Separation and Characterization of Urinary Indoles Resembling 5-Hydroxytryptamine and Tryptamine

By R. RODNIGHT

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, London, S.E. 5

(Received 10 May 1956)

The occurrence of 5-hydroxytryptamine (serotonin) in normal human urine is suggested by the experiments of Page and his co-workers. Twarog & Page (1953) examined acetone extracts of urine by a pharmacological method (isolated heart of *Venus mercenaria*) and reported a 'serotonin-like activity' equivalent to 0.1–1.0 μ g. of 5-hydroxytryptamine/ml. of urine. Later, Bumpus & Page (1955) described indoles with R_f values similar to 5-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine and *NN*-dimethyl-5-hydroxytryptamine (bufotenin) in similar extracts. In neither of these studies was the identity of the indoles confirmed by more than one test.

In the present work, a preliminary report of which has already been given (Rodnight, 1955), the ion-exchange resin Zeo-Karb 226 has been used to examine human urine for the presence of 5-hydroxytryptamine and related indoles. Zeo-Karb 226 is a carboxylic acid-type resin with similar properties to the resin Amberlite IRC 50, recently used to determine histamine in rat urine (Wilson, 1954). Weakly acidic resins of this type have several features which render them suitable for extracting amines from body fluids under mild conditions. In particular, they can be buffered, enabling columns to be operated at a neutral pH. Bases such as 5-hydroxytryptamine are then retained by the resin, whereas neutral ampholytes like tryptophan are not. Moreover, the absorbed bases can be readily displaced by weakly acidic solutions, in which they may be more stable than in the alkaline solutions often used with strongly acidic cation exchangers.

METHODS

Materials. 'AnalR' grade solvents and water were redistilled in an all-glass still. Sodium phosphate buffer (0.5M), pH 6, was prepared from $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, with conc. HCl to adjust the pH (glass electrode). Acetic acid (0.02N) in 75% (v/v) ethanol (eluting agent) was prepared daily from 95% (v/v) ethanol and 2N acetic acid. The ethanol was incorporated to reduce the quantity of buffer salt displaced from the resin along with urinary constituents.

Preparation of resin. Zeo-Karb 226 (H form) was obtained from The Permutit Co., Ltd. At first it was used in the mesh supplied (16–60), but later, better columns were obtained

with resin of mesh 90–120, prepared by grinding and sieving the coarse material.

New resin was stirred in a beaker with about 20 times its weight of N-NaOH, and allowed to settle for 5 min. The cloudy supernatant was decanted. The resin was washed free of alkali and fine particles by stirring in and decanting large volumes of water. Next the process was repeated with N-H₂SO₄ and the resin again washed. Finally it was stirred with about five times its weight of 75% (v/v) ethanol, filtered through sintered glass and dried at room temp. Buffering was carried out in the columns as described below.

Column chromatography

Preliminary experiments. Columns were prepared in water with resin buffered at pH 6. In a typical experiment 250 ml. of urine was brought to pH 6 and run through a column of resin 18 mm. $\times$ 240 mm. in 2 hr. This was washed with water and aqueous ethanol solutions and absorbed bases were eluted with 750 ml. of eluting agent overnight. Recovery of added 5-hydroxytryptamine was about 50%.

Routine procedure. The apparatus is illustrated in Fig. 1. The columns (Quickfit and Quartz Ltd.) were 18 mm $\times$ 400 mm.; the resin was supported by a sintered-glass disk *S*. The B 19 standard ground-glass cone below the disk fitted into the socket of a unit incorporating two stopcocks; the lower one, *A*, allowed liquid to flow straight through, the other, *B*, led to the raised outlet *C*. Stopcocks were not lubricated.

Each column contained 4 g. of resin (H form, 90- to 120-mesh). Regeneration and buffering were effected by filling the column with reagent from a reservoir through *B*, the raised outlet *C* being removed for this purpose; after sedimentation the reagent was run out through *A*. Three fillings (350 ml.) of N-H₂SO₄ and three of 0.1N-NaOH were necessary; several washes with water followed each reagent. Treatment with buffer was then carried out in the same way until the pH of the effluent fell to 6. Excess of buffer was run out, leaving a few millilitres covering the resin, and the column was then ready for use. No deterioration in performance after eight cycles has been noticed.

In operation stopcock *A* was closed and *B* open. The urine and all subsequent reagents were allowed to flow down the side of the column so that they layered on to the liquid above the resin. Rate of flow was controlled entirely by adjustment of the outflow from the reservoir, by a screw-clip on a rubber sleeve.

According to its specific gravity and 24 hr. volume, 75–120 ml. of urine was taken, adjusted to pH 6 (glass electrode), diluted to 250 ml. with water to reduce interference by salts, and run through a column at about 100 ml./hr. The resin was

washed with 125 ml. of de-gassed water (1 hr.), followed by 125 ml. of de-gassed 50% (v/v) ethanol (2 hr.). De-gassing was necessary to avoid the formation of bubbles in the column; the longer time allowed for the ethanol wash ensured regular shrinkage of the resin. Bases were eluted with 350 ml. of eluting agent, generally overnight, but sometimes in 4 hr. on the following day.

Concentration of eluates

Eluates contained, besides organic bases, salts mainly derived from the buffered resin. The eluate was rapidly (1 hr.) concentrated *in vacuo* (N_2) to less than 2 ml. with an oil pump and a condenser cooled with solid CO_2 in ethanol; the bath temp. was always below 30° . Ethanol (3 ml.) was added, and then 50–60 ml. of acetone in 10 ml. volumes. The precipitated salts were filtered off. The acetone and most of the ethanol were removed *in vacuo* (water pump) and the remaining liquid was quantitatively transferred with the aid of ethanol to a 150 mm. glass-stoppered tube (B24 standard ground-glass joint). The solution was evaporated to dryness *in vacuo* and the last traces of water were removed by the addition and evaporation of 1 ml.

Fig. 1. Type of column unit used for chromatography of urine on Zeo-Karb 226. For description see text.

volumes of ethanol. The dried deposit was then extracted twice with 5 ml. of acetone containing 1% (v/v) of ethanol and the extracts were filtered, combined and evaporated to dryness *in vacuo*. The residue was dissolved in 200 μ l. of 95% (v/v) ethanol and stored at -20° .

Paper chromatography

Ascending runs in one dimension on Whatman no. 1 paper, or more often no. 20, were generally used. Occasionally descending or two-dimensional techniques were employed. The solvents are given in Table 1.

Detection of indole spots. The best results were obtained by dipping the papers in Ehrlich's reagent. The stock solution was 1% (w/v) *p*-dimethylaminobenzaldehyde in acetone-light petroleum (b.p. $60-80^\circ$) (9:1); 7% (v/v) conc. HCl was added immediately before use. The dipped papers were allowed to dry for 3 min. and then placed in a strong current of warm air (40°) until they no longer smelt strongly of HCl (7–8 min.). The method could detect 0.05 μ g. of 5-hydroxytryptamine on 8 in. runs in the aqueous propan-1-ol or butanol-acetic acid solvents.

Quantitative methods. Data were obtained from chromatograms treated with Ehrlich's reagent. Approximate estimates were first made on one- or two-dimensional runs by visually comparing the intensity of the spots with spots of pure standard solutions run in parallel. A volume of extract containing 0.05–0.25 μ g. of amine was chosen. Spots of this volume and an equal number of twice this volume were applied alternately to sheets of Whatman no. 20 paper with a syringe pipette. On the lower volume spots on each sheet standards from 0.025 to 0.15 μ g. were superimposed. After ascending chromatography (7–8 in.) in aqueous propan-1-ol or butanol-acetic acid-water the spots were revealed by dipping in the Ehrlich's reagent. Occasionally, when using these solvents, masking and distortion of the spots made the estimate unsatisfactory; chromatograms were then run in propan-1-ol- NH_3 soln. (Table 1), although this solvent was generally avoided since it gave diffuse spots. Comparisons were made (a) visually or (b) photometrically.

(a) In a typical example 16 spots of 4 and 8 μ l. were run. The 8 μ l. spots most nearly matched the 4 μ l. spots to which 0.1 μ g. of 5-hydroxytryptamine had been added; they were clearly differentiated from the adjacent 4 μ l. spots which had received 0.075 and 0.125 μ g. of 5-hydroxytryptamine. The extract was therefore estimated to contain 0.2 μ g. of 5-hydroxytryptamine $\pm 25\%$ in 8 μ l.

(b) The maximum density of the spots was determined at 560 $m\mu$. in a Unicam spectrophotometer with a specially constructed adaptor. Measurements were made within 0.5 hr. of dipping. Compact and regular spots were essential for good results; densities were then proportional to 5-hydroxytryptamine content up to 0.25 μ g. The mean densities of the spots containing standard were plotted and the amine content of the extract was calculated from the mean density of the unknown spots. The accuracy varied from ± 8 to $\pm 15\%$, depending on the number of spots run and the scatter of their densities. In one instance 30 spots were run on five sheets. The 5-hydroxytryptamine content of the urine was calculated from the mean densities on each sheet as 51, 58, 64, 68 and 70 μ g. of 5-hydroxytryptamine/l., mean $62 \pm 15\%$.

RESULTS

Qualitative observations

Extracts prepared from 100 ml. of urine usually had a dry wt. of 5–10 mg.; 5% (w/v) solutions in ethanol were clear and pale yellow. Chromatograms of extracts, equivalent to 1–5 ml. of urine and developed with Ehrlich's reagent, always revealed two main bluish spots with R_F values in six solvent systems which corresponded to those of authentic 5-hydroxytryptamine and tryptamine obtained in parallel runs (Table 1). When 5-hydroxytryptamine and tryptamine were added to extracts complete identity of spots was obtained in all the solvents. Similar results were obtained on two-dimensional chromatograms. Both spots gave a greenish fluorescence with acid ninhydrin (Jepson & Stevens, 1953) and with potassium dichromate and formaldehyde (Shepherd, West & Erspamer, 1953); neither of these reactions is given by *N*-methyl-5-hydroxytryptamine or bufotenin. On the other hand, only the spots with R_F value corresponding to that of 5-hydroxytryptamine reacted with diazotized sulphanilamide (Block, Durrum & Zweig, 1955) or rapidly reduced ammoniacal silver nitrate (Dalglish, 1955). This indicated that one indole was phenolic and the other non-phenolic in character. Further evidence as to the identity of the spots was obtained by examining their action on guinea-pig ileum, since 5-hydroxytryptamine is a powerful stimulant of smooth muscle, and tryptamine only stimulates in high doses (Gaddum, 1953). To do this it was necessary to separate the two indoles.

Fractionation on cellulose powder. Extracts of several urine specimens were pooled and dried *in vacuo*. The residue (225 mg.) was dissolved in 2.5 ml. of propan-1-ol. The extract solution was pipetted on to a column of Whatman ashless cellulose powder, 10 mm. $\times$ 600 mm., prepared in 95% (v/v) propan-1-ol. The solution was allowed to soak into the column in about 2 hr.; it was then washed in at about 6 ml./hr. by successive additions of 2 ml. volumes of 92.5, 90 and 87.5% (v/v)

propan-1-ol, followed by 5 and 10 ml. volumes of 85 and 82.5% (v/v) propan-1-ol. Propan-1-ol (80%, v/v) was then run through at 2.5 ml./hr. and a total of 75 fractions each of 1 ml. collected on a fraction-collector. All fractions were examined by paper chromatography and Ehrlich's reagent. Fractions 3–7 contained fast-running material. The pooled fractions 14–16 (*A*) contained about 90% of the non-phenolic indole and all the phenolic indole was in the pooled fractions 27–40 (*B*). The pooled fractions *A* and *B* were each evaporated to dryness *in vacuo* and dissolved in 8–10 ml. of 0.9% (w/v) NaCl.

Tests on guinea-pig ileum. Up to 0.5 ml. of each fraction was added to guinea-pig ileum suspended in 18 ml. of Tyrode solution at 34°. Only fraction *B*, which contained the indole resembling 5-hydroxytryptamine, caused a contraction; this was unaffected by mepyramine, but was partially antagonized by a large dose of tryptamine (Gaddum, 1953). The height of the contraction caused by 0.1 ml. of fraction *B* was approximately matched by 1 μ g. of 5-hydroxytryptamine (Fig. 2), an estimate which corresponded to that obtained by paper chromatography. Fraction *A* had no action on the tissue. These observations, together with those made by paper chromatography, justify reference to the phenolic indole as 'urinary 5-hydroxytryptamine' and to the non-phenolic indole as 'urinary tryptamine'.

Other indole spots. In about one-quarter of the urine specimens, chromatograms of extracts run in butanol-acetic acid-water or aqueous propan-1-ol showed a very faint indole spot in the bufotenin position; bufotenin added to extracts also gave identity with this spot. Further evidence as to its nature has not been obtained. It gave no detectable colour with diazotized sulphanilamide, but this could be due to the low sensitivity for bufotenin of this reagent, which requires about 1 μ g. compared with 0.05 μ g. for the Ehrlich's reagent. Fast-running material reacting with Ehrlich's reagent was seen in most extracts; it usually appeared as

Table 1. R_F values of indoles run on ascending chromatograms for 7–8 in. at 16–20°

Solvent system	R_F values			
	5-Hydroxytryptamine	<i>N</i> -Methyl-5-hydroxytryptamine	Bufotenin	Tryptamine
Propan-1-ol-water (3:1)	0.48	0.53	0.59	0.69
Propan-1-ol-NH ₃ soln. (sp.gr. 0.88)-water (16:1:3)	0.47	0.66	0.86	0.71
<i>n</i> -Butanol-acetic acid-water (4:1:5)*	0.44	0.47	0.51	0.67
<i>n</i> -Butanol saturated with <i>N</i> -HCl†	0.18	—	—	0.43
<i>n</i> -Butanol-methylamine (3:1)†	0.56	—	—	0.89
<i>n</i> -Pentanol-pyridine-water (2:2:1)†	0.43	—	—	0.53

* Partridge (1946).

† After Erspamer (1955).

a diffuse streak beyond R_f 0.75, but sometimes formed two distinct spots at R_f 0.75 and 0.85. Other blue spots were occasionally noted, particularly with urine specimens from hospital patients.

Quantitative observations

Values for the recovery of some indoles added to urine are given in Table 2. The figures for 5-hydroxytryptamine are taken from a series of 10 experiments in which the mean recovery was 71 % (S.D. $\pm$ 13); those for bufotenin and *N*-methyl-5-hydroxytryptamine are the only values available. Tryptamine recovery in several experiments was between 60 and 90 %.

Results for the daily excretion of 'urinary 5-hydroxytryptamine' in normal adults are given in Table 3. They indicate a normal rate of excretion in these individuals of 40–150 μ g. of 'urinary 5-hydroxytryptamine'/24 hr.; 'urinary tryptamine' excretion was of the same order or somewhat lower. No obvious correlation with urine volume can be seen.

Daily variation in output; influence of oral administration of tryptophan. 5-Hydroxytryptamine in the body is probably derived from dietary tryptophan via the intermediate 5-hydroxytryptophan (Clark, Weissbach & Udenfriend, 1954); an adult diet supplies about 1 g. of tryptophan/24hr.

Fig. 2. Responses of guinea-pig ileum suspended in 18 ml. of Tyrode soln. at 34°; 3 min. cycle, 30 sec. contact time. A, Action of urinary fraction B (see text) in the presence of mepyramine. At *M* 0.5 μ g. of mepyramine was added to the bath, followed in 15 sec. by doses of histamine (1 μ g.), 5-hydroxytryptamine (5-HT) (1 μ g.) or fraction B (0.1 ml.) at *H*, 5-hydroxytryptamine and *B* respectively. B, Action of fraction B in presence of tryptamine (500 μ g.). The tryptamine (at *T*) was not washed out before addition of the stimulating agent (Gaddum, 1953).

Table 2. *Recovery of indoles added to human urine*

Results were obtained by estimating spot intensities on paper chromatograms by a photometric method to an accuracy of $\pm$ 12 % (see text).

Amine	Present in urine (μ g./l.)	Added (μ g./l.)	Total found (μ g./l.)	Recovery (%)
5-Hydroxytryptamine	32	133	117	71
5-Hydroxytryptamine	19	100	80	67
5-Hydroxytryptamine	18	100	105	89
<i>N</i> -Methyl-5-hydroxytryptamine	Not detected	420	300	71
Bufotenin	Not detected	200	130	65
Tryptamine	36	100	100	74

Table 3. *Daily excretion of 'urinary 5-hydroxytryptamine' and 'urinary tryptamine' in normal male adults*

Results were obtained by estimating spot intensities on paper chromatograms, either by visual inspection (accuracy $\pm 25\%$) or by photometric determination of spot density ($\pm 15\%$).

Subject	Urine vol. (ml.)	'Urinary 5-hydroxytryptamine' output ($\mu\text{g./24 hr.}$)	'Urinary tryptamine' output ($\mu\text{g./24 hr.}$)
Visual method			
1	1500	80	60
2	1820	70	70
3	1040	50	50
4	1820	100	110
5	1440	60	30
6	2300	115	50
7	950	120	—
8a*	1390	110	—
8b*	1390	100	—
Photometric method			
9	1350	116	—
10	1660	75	—
11	610	101	—

* Independent analyses on separate samples of the same 24 hr. urine by two workers.

Table 4. *Daily excretion of 'urinary 5-hydroxytryptamine' in a normal adult male, showing the effect of extra dietary tryptophan*

Results were obtained by estimating spot intensities on paper chromatograms ($\pm 15\%$). On day 5 L-tryptophan (2 g.) was administered.

Day	Urine vol. (ml.)	'Urinary 5-hydroxytryptamine' output ($\mu\text{g./24 hr.}$)
1	2010	54
2	940	48
3	1130	62
4	2050	50
5	920	106
6	1300	59
7	1360	45

Urine was collected in 24 hr. periods for seven consecutive days; 2 g. of L-tryptophan was administered in a single dose on the fifth day. All specimens were analysed in parallel and 'urinary 5-hydroxytryptamine' in the extracts was estimated. On a normal diet the output ranged from 45 to 62 $\mu\text{g./24 hr.}$, whereas during the period after ingestion of extra tryptophan it rose to 106 $\mu\text{g./24 hr.}$ (Table 4). In another experiment urine was collected for two 24 hr. periods; on the first day, diet was normal, on the second, 2 g. of L-tryptophan was given. Values for 'urinary 5-hydroxytryptamine' output were 80 and 160 $\mu\text{g./24 hr.}$ respectively.

DISCUSSION

These experiments strongly support the conclusion that 5-hydroxytryptamine occurs in normal human urine, as was suggested by Twarog & Page (1953). They also adduce evidence for the excretion of tryptamine in urine. No account of tryptamine in normal urine has been found in the literature, but Sullivan (1922) isolated a tryptamine-like base from 40 l. of urine obtained from cases of pellagra.

Methylated derivatives of 5-hydroxytryptamine in urine. Evidence for the occurrence of these indoles in urine has been given by Bumpus & Page (1955) who detected on chromatograms by Ehrlich's reagent spots with R_F values similar to those of 5-hydroxytryptamine and its *N*-methyl derivative and bufotenin. No attempt, however, was made to characterize the spots further by other tests such as the diazo reaction for phenols; consequently the presence of tryptamine, which in alkaline solvents such as Bumpus & Page used has an R_F value near to that of bufotenin, was not excluded. In the present work no satisfactory evidence for the occurrence of *N*-methyl-5-hydroxytryptamine or bufotenin in urine has been obtained, although both amines were recovered from urine in yields similar to those of added 5-hydroxytryptamine, and consequently an excretion greater than about 5 $\mu\text{g./24 hr.}$ would presumably have been detected. Bumpus & Page (1955) gave no indication of the relative amounts of 5-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine and bufotenin occurring in their extract.

Quantitative observations. Estimation of spot intensities of the indoles on paper chromatograms was the most sensitive chemical method available. Thus extracts prepared from 100 ml. of urine usually contained 3–10 $\mu\text{g.}$ of 'urinary 5-hydroxytryptamine', which is well below the range of the colorimetric method of Udenfriend, Weissbach & Clark (1955). The disadvantage of a large error was not serious since detection of small changes in output was not sought. On the basis of a recovery of 70 % by the method, the data in Tables 4 and 5 suggest a regular excretion of 'urinary 5-hydroxytryptamine' in normal subjects ranging from 60 to 150 $\mu\text{g./24 hr.}$ Since Humphrey & Jaques (1954) found a very low level of free 5-hydroxytryptamine in the plasma (about 0.2 $\mu\text{g./100 ml.}$) this estimate of 'urinary 5-hydroxytryptamine' output suggests that either the renal clearance of the compound is high or that platelet-bound 5-hydroxytryptamine is released into the plasma at or near the sites of urine formation. In either event the platelets, by their capacity to bind 5-hydroxytryptamine, are apparently functioning to conserve the compound for the organism. However, the possibility that part of the 'urinary 5-hydroxytryptamine' is contributed directly by kidney metabolism must also be

considered; preparations of kidney tissue rapidly form 5-hydroxytryptamine from added 5-hydroxytryptophan (Clark *et al.* 1954).

SUMMARY

1. The occurrence of indole bases in human urine has been studied by chromatography of urine on Zeo-Karb 226, elution of absorbed bases with acid ethanol and concentration to 0.002 of the volume of urine used. Indoles were investigated by paper chromatography and tests on guinea-pig ileum.

2. Urine extracts contained two indoles. Evidence strongly supporting the identity of one of these with 5-hydroxytryptamine and the other with tryptamine was obtained.

3. *N*-Methyl-5-hydroxytryptamine or bufotenin was not detected in the extracts.

4. By estimation of spot intensities on paper chromatograms recovery of 5-hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, bufotenin and tryptamine added to urine was of the order of 70 %.

5. The excretion of 'urinary 5-hydroxytryptamine' in twelve normal adults ranged from 45 to 120 $\mu\text{g.}/24 \text{ hr.}$; similar values were found for 'urinary tryptamine' output (six subjects).

The author is indebted to Professor H. McIlwain for encouragement and advice; to Mr E. K. Aves for excellent technical assistance; and to the Board of Governors of the Bethlem Royal Hospital and Maudsley Hospital for a grant from the Hospital Research Fund. Facilities and guidance

in the pharmacological experiments were very kindly given by Dr M. F. Lockett of the Chelsea Polytechnic. Gifts of 5-hydroxytryptamine creatinine sulphate from Dr B. A. Hems of Glaxo Laboratories, *N*-methyl-5-hydroxytryptamine oxalate from Dr J. B. Jepson of the Courtauld Institute and bufotenin from Dr M. E. Speeter of the Upjohn Co. are gratefully acknowledged.

REFERENCES

- Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Paper Electrophoresis*, 1st ed. New York: Academic Press Inc.
- Bumpus, F. M. & Page, I. H. (1955). *J. biol. Chem.* **212**, 111.
- Clark, C. T., Weissbach, H. & Udenfriend, S. (1954). *J. biol. Chem.* **210**, 139.
- Dalgliesh, C. E. (1955). *J. clin. Path.* **8**, 73.
- Erspamer, V. (1955). *J. Physiol.* **127**, 118.
- Gaddum, J. H. (1953). *J. Physiol.* **119**, 363.
- Humphrey, J. H. & Jaques, R. (1954). *J. Physiol.* **124**, 305.
- Jepson, J. B. & Stevens, B. J. (1953). *Nature, Lond.*, **172**, 772.
- Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.
- Rodnight, R. (1955). *Résumés des Communications*, 3rd Int. Congr. Biochem., Brussels, 15-9.
- Shepherd, D. M., West, G. B. & Erspamer, V. (1953). *Nature, Lond.*, **172**, 357.
- Sullivan, M. X. (1922). *J. biol. Chem.* **50**, xxxix.
- Twarog, B. M. & Page, I. H. (1953). *Amer. J. Physiol.* **175**, 157.
- Udenfriend, S., Weissbach, H. & Clark, C. T. (1955). *J. biol. Chem.* **215**, 337.
- Wilson, C. W. M. (1954). *J. Physiol.* **125**, 534.

The Prosthetic Group of Cytochrome a_2

By J. BARRETT*

Institute of Medical Research, Royal North Shore Hospital of Sydney, Australia

(Received 20 February 1956)

Cells of *Escherichia coli* and *Shigella dysenteria* which had been grown under aerobic conditions were shown by Yaoi & Tamiya (1928) to possess an absorption band which differed from those of previously described cytochromes in that it lay well within the red region of the spectrum. Keilin (1933) attributed this band to a component of the cytochrome system in these bacteria. Negelein & Gerischer (1934) and Fujita & Kodama (1934) independently published spectroscopic evidence that cytochrome a_2 , as it had been designated, was autoxidizable and could combine with carbon monoxide and cyanide. Unlike other cytochromes that had been observed the oxidized form showed a

band in the visible region of the spectrum, at 645 $m\mu$. Fujita & Kodama (1934) also showed that this cytochrome was widely distributed amongst other bacteria, e.g. *Azotobacter chroococcum*, *Proteus vulgaris*, *Acetobacter pasteurianum*, *Eberthella typhosa* and *Salmonella paratyphi*. The spectroscopic evidence of the properties of cytochrome a_2 led to the assumption that in these organisms it had the function of a cytochrome oxidase. This view remained current until the recent work of Tissières (1952), Moss (1952) and Chance (1953) threw doubt on it.

No attempt seems to have been made to establish the nature of the prosthetic group of cytochrome a_2 ; though Negelein & Gerischer (1934) suggested that it might be related to the ferrophaeophorbides, and

* Working under a grant from the National Health and Medical Research Council of Australia.