as conversion into the oxime results in a stronger β-band. The apoprotein of the cytochrome must be responsible for the significant difference between the position of the absorption bands of cytochrome *a* and pyridine-haemochrome *a*.

SUMMARY

1. Two different methods of preparation have yielded porphyrin *a* with identical characteristics.

2. The quantitative absorption spectra of porphyrin *a* in chloroform, acetic acid, pyridine and dioxan are given and discussed.

3. The spectral changes brought about by the formation of both oxime and copper derivatives of porphyrin *a* have been measured.

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Ultrafiltration of the Parathyroid Hormone

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The aim of the present work has been to ascertain whether ultrafiltration through cellophan can usefully be employed as a stage in the purification of the parathyroid hormone. This possibility was suggested by the dialysis experiments of Davies & Gordon (1953), who showed that material able to stimulate the excretion of phosphate in the urine of rats could pass through such membranes. Before such a technique can be applied some means must be employed to extract the hormone from the glands.

Since Ellingson, Bell & Hanson (1924) and Collip (1925) first established that active extracts can be obtained by treatment of parathyroid glands with HCl at 100°, very few attempts have been made to vary the solution used for extraction, or even the time and temperature employed.

The conditions required should evidently be such as to free the hormone from associated material and especially from other hormones, if any such are secreted by the same gland, without modifying any of the properties of the active molecule itself. If, however, several hormones exist in the gland as a single large molecule or as a complex of molecules depending for stability on forces weaker than covalencies, then, in one sense, such conditions may be mutally incompatible. An example of this type of behaviour has been given by Van Dyke, Chow, Greep & Rothen (1942), who showed that the oxytocic and antidiuretic hormones of the posterior pituitary are associated more or less firmly with one another in the juice expressible from the gland.

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