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 oxy-haemoglobin. 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 m μ .) longer than that of copper-porphyrin *a* and pyridine-haemochrome *a* (598 and 587 m μ . 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 mutually 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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The vigorous extraction with hot HCl which has been used so unvaryingly since its introduction by Ellingson *et al.* (1924) and Collip (1925) has the great advantage that it most certainly inactivates proteolytic enzymes which might otherwise destroy the hormone. The sensitivity of parathyroid hormone to pepsin has been clearly demonstrated by Ross & Wood (1942). Thus, so long as the aim of the work is to obtain the smallest molecule still retaining hormonal activity, the HCl extraction procedure might be acceptable were it not for certain recent findings suggesting that parathyroid hormone, at least as prepared by HCl extraction, is liable to form complexes with other protein molecules. Thus Handler, Cohn & Dratz (1954) found that proteins of very different properties eluted from an ion-exchange chromatogram were all active as the hormone.

Unfortunately, the earlier attempts to extract parathyroid glands with reagents milder than hot dilute HCl have been uniformly unsuccessful (Hjort, Robison & Tendick, 1925). More recently, the only attempt to avoid the use of HCl had been that mentioned above by Davies & Gordon (1953), who obtained active solutions by extraction of acetone powders of the gland with 80% acetic acid. Even this extraction was done at 75°. For these reasons, and because glacial acetic acid has been used successfully by Payne, Raben & Astwood (1950) for extraction of corticotropin from the pituitary, it was decided to use 80% acetic acid for the present experiments.

Because it had been noticed that these extracts are rather unstable, it was decided to carry through a preliminary precipitation with acetone before going on to the ultrafiltration stage. By this means all the material insoluble in acetone-acetic acid-water (70:24:6 by vol.) was rejected. Some additional purification was also obtained by dialysis against water. This was followed by reacidification to approx. 2N with respect to acetic acid and ultrafiltration. The conditions used were rather similar to those described by Cortis-Jones, Crooke, Henly, Morris & Morris (1950), who found that corticotropin could pass through a cellophan membrane to a maximum extent at pH 2.

EXPERIMENTAL

Materials. Ox parathyroid glands were collected on solid CO₂ at the time of slaughter and kept at -5° until used. Acetic acid and acetone were both redistilled before use.

Biological testing. The mouse urine-phosphate assay method for parathyroid hormone of Davies, Gordon & Mussett (1955) was used at each stage of the preparation. Since one batch of Eli Lilly Parathormone was used as standard this gave values of 'urine phosphate activity' in terms of this material, which was assumed for the purposes of these experiments to have 100 phosphate units/ml. The results obtained are given in Tables 1-3.

Solution I^B was also assayed by the rat plasma-calcium method of Davies, Gordon & Mussett (1954). The results of this assay are given in Table 3. Insufficient I^{DP} was available for a full assay, but some information about the plasma calcium-raising activity of this material was obtained by injecting it subcutaneously into parathyroid-ectomized rats at a dose level of 5 mg./rat. Five similar rats received 50 U.S.P. units each of Parathormone at the same time. The groups of rats were exchanged on a subsequent day when the test was repeated. The increases in plasma calcium thus obtained are given below (p. 648).

Preparation of 'acetone powder'. Acetone powder was made from the glands as described by L'Heureux, Tepperman & Wilhelmi (1947), except that only a single mincing was done. This was carried out with the fine plate (2.0 mm. holes) on the glands as soon as they had warmed up sufficiently to be separable from the frozen mass. As soon as it emerged from the mincer the mince was refrozen by mixing with solid CO₂. A portion (820 g.) of glands thus treated yielded 158 g. of acetone powder. This was kept at -5° and worked up as described below. Preparations I, II and III were all made from this same acetone powder.

Extraction of acetone powder with acetic acid. Preparation I: 40 g. of acetone powder were suspended in 600 ml. of 80% (v/v) aqueous acetic acid. By stirring in a water bath at about 90° the temperature of the suspension was quickly raised to 70° and maintained between 70 and 75° for 5 min. Next the flask was gently shaken for 1 hr. on a mechanical shaker. At the end of this period the undissolved material was removed by filtration through a Büchner funnel, covered with the cellulose powder Solka Floe (Brown Co., Berlin, New Hampshire). The filtrate (385 ml.) obtained was transferred to a beaker which was cooled externally with crushed ice. Next acetone was added with stirring to a concentration of 70% (v/v). The final temperature was 15°. After standing for 10 min. in the cold the precipitate was removed at the centrifuge and discarded. Further addition of acetone to a concentration of 86% (v/v) brought down a second precipitate, which was again removed at the centrifuge. After two washes with acetone the resultant material was dried *in vacuo* at 0° over H₂SO₄. A light-brown powder (2.21 g.) was obtained.

Dialysis against water. The powder was suspended in water and dissolved by the addition of just sufficient 0.1N-HCl; the resultant solution was then diluted with water to 60 ml. After adjustment of the pH to 5.5 by means of 0.1N-NaOH, this solution was dialysed in cellophan at 0° against 2 l. of water. Dialysis was continued for 4 days, during which time the water was once renewed. After dialysis the solution from inside the membrane was freed from a light precipitate at the centrifuge. Samples were removed and tested for biological activity/unit of dry weight as shown in Tables 1 and 3.

Ultrafiltration. After dialysis at neutrality, 15.2 ml. of glacial acetic acid were added to the solution, thus giving 117 ml. of solution approx. 2.2N with respect to acetic acid. This was now ultrafiltered through a Visking cellophan dialysis membrane (1.7 cm. diameter when dry) maintained at 2° in the cold room. A pressure of 10 lb./sq.in. applied for 90 hr. resulted in the formation of 77 ml. of ultrafiltrate I^{UF}. At the end of the period, 31 ml. (I^B) were recovered from inside the sack, representing a loss of 9 ml. by evaporation. The ultrafiltrate I^{UF} was evaporated to dryness *in vacuo* in the presence of octan-2-ol. The bath temperature was not allowed to exceed 40° and the last

stages of the evaporation were carried out by shaking the flask manually. This was repeated after the addition of a few ml. of water to ensure the removal of most of the acetic acid. After drying *in vacuo* over NaOH to complete the removal of acetic acid, the solid was dissolved in water by the addition of 0.2 m-mole of HCl. Approximately this amount of HCl was required to effect solution, which occurred at about pH 3.5. Finally the solution was freeze-dried and the product I^{UF} stored at 2° *in vacuo*.

Repeat preparations. Preparations II and III were made just as described above for preparation I, except that preparation III was made from 27 g. of the acetone powder. In addition, after the first ultrafiltration the residue of preparation III (vol. 20 ml.) which had been retained by the membrane was diluted with acetic acid so that its volume and acetic acid content (approx. 2N) were the same

as before the ultrafiltration procedure. Ultrafiltration was then repeated in the same manner and ultrafiltrate (2) (see Table 1) was thus obtained.

Investigation of solutions obtained by ultrafiltration

Activity of I^{UF} in respect to plasma-calcium level of rats. In the test carried out as referred to above, the average increase for the 2 days in plasma calcium was 1.7 mg./100 ml., whereas the corresponding figure due to 50 U.S.P. units of Parathormone was 1.0 mg./100 ml. If the plasma initial levels are taken into account (Davies *et al.* 1954), this gives $z=6.17$ for the dose of 5 mg. of I^{UF} and $z=5.54$ for the 50 units of Parathormone. An analysis of variance shows that these values were significantly different ($P<0.05$). Previous work at this season of the year yielded an average slope of 2.0 for the Parathormone log dose-response (z)

Table 1. *Parathyroid activity before and after passing through dialysis membrane*

Units are units of mouse urine-phosphate activity, Parathormone being assumed to have 100 phosphate units/ml.

Preparation ...	Volume (ml.)			Total dry wt. (mg.)			Units/mg. dry wt.			Total units		
	I	II	III*	I	II	III	I	II	III	I	II	III
Before dialysis against water	—	66	34.3	—	—	1 010	—	—	9.1	—	—	9 190
After dialysis against water	102†	101†	51.4†	1 400	1 333	954	—	8.2	—	—	10 200	—
Ultrafiltrate (1)	77	82	34	120	128	62	43	34.4	42	5 140	4 410	2 600
Ultrafiltrate (2)	—	—	30	—	—	29	—	—	18	—	—	520
Solution retained by membrane	31	30	—	—	1 208	710†	7.8	9.9	5.1†	10 130	11 960	3 620

* Made from 27 g. of acetone powder.

† Volume before addition of glacial acetic acid.

‡ Solution after second ultrafiltration.

Table 2. *Recovery of urine-phosphate activity after ultrafiltration*

Preparation ...	Dry wt. (mg.)			Units of mouse urine-phosphate activity		
	I	II	III	I	II	III
Ultrafiltrates as % of original solution	8.6	9.6	6.5*	34†	27†	41
Values for ultrafiltrates expected if whole volume had passed through the membrane (% of original solutions)	12.6	12.6	10.3*	50†	37.5†	67

* 1st ultrafiltrate only.

† Calculated as percentage of activity of (ultrafiltrate + solution retained by membrane).

Table 3. *Assay results*

Material	Top dose level (µg./animal)	Potency	Units/mg. dry wt.	% Limits of error, calculated at the 5% level
Mouse urine-phosphate method*				
III Before dialysis against water	330	2.01	9.1	72-158
III ^R	330	1.13	5.1	77-133
III ^{UF} (1)	33	0.92	42	55-174
III ^{UF} (2)	50	0.60	18	37-181
II After dialysis against water	180	0.98	8.2	58-170
II ^R	160	1.05	9.9	71-143
II ^{UF}	60	1.37	34	71-151
I ^R	250	1.30	7.8	67-160
I ^{UF}	42	1.19	43	54-213
Rat plasma-calcium method†				
I ^R	35	2.05	7.3	53-258

* Twin crossover design against 0.5 and 1.5 units of Eli Lilly Parathormone.

† Triplet crossover design against 20, 50 and 125 units of Parathormone using 18 parathyroidectomized rats.

Fig. 1. Ultracentrifugal diagrams of I^{UF} and I^R . *a, b, c* and *d* are I^{UF} after respectively 48, 128, 208 and 288 min. at 260 000 *g*. *e* and *f* are I^R after respectively 32 and 160 min. at 260 000 *g*. See Table 4 for further details.

curve. Therefore, assuming this to be the slope, I^{UF} would contain 20.7 units/mg. dry wt.

Ultracentrifuge results. The material I^{UF} , which passed through the membrane in preparation I, and that, I^R , which was found inside the sac, were separately dissolved in 0.1M-NaCl, 0.05N acetic acid and examined in the Spinco analytical ultracentrifuge Model E. As is apparent from Fig. 1, only a single boundary was observed in each case. The details of the conditions under which these investigations were carried out are given in Table 4, together with the calculated sedimentation constants.

Electrophoresis. III^{UF} at a concentration of 0.8% of dry matter in 0.1M-NaCl, 0.05N acetic acid was examined in the Perkin-Elmer Tiselius apparatus. The mobilities of the three components observed are shown in Table 5 (see also Fig. 2). After recovery from the cell, the solution was freeze-dried and the solid redissolved in 0.08M calcium acetate at pH 5.1. After dialysis for 20 hr. against the same solution, a second electrophoresis was carried out. As shown in Table 5, three components were again observed.

DISCUSSION

Although all preparations of parathyroid hormone so far obtained have been shown to contain more than one molecular species, some data has already

Fig. 2. Electrophoresis of III^{UF} in 0.1M-NaCl, 0.05N acetic acid (pH 3.0). Descending side after 84 min. (cf. Table 5 for further details).

Table 4. Ultracentrifuge results

For conditions see text.							
Preparation	Dry matter in solution (%)	pH	Time at 260 000 <i>g</i> (min.)	Rotor temp. (free couple)		<i>S</i> ₂₀ '	<i>S</i> ₂₀ *
				Initial	Final		
I^{UF}	1.0	2.9	272	18.0°	21.0°	0.98	0.99
I^{UF}	1.2	3.0	224	20.0°	23.3°	0.98	0.99
I^R	1.0	2.9	224	20.0°	20.0°	1.43	1.45

* Not corrected for partial specific volume of hormone.

Table 5. Electrophoretic results on solution III^{UF}

For conditions see text.								
Solvent	pH	Time (min.)	Current (A)	Potential gradient (v/cm.)	Mobilities* (10 ⁻⁵ cm. ² v ⁻¹ sec. ⁻¹)			
					α	β	γ	
0.1M-NaCl, 0.05N acetic acid	3.0	84	0.015	6.9	-4.4	-3.1	1.2†	
0.08M calcium acetate buffer	5.1	46	0.013	8.2	-6.1	-2.8	0.0	

* Measured on descending side.
† Since no attempt was made to dialyse at pH 3 it seems probable that the height of this boundary may have been artificially increased.

accumulated on the molecular size of the hormone. Thus material prepared by Ross & Wood (1942) showed two components in the ultracentrifuge, the smaller having $S_{20}=1.6$. Also an active solution made by L'Heureux *et al.* (1947) by acetone precipitation, when subjected to 65 400 g for an hour, did not show any sedimenting component. The present extracts, which were obtained from an acetone powder of the gland by means of 80% acetic acid, have proved to consist mainly of molecules of about the size of the smaller component reported by Ross & Wood (1942), together with some yet smaller, capable of passing through cellophan. Such ultrafiltrates have been found to be active both in respect to plasma-calcium-raising ability and stimulation of urine-phosphate excretion. By the phosphate assay, this material has been shown to be 4–5 times as active on a dry-weight basis as the solution before ultrafiltration and to represent about half of the total activity previously present. Examination at pH 2.9 in the ultracentrifuge revealed only a single very slowly sedimenting boundary ($S_{20}=1.0$). On electrophoresis, however, at the same pH three separate components were apparent. In view of this complexity no measurements of diffusion constant or partial specific volume have yet been made. It may be of interest, however, to note that $S_{20}=1.0$ corresponds to a molecular weight of approximately 6000 for molecules of $D_{20}=13.6$ and $V=0.709$, the values obtained by Rothen (1940) for ribonuclease. This size of molecule is quite small enough to have passed through Visking cellophan membrane under these conditions [cf. Cortis-Jones *et al.* (1950) and Tallan & Stein (1953)], who reported that lysozyme (mol.wt. 14 000–17 000) is able to diffuse through Visking cellophan membrane. Most probably, however, the diffusion constant of this material which has a sedimentation constant so much below that of ribonuclease ($S_{20}=1.85$) is well above 13.6, in which case the molecular weight can be expected to be below 5000.

Since on ultrafiltration about half of the activity always failed to pass through the membrane, the question arises as to whether the hormonal activity is associated with only one or with more than one of the smaller molar species or, on the other hand, whether it is also a property of the larger molecules with sedimentation constant $S_{20}=1.45$. That this latter hypothesis may be true is suggested by the results with preparation III, in which the solution retained by the membrane during the ultrafiltration was rediluted with 2N acetic acid and then subjected to a second ultrafiltration procedure. Only a further 520 units of activity were thus obtained. In addition, this result suggests that if the molecules ($S_{20}=1.45$) are

really aggregates of small active molecules with rather larger inactive molecules then they are not freely dissociable on dilution with 2N acetic acid.

It is of some interest that the present most active material has an activity of just over 40 units/mg. in the mouse urine-phosphate assay. This is very close to the figure of 44 units/mg. found by A. H. Gordon (unpublished) for material purified by electrophoresis in 0.1M calcium acetate buffer at pH 5.8. In addition, the activity of I^{45} in respect to the plasma-calcium level of rats has been roughly assessed. Assuming it to be some 20 units/mg. (see p. 648 for the assumptions involved) we have a urine-phosphate/plasma-calcium activity ratio of about 2. Since this ratio was determined only once and the calcium activity measured only approximately, this result taken by itself is not important; however, it acquires interest when compared with the findings of Davies *et al.* (1955), who obtained a urine-phosphate/plasma-calcium activity ratio of 2.6 for a preparation made according to L'Heureux *et al.* (1947), and of Kenny, Vine & Munson (1954), who report a ratio of 3.6 for a 'crude hot acid extract of bovine glands'.

Apparently, preparations of the hormone in which the ratio of urine-phosphate to plasma-calcium activity is higher than that characteristic for Parathormone can easily be obtained by a number of different methods.

The electrophoretic heterogeneity of the present ultrafiltrates prevents any decision as to whether both the biological effects of the hormone are due to a single molecular species. The very low isoelectric point of one of the components present (below pH 3) should, however, facilitate separation. Meanwhile, it is of interest that these ultrafiltrates contain only molecules considerably smaller than any previously shown to carry the hormonal activities. It thus appears that the chemical structures necessary for parathyroid activity form part of a molecule or molecules of about the same size as that of corticotropin.

SUMMARY

1. A method for extraction of hormone from acetone powders of the parathyroid gland by the use of acetic acid is described.
2. The extracts thus obtained have been fractionated by precipitation with acetone and then subjected to ultrafiltration through cellophan.
3. Assays based on the ability of the ultrafiltrate to stimulate the excretion of urinary phosphate in mice show that a four- to five-fold purification is achieved in the ultrafiltration step.
4. Preliminary ultracentrifugal and electrophoretic examination of this material has shown it to consist of more than one molecular species.

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Radioactive Compounds Excreted by Rats Treated with ³⁵S-labelled British Anti-Lewisite. Preliminary Studies

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Although British Anti-Lewisite (BAL) has been used, since its discovery, for the treatment of cases of poisoning by heavy metals (Stocken & Thompson, 1949), and more recently as a chemotherapeutic reagent in neurological disorders (Cumings, 1951; Streifler & Feldman, 1953), little is known of its metabolism in the mammal. Stocken & Thompson (1946) reported that the urine of rats and rabbits which had been given BAL by injection showed a strong nitroprusside reaction and an increased iodine uptake during the first few hours after administration, indicating the possible presence of a thiol. Evidence was adduced by Spray, Stocken & Thompson (1947) to show that in rabbits injected with BAL a dithiol compound was excreted which was very similar to BAL but not identical with it. Simpson & Young (1950) were also unable to detect the presence of BAL in the urine of rats treated with BAL. In further experiments, Spray (1947) concluded that BAL is not conjugated with sulphuric acid and he obtained indirect evidence that BAL might be excreted in conjugation with glucuronic acid.

Studies with ³⁵S-labelled BAL have shown that, in rats, most of the ³⁵S is excreted in the neutral sulphur fraction of the urine (Peters *et al.* 1947; Simpson & Young, 1950). Similar results were obtained in the present work, which showed

further that the greatest excretion occurred during the first 6 hr. after administration of the BAL. Because the sulphur of the BAL appeared to be excreted in an organically bound form, it was felt that an investigation of this sulphur-containing material would provide information concerning the manner in which BAL is metabolized. It was first necessary to determine whether one or more compounds derived from BAL were excreted and this was done mainly by paper chromatography. A system was developed for the detection of BAL on paper chromatograms and this was applied to the urines of rats which had been injected with radioactive BAL. The chromatograms were scanned for radioactive areas. It appeared that at least two radioactive materials were excreted; although these have not yet been identified, it appeared that one of the compounds is the chief excretory product of BAL. The second compound behaved like inorganic sulphate. A third material, detected in acidified urine, appeared to be a derived product resulting from the action of acid on other radioactive substances in the urine. Free BAL was not detected.

METHODS

Determination of radioactivity. To determine the radioactivity in the total sulphur of urine or other materials containing organically bound sulphur, the material was oxidized in a Carius bomb (cf. Young, Edson & McCarter, 1949). After heating, the bomb was cooled and opened,

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