Comparison of the isolated and synthetic compounds.

Both substances had the same melting-point and no depression was observed in a mixture of the two. They showed no differences in solubility and both gave the same colour reaction with nitrite. Dr F. B. Strauss (Oxford) compared the absorption spectra of the isolated and synthesized compounds and gave the following opinion: 'both spectra are approximately identical; moreover the wave-lengths of maximum absorption as well as the molar extinction coefficients of maximum absorption are identical within the limits of experimental error.'

The colour reaction. 2-Acetamido-7-hydroxyfluorene (1 mg.) was dissolved in 5 ml. glacial acetic acid. 0.5 ml. of this solution was diluted with an equal volume of water, and a drop of 2N-HCl and four drops of a freshly prepared 0.1% solution of sodium nitrite were added. A deep purple colour developed. The dye could be extracted with amyl alcohol. Amounts as small as 20 μ g. are still detectable with this reaction.

The ether extract of the urine of rats receiving a diet containing 2-acetamidofluorene showed a strong nitrite reaction: 250 ml. of urine were extracted as described above, the ether was dried over sodium sulphate, evaporated and the residue dissolved in

5 ml. of glacial acetic acid. Of this extract 0.1 ml. still showed a positive nitrite reaction. When collection of the urine of rats was commenced 48 hr. after the withdrawal of the drug from the diet and the urine was treated in the same way, the extracts did not show a positive test, even when amounts corresponding to 50 ml. of urine were taken.

DISCUSSION

The isolation of 2-acetamido-7-hydroxyfluorene from the urine of rats receiving 2-acetamidofluorene suggests that the observation of Berenblum & Schoental (1943) is not confined to benzanthracene and dibenzanthracene. When 2-aminofluorene is treated with sulphuric acid 2-amino-7-fluorene-sulphonic acid is formed (Courtot, 1930). The biological hydroxylation has thus taken place in the same position as that in which sulphonation occurs *in vitro*.

SUMMARY

One metabolite of 2-acetamidofluorene has been isolated from the urine of rats, and has been identified as 2-acetamido-7-hydroxyfluorene.

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REFERENCES

- Armstrong, E. C. & Bonser, G. M. (1944). *J. Path. Bact.* **56**, 507.
Berenblum, I. & Schoental, R. (1943). *Cancer Res.* **3**, 686.
Bielschowsky, F. (1944). *Brit. J. exp. Path.* **25**, 1.
Boyland, E. & Levi, A. A. (1935). *Biochem. J.* **29**, 2679.
Chalmers, J. G. & Peacock, P. R. (1936). *Biochem. J.* **30**, 1242.
Courtot, C. (1930). *Ann. Chim. (Phys.)*, **14**, 122.
Diels, O., Schill, E. & Tolson, S. (1902). *Ber. dtsch. chem. Ges.* **35**, 3288.
Fieser, L. F. (1941). *Cause and Growth of Cancer*. Philadelphia: University of Pennsylvania Press.
Kennaway, E. L. (1930). *Biochem. J.* **24**, 497.
Wilson, R. H., DeEd, F. & Cox, A. J. (1941). *Cancer Res.* **1**, 595.

Purification and Properties of Cytochrome c

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It was previously shown that cytochrome *c* can be extracted in a soluble form from baker's yeast (Keilin, 1930) or from heart muscle of horse or ox (Theorell, 1935; Keilin & Hartree, 1937), and that fractionation of the cytochrome *c* from heart muscle gives rise to a preparation containing 0.34% Fe. A very simple method of preparation of cytochrome *c* of this purity was described (Keilin & Hartree, 1937), and this method has been generally adopted by other workers in their studies of this substance.

Attempts to increase the iron content above 0.34% were unsuccessful, and the protein appeared to be homogeneous in cataphoretic experiments.

While determining the isoelectric point of cytochrome *c* in the Tiselius apparatus, Theorell & Åkesson (1939) noticed that at pH slightly above the isoelectric point cytochrome *c* containing 0.34% Fe no longer migrated as a homogeneous protein. The isoelectric point of cytochrome *c* was given as 10.65 at 0°, and at pH 10.68 the red cytochrome *c* moved slowly towards the anode while a colourless boundary moved more rapidly (Theorell & Åkesson, 1941). By the use of a large Tiselius (1938) apparatus and continuous electrophoresis of 9–10 days' duration, Theorell & Åkesson were able to separate the colourless fraction from a considerable quantity of

cytochrome *c* containing 0.258–0.31 % Fe and to obtain material with an iron content of 0.43 %. This fraction appeared to be homogeneous by cataphoresis at all pH values. Although the amino-acid analyses and some physical properties of this purified cytochrome were recorded by Theorell & Åkesson (1941), the most fundamental property of cytochrome, viz. its activity as a biological oxidation catalyst, was not examined by them.

The object of this investigation was to compare the catalytic activity of this new preparation (0.43 % Fe) with that of our usual purified preparation (0.34 % Fe), especially as it was suggested by these workers that only the electrophoretically purified cytochrome *c* should be used in enzyme studies. For this purpose cytochrome *c* containing 0.43 % Fe was prepared by a new method not requiring the large-scale Tiselius apparatus, which is not usually available to workers engaged in the study of intracellular catalysts.

After several years' experience in the preparation of cytochrome *c* containing 0.34 % Fe* we have been led to introduce a few minor modifications in our original method of preparation, which will therefore be redescribed.

METHODS

Estimation of iron. 1–3 ml. cytochrome solution is combusted in a Kjeldahl flask with H_2SO_4 plus the occasional addition of a drop of KClO_3 solution. The acid is evaporated almost to dryness, treated with 5 ml. distilled water, 5 mg. $\alpha\alpha'$ -dipyridyl and 1 g. $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$. The pink colour develops on standing 30 min. The solution is diluted to 10 ml. and either (1) compared in a colorimeter with a standard iron-dipyridyl solution, or (2) estimated by measuring the extinction coefficient at $521\text{ m}\mu$, the wave-length of maximum absorption. By working with known amounts of iron it was found that the iron concentration was related to the extinction coefficient by the equation $c = 0.00635 K_{521}$, where c = concentration of Fe in mg./ml. and $K = 1/d \log_{10} I_0/I$. The second method was found to be the more reliable one.

Dry weight of cytochrome in presence of NaCl or KCl was estimated by evaporating the solution at 100° to constant weight in a platinum crucible and measuring the loss in weight after ignition for 1 hr. at 500° . Dry weight of cytochrome = loss in weight + weight of Fe_2O_3 or loss in weight + $1.4 \times (\text{weight of Fe})$, the latter having already been determined. The second term (Fe_2O_3) is almost within the experimental error; hence the actual constitution of the oxide is unimportant although the brown colour indicates that it is Fe_2O_3 . In many cases iron was estimated directly in the residue from the incineration, which dissolved readily in hot dilute H_2SO_4 .

The reliability of this method was confirmed on preparations the dry weight of which was subsequently redetermined after dialysis against distilled water until salt-free.

* It was stated by Theorell & Åkesson (1941) that our method normally yields material containing less iron than 0.34 %, though in our hands the method, even without the newer modifications, almost always yields cytochrome with an iron content of 0.34 %.

Preparation of cytochrome *c* from horse heart

(a) **Preparation containing 0.34 % Fe.** A horse heart is cut open as soon as possible after collection from the slaughter-house and the blood allowed to drain out. The muscle is freed from fat and ligaments and finely minced. To the minced muscle is added 0.145 N-trichloroacetic acid at the rate of 1 l./kg. mince. The acid is added in three or four lots with adequate mixing after each addition. The trichloroacetic acid is prepared by diluting a 20 % solution, the exact strength of which has been determined by titration with N-NaOH, using phenolphthalein as indicator. After standing 3–4 hr. the acid mixture is squeezed out in a press and the cloudy fluid brought to pH 7.3 with 10 % NaOH. Ammonium sulphate is now added at the rate of 500 g./l., and when it is dissolved the mixture is placed on large fluted filter papers and left overnight to drain completely. The clear pink filtrate, which is free from all pigments except cytochrome *c*, is treated again with $(\text{NH}_4)_2\text{SO}_4$ at the rate of 50 g./l. and left overnight at $0-5^\circ$. If any precipitate forms it is filtered off and the cytochrome in the cold filtrate is precipitated by the addition of 20 % trichloroacetic acid (25 ml./l.). Within a few minutes the cytochrome aggregates and is centrifuged down. The red precipitate is suspended in about 300 ml. saturated $(\text{NH}_4)_2\text{SO}_4$ and centrifuged again. It is now suspended in the minimum amount of distilled water and transferred to a cellophan sac in which it is dialyzed against 0.5 % NaCl until free from $(\text{NH}_4)_2\text{SO}_4$. The suspension is shaken with a few drops of chloroform and the precipitate is filtered off. 30–40 ml. of a dark red solution of cytochrome *c* are obtained, containing about 1 % protein. The iron content is usually 0.34 %, but in cases where it is lower it can be brought up to this value by diluting the solution to 500–1000 ml. and repeating the ammonium sulphate-trichloroacetic acid precipitation. While saturated with chloroform the solution of cytochrome thus obtained keeps indefinitely at 5° in a well-stoppered bottle.

Some workers in this field have preferred to dialyze cytochrome against distilled water rather than against dilute NaCl. In our experience the cytochrome in complete absence of salts tends to become absorbed by the cellophan. The estimation of dry weight of protein in presence of salts is described above.

(b) **Preparation containing 0.43 % Fe.** 120 ml. of the above solution of cytochrome *c* (0.34 % Fe) are treated with $(\text{NH}_4)_2\text{SO}_4$ until a small quantity of the latter remains undissolved. 20 ml. ammonia, sp.gr. 0.880, are added drop by drop to give pH 10 (10.06 as measured with a glass electrode) and the mixture left overnight at 5° . Next day the precipitate *A* is removed and the filtrate is freed from

Table 1. *Analyses of various cytochrome c fractions*(Average for B_1 , B_2 and $B_3=0.426\%$ Fe)

Preparation	Dry wt. (mg./ml.)	Fe (mg./ml.)	Fe (%)	Total vol. (ml.)	Yield in terms of Fe (%)
Original solution cytochrome <i>c</i>	7.2	0.0247	0.34	120	(100)
Solution B_1	9.39	0.0402	0.42 ₉	60	81
Solution A_1	15.25	0.030	0.20	10.5	11
Fractionation of 40 ml. B_1 : B_2	6.24	0.0265	0.42 ₅	30	49
B_3	3.04	0.0129	0.42 ₄	9	7
Fractionation of 9.5 ml. A_1 : A_2	2.89	0.0117	0.40 ₅	10	40

$(\text{NH}_4)_2\text{SO}_4$ by dialysis in a cellophan tube for 3 hr. against running tap water and finally against 0.5% KCl. The resulting solution (250 ml.) is reduced in bulk to about 50 ml. by suspending the cellophan tube in a current of air. After a further dialysis against KCl the solution B_1 (60 ml.) is collected. The cytochrome of this fraction contains 0.43% Fe (Table 1).

The homogeneity of this fraction was tested in three ways:

(1) When tested in the Tiselius apparatus it showed only one moving boundary, both at pH 7.3 and at pH 10.7.

(2) A diffusion experiment at pH 7.3 also indicated a pure substance.

(3) Fractionation of the solution by absorption on and elution from calcium phosphate gel yielded cytochrome fractions, B_2 and B_3 , with unchanged iron contents (Table 1).

The precipitate *A* is washed with water containing $(\text{NH}_4)_2\text{SO}_4$ and NH_3 in concentrations equal to those added to the original cytochrome solution. When the washings become almost colourless the precipitate is dissolved in a little water and dialyzed against tap water and finally distilled water. A small quantity of denatured protein is filtered off to give solution A_1 (10.5 ml.). This fraction contains 0.20% Fe. It is decidedly more brown than B_1 or the original solution and contains some modified pigment.

Attempts to isolate an appreciable amount of the colourless fraction from A_1 were not successful. 90% saturation with $(\text{NH}_4)_2\text{SO}_4$ yielded a pale pink-brown precipitate which could not be sharply separated into coloured and colourless fractions. After removal of the precipitate the cytochrome still in solution was thrown down by saturating with $(\text{NH}_4)_2\text{SO}_4$ at 60°. It contained 0.40% Fe (A_2) and would therefore appear to consist essentially of the cytochrome containing 0.43% Fe.

Catalytic activity of the two cytochrome preparations

The catalytic activities of the original cytochrome *c* (0.34% Fe) and of the preparation B_2 (0.43% Fe) were compared in two systems obtained from a kidney preparation: (1) the system oxidizing ascorbic acid and (2) the system oxidizing succinic acid. Kidney preparation, which we have previously

described (Keilin & Hartree, 1940), has the advantage in such experiments of being deficient in

Fig. 1. Comparison of the catalytic effects of two cytochrome *c* fractions containing 0.34% Fe (full lines) and 0.43% Fe (dotted lines) in the system cytochrome oxidase (0.1 ml. sheep kidney extract) + ascorbic acid (15 mg.). Temp. = 39°; pH = 7.3.

Fig. 2. Comparison of the catalytic effects of two cytochrome *c* fractions in oxidation of succinate (0.04M) by kidney extract (0.25 ml.). Experimental conditions as for Fig. 1.

cytochrome *c*, hence the oxidation of ascorbic acid, succinic acid, and other substances is very slow in

the absence of added cytochrome *c*. Experiments were carried out at 39° and pH 7.3 in Barcroft differential manometers under the usual conditions. The components of the systems investigated are:

(1) Cytochrome oxidase + cytochrome *c* + ascorbic acid.

(2) Cytochrome oxidase + cytochrome *c* + succinic dehydrogenase + succinic acid.

Cytochrome oxidase and succinic dehydrogenase are represented by the kidney preparation. When the enzymes and the substrate are in excess the O_2 uptakes of the systems serve as measures of the catalytic power of cytochrome *c*. The results of these experiments, which are shown in Figs. 1 and 2, clearly demonstrate that the catalytic activities of the iron in the two cytochrome preparations are identical, and that both preparations can equally well be used in work with enzymes.

The comparative study of other properties of these two preparations, namely, their absolute absorption spectra and their reactions, at varying pH values with O_2 and CO, did not reveal any noticeable differences.

DISCUSSION

The increase in the iron content of cytochrome *c* from 0.34 to 0.43%, which can be obtained either by a prolonged cataphoresis at pH > 10.65 (Theorell & Åkesson, 1941), or by fractionation at pH 10 as described above, indicates a proportional increase in the purity of the compound. This has been corroborated by studying the catalytic activities per atom of iron of these two preparations, which were found to be equal. Hence removal of about 20% of the protein of the molecule has not affected the properties of the prosthetic group. On the other hand, this final step of purification cannot be considered as a removal of some indifferent impurities which arbitrarily become associated with cytochrome *c* during its preparation. It is conceivable that the protein fraction separated at this very alkaline pH forms part of the original intracellular cytochrome *c*. In fact, cytochrome preparations containing 0.34% Fe were obtained by two different methods (Theorell, 1935; Keilin & Hartree, 1937). Moreover, these preparations behave in every respect as homogeneous proteins at pH below 10, and only at pH > 10.65 in electrophoretic experiments does the protein of cytochrome (0.34% Fe) dissociate into two fractions: a colourless fraction representing about 20% of the total solids, and the

remaining red cytochrome *c* containing 0.43% Fe. It seems unlikely that cytochrome *c* obtained by different methods should combine in the same proportions with accidental impurities. The dissociation of such a complex at a very alkaline pH resembles the dissociation of other apparently pure proteins such as haemocyanin at extreme pH (Redfield, 1934). It must also be remembered that whereas cytochrome containing 0.34% Fe corresponds to a molecular weight of 16,500 which has been found for several proteins (Svedberg & Pedersen, 1940), the iron content of 0.43% indicates a molecular weight of about 13,000 which is unprecedented among proteins.

As the two cytochrome preparations show no marked differences in their properties, both can be used equally well in experiments with enzymes or in the reconstruction of certain intracellular catalytic systems. It is conceivable that the colourless protein forms an integral portion of the intracellular cytochrome molecule, playing some part in its catalytic activity within cells, where this component exhibits a much greater efficiency than it does in any experiments *in vitro* (Keilin & Hartree, 1940).

SUMMARY

1. By slight modifications of our original method the preparation of cytochrome *c* containing 0.34% Fe has been simplified.

2. By submitting this compound to electrophoresis Theorell & Åkesson (1941) found that below its isoelectric point (pH 10.65) it appears to be homogeneous, whereas at a higher pH (10.68) it dissociates into a colourless protein (20% of the total) and red cytochrome *c* containing 0.42–0.43% Fe. This raising of the iron content of cytochrome *c* has been confirmed by fractional precipitation of the preparation (0.34% Fe) at pH 10.0, i.e. a method not requiring specialized apparatus.

3. Both cytochrome preparations containing 0.34 and 0.43% Fe show the same catalytic activity per atom of iron; hence the preparation containing 0.34% Fe is suitable for experiments with enzymes and the reconstruction of intracellular oxidizing systems.

4. The significance of this final step of purification of cytochrome *c* is discussed.

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REFERENCES

- Keilin, D. (1930). *Proc. Roy. Soc. B*, **106**, 418.
 Keilin, D. & Hartree, E. F. (1937). *Proc. Roy. Soc. B*, **122**, 298.
 Keilin, D. & Hartree, E. F. (1940). *Proc. Roy. Soc. B*, **129**, 277.
 Redfield, A. C. (1934). *Biol. Rev.* **9**, 175.
 Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*. Oxford: Clarendon Press.
 Theorell, H. (1935). *Biochem. Z.* **279**, 463.
 Theorell, H. & Åkesson, Å. (1939). *Science*, **90**, 67.
 Theorell, H. & Åkesson, Å. (1941). *J. Amer. chem. Soc.* **63**, 1804, 1812, 1818, 1820.
 Tiselius, A. (1938). *Kolloidzshr.* **85**, 129.