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The Cytochrome System of Adrenal Medulla

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Huszk (1932, 1942) examined the cytochrome system of the medulla of the adrenal gland and reached the conclusion that, unlike all other animal tissues, it does not contain a cytochrome oxidase. The oxidation of its reduced cytochrome was said to be carried out by a peroxidase, together with hydrogen peroxide produced by the autoxidation of substances such as ascorbic acid or adrenaline. In the present investigation, however, it is shown that the adrenal medulla contains a perfectly normal cytochrome oxidase.

EXPERIMENTAL

Adrenal medulla preparation. Adrenal glands from ten or twelve horses at a time were obtained directly from the slaughter-house. The medulla (10 g. wet weight) was carefully cleaned from all traces of cortex, cut into small pieces about $5 \times 5 \times 1$ mm., and rapidly homogenized for 3 min. in a Waring blender with 100 ml. of cold 0.02 M-phosphate buffer, pH 7.3. The homogenate was then centrifuged at 2000 rev./min. for 10 min. and the supernatant was again centrifuged at 12,000 rev./min. for 25 min. The precipitate from the latter operation was then washed twice with 50 ml. of cold 0.01 M-phosphate buffer, pH 7.3, collected by centrifugation (12,000 rev./min., 25 min.) after each washing, and the final precipitate was suspended in an equal volume of 0.1 M-phosphate buffer, pH 7.3.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) and contained 0.34% Fe.

Ferrocycytochrome *c* was prepared immediately before use by reducing ferricytochrome *c* in 0.1 M-phosphate buffer, pH 7.3, with a current of hydrogen in presence of platinum black. Ferrocycytochrome *c* thus prepared was slowly oxidized by the H_2O_2 formed in the solution from the Pt-catalysed reduction

of dissolved O_2 by H_2 . To prevent this oxidation, a few drops of dilute catalase solution were added immediately after the current of H_2 had been stopped. The Pt black was then removed by filtration.

Catalase was prepared by the method of Keilin & Hartree (1937).

Oxidation of ferrocycytochrome *c*. The oxidation of ferrocycytochrome *c* by the washed medulla preparation was followed by measuring the intensity of its α -band at 550 m μ . in a Beckman spectrophotometer. Each 1 cm. cell contained 1.7×10^{-5} M-ferrocycytochrome *c*; 0.1 M-phosphate buffer, pH 7.3; 0.1–0.2 mg. enzyme preparation; and 1 μ g. catalase in a total volume of 3 ml. With gas mixtures other than air, 2 cm. cylindrical cells, provided with ground-glass stoppers, were used. These cells, with a total capacity of 5.5 ml., contained the same concentration of all the above reagents except that the cytochrome concentration was 0.85×10^{-5} M. The required gas mixture was bubbled through the solution in these cells in presence of a drop of capryl alcohol before the addition of the enzyme. The concentration of ferrocycytochrome at time *t* is given by $(D_t - D_\infty)/(1.92 \times 10^4)$, where D_t and D_∞ are the density at time *t* and after complete oxidation respectively and 1.92×10^4 is the difference between the molecular extinction coefficients of reduced and oxidized cytochrome *c* at 550 m μ .

The activities of cytochrome oxidase, succinic dehydrogenase, succinic oxidase and dihydrocozymase oxidase were measured as described by Slater (1949*a*, *b*, 1950).

Peroxidase activity was measured either as described by Keilin & Mann (1937) or by a spectrophotometric method of Morell (1951).

RESULTS

Spectroscopic observations. Intact adrenal medulla tissue, as well as the washed preparation, showed a diffuse *d*-band of cytochrome at 530 m μ . and a

strong asymmetric *b*-band at 561 $m\mu$, which was shaded towards the blue end of the spectrum. Unlike the cytochrome bands of heart muscle and kidney, the band at 561 $m\mu$ remained in the reduced state despite thorough washing and vigorous shaking of the preparation in air. On the addition of a drop of a cytochrome *c* solution, the band at 561 $m\mu$ was oxidized leaving a broad shading with its centre at about 556 $m\mu$. If succinate was then added, both the cytochrome *b* originally present and the added cytochrome *c* were reduced. The oxidation as well as the reduction of cytochrome *b* in the adrenal medulla were very slow. The reduction could be hastened, however, by the addition of cyanide together with succinate. The medulla cytochrome *b* did not combine with carbon monoxide.

Fig. 1. Oxidation of ferrocytochrome *c*. Ferrocytochrome *c* was prepared by reduction with hydrogen in presence of Pt black. Curve *B* shows the slow oxidation by hydrogen peroxide formed from catalytic reduction of dissolved oxygen. In presence of catalase (curve *A*) there was no oxidation. Curves *C* and *D* show the oxidation of ferrocytochrome *c* by adrenal medulla preparation (0.31 mg.) in presence and absence of catalase respectively. Temp. 22°, 0.06 M-phosphate buffer, pH 7.3.

On cooling the washed preparation to the temperature of liquid air, a band was visible at 595 $m\mu$, which is the position of the combined cytochromes *a* and *a*₃ band of heart-muscle preparation at this temperature (Keilin & Hartree, 1949). The band at 561 $m\mu$ was moved towards the blue end of the spectrum and split into two bands at 552 and 558 $m\mu$, respectively. The former band is not ascribed to cytochrome *c*, since at liquid air temperature this component is characterized by a main band at

547 $m\mu$, together with a satellite band (Keilin & Hartree, 1949).

Cytochrome oxidase. The presence of a combined cytochrome *a* and *a*₃ band in adrenal medulla indicated that this tissue contained a cytochrome oxidase. Moreover, the adrenal medulla preparation catalysed the oxidation of ferrocytochrome *c* and the oxidation of ascorbic acid or *p*-phenylenediamine in presence of added cytochrome *c*.

Fig. 1 shows that in presence of the medulla preparation, ferrocytochrome *c* was rapidly oxidized by air. In addition, although ferrocytochrome *c* was very slowly oxidized by hydrogen peroxide, the rate of the rapid, medulla-catalysed oxidation was not affected by hydrogen peroxide which clearly indicates that the enzyme responsible for this catalysed

Fig. 2. Oxidation of *p*-phenylenediamine. O₂ uptake measured at 38°. Contents of the manometric flasks: 0.06 M-phosphate buffer, pH 7.3; 0.92 mg. fat-free dry wt. of adrenal medulla preparation; 50 μ M-cytochrome *c*; 55 mM-*p*-phenylenediamine. Curve *A*, adrenal medulla preparation alone; curve *B*, as *A* with *p*-phenylenediamine; curve *C*, as *A* with *p*-phenylenediamine and cytochrome *c*.

oxidation of ferrocytochrome *c* is an oxidase and not a peroxidase.

The oxidation of *p*-phenylenediamine by the adrenal medulla preparation and added cytochrome *c* is shown in Fig. 2. The preparation alone or with added *p*-phenylenediamine gave no appreciable oxygen uptake, whereas a rapid oxygen uptake was obtained when both cytochrome *c* and *p*-phenylenediamine were added. Essentially the same results were obtained with ascorbic acid in place of *p*-phenylenediamine. The cytochrome oxidase activity

(Q_{O_2} expressed as $\mu\text{l. O}_2/\text{mg. dry wt./hr.}$) of different medulla preparations was usually between 400 and 600, as compared with 3000–3500 for heart-muscle preparation (Slater, 1949a).

Inhibition by carbon monoxide. Carbon monoxide inhibited both the oxidation of ferrocytochrome *c* by the preparation and the oxidation of ascorbic acid in presence of the preparation and cytochrome *c*. The former inhibition is shown in Fig. 3. It will also be noted that a change from air to a gas mixture of 95% N_2 and 5% O_2 did not affect the rate of oxidation of ferrocytochrome *c* catalysed by the preparation.

Fig. 3. Carbon monoxide inhibition of the oxidation of ferrocytochrome *c* by adrenal medulla preparation. Curve *A*, ferrocytochrome *c* alone; curves *B*, *C* and *D*, ferrocytochrome *c* in presence of adrenal medulla preparation (0.31 mg.). Curves *A* and *D*, air; curve *B*, 95% CO and 5% O_2 ; curve *C*, 95% N_2 and 5% O_2 . Experimental conditions as for Fig. 1.

It has not been possible to demonstrate spectrophotometrically the reversal by light of the carbon monoxide inhibition of the oxidation of ferrocytochrome *c*. It is known that ferrocytochrome *c* shows strong absorption in the violet region of the spectrum, and being present in great excess over the oxidase it presumably acted as a filter which removed the greater part of the light at those wavelengths which are most effective in dissociating the oxidase-carbon monoxide complex. This is fully supported by the manometric experiments shown in Fig. 4. Under the experimental conditions of Fig. 4, the enzyme:cytochrome *c* ratio was increased 28-fold as compared with the experiments shown in Fig. 3,

and irradiation by mercury-vapour light led to a considerable increase in the rate of oxygen uptake when carbon monoxide was present.

Effect of other inhibitors. The effect of various inhibitors on the oxidation of ascorbic acid by the medulla preparation and added cytochrome *c* is shown in Table 1. All the known inhibitors for haematin enzymes such as cyanide, azide, sulphide and hydroxylamine inhibited this reaction strongly. Diethyldithiocarbamate and phenylthiourea, both of which are powerful inhibitors for copper enzymes, did not inhibit this reaction. Fluoride had practically no effect.

Fig. 4. Carbon monoxide inhibition of the oxidation of ascorbic acid in presence of adrenal medulla preparation and cytochrome *c* at 30° . Reaction mixture contained ascorbic acid, 0.026 M ; $0.7 \times 10^{-5}\text{ M}$ -cytochrome *c*; 0.06 M -phosphate buffer, pH 7.3; and enzyme, 3.68 mg. fat-free dry wt. Curve *A*, in 95% N_2 and 5% O_2 ; curves *B*, *C* and *D*, in 95% CO and 5% O_2 . For curves *B*, *C* and *D*: —, reaction in light; - - -, reaction in dark.

Oxidation of succinate and dihydrocozymase. The activities of succinic and dihydrocozymase oxidase systems in the medulla preparation are compared in Table 2 with those of heart-muscle and kidney preparations (Slater, 1949b, 1950). Like that of the heart-muscle and kidney preparations (Slater, 1950), the dihydrocozymase oxidase system of the medulla is also sensitive to BAL (2:3-dimercaptopropanol).

Although adrenal medulla has a strong cytochrome *b* band, it showed very little succinic dehydrogenase, whereas in most other tissues and micro-organisms, these two are usually closely associated (Table 3).

Table 1. *Effect of inhibitors on the oxidation of ascorbic acid by adrenal medulla preparation and added cytochrome c*

(Oxygen uptake measured at 38°. Reaction mixture contained 0.92 mg. fat-free dry wt. of enzyme; 0.026 M-ascorbic acid; 5×10^{-6} M-cytochrome c; 0.06 M-phosphate buffer, pH 7.3, and inhibitors as specified in the table. Percentage inhibition calculated on the basis of initial rates.)

Inhibitor	Concn. (M)	Inhibition (%)
Cyanide	5×10^{-7}	17
	5×10^{-6}	42
	5×10^{-5}	75
	5×10^{-4}	98
	5×10^{-3}	99
Sulphide	1×10^{-5}	62
	1×10^{-3}	87
Hydroxylamine	2×10^{-3}	32
	1×10^{-2}	55
	4×10^{-2}	72
Azide	2×10^{-5}	2
	2×10^{-4}	36
	2×10^{-3}	45
Fluoride	2×10^{-3}	0
	1×10^{-2}	5
Sodium diethyldithiocarbamate	3×10^{-3}	0
Phenylthiourea	3×10^{-3}	0

Peroxidase activity. Owing to the presence of a considerable amount of fat which interferes with the ether extraction, accurate determinations of peroxidase activity of the homogenate and of the washed preparation by Willstätter's method were impracticable. However, visual comparison of the purpurogallin colour formed in the reaction mixture with pyrogallol as substrate indicated that the 'Purpurogallinzahl' (P.Z.) (Willstätter & Stoll, 1918) of the homogenate was of the order of 0.001–0.01. Measured by a very sensitive spectrophotometric method (Morell, 1951), an alkaline phosphate extract, prepared exactly according to Huszak (1942), showed no peroxidase activity with pyrogallol as substrate.

DISCUSSION

One can postulate three possible mechanisms for the oxidation *in vivo* of cytochrome in the adrenal medulla: (1) a peroxidase acting in conjunction with hydrogen peroxide produced by the autoxidation of substances such as adrenaline; (2) a copper-protein enzyme; and (3) a haem-protein cytochrome oxidase, as in other tissues. These possibilities will now be examined in the light of the present study.

Table 2. *Succinic oxidase and dihydrocozymase oxidase activities of medulla preparation as compared with those of heart-muscle and kidney preparations*

(Succinic oxidase activity measured at 38° using the method described by Slater (1949b) except that the amount of enzyme used was 10.4 mg. fat-free dry wt. Dihydrocozymase activity determined at room temperature and calculated to 38°, assuming the same temperature coefficient as the heart-muscle enzyme (Slater, 1950).)

System	Added cytochrome c (M)	Activity of enzyme preparation (Q_{O_2})*		
		Adrenal medulla	Heart muscle†	Kidney†
Succinic oxidase	—	1	500	60
	6×10^{-5}	30	650	200
Dihydrocozymase oxidase	—	—	870	50
	4×10^{-5}	80	1000	270

* Average values of different preparations.

† Figures taken from Slater (1949b, 1950).

Table 3. *Relationships between cytochrome b content and succinic dehydrogenase activity*

(The succinic dehydrogenase (Suc. D.) activity is expressed as Q_{O_2} measured in presence of methylene blue as described by Slater (1949b). Cytochrome b (Cyt. b) content was measured by comparison with a standard pyridine haemochromogen solution and expressed in μ mol. cytochrome b haematin/g. fat-free dry wt. of preparation. Figures for heart-muscle and kidney preparations are taken from Slater (1949b).)

Preparation	Suc. D.	Cyt. b	Ratio Suc. D./Cyt. b
Heart muscle	240	0.64	375
Kidney	67	0.27	248
Adrenal medulla	32	3.3	9.7

Peroxidase. It is clear that this tissue contains very little peroxidase activity. Moreover, whatever little peroxidase activity it has, can probably be accounted for by the presence of haemoglobin and cytochrome which are known to possess some peroxidase activity. The facts that (1) the rate of oxidation of ferrocytochrome c is not dependent on the presence of hydrogen peroxide, and (2) the catalysed oxidation of ascorbic acid in presence of the enzyme and added cytochrome c is inhibited by carbon monoxide definitely show that the oxidation of cytochrome is not carried out through a peroxidase.

Copper-protein enzyme. It has been claimed that a copper-protein enzyme, laccase, is capable of

catalysing the oxidation of cytochrome *c* (Bertrand, Belval & Legrand, 1947), although this has not been confirmed by Tissières (1949). In the present case, however, the possibility that a copper-enzyme is involved does not appear to be likely since two powerful copper-enzyme inhibitors, phenylthiourea and diethyldithiocarbamate, do not affect the action of the enzyme and, in addition, the carbon monoxide inhibition is light sensitive.

Cytochrome oxidase. All the experimental data brought forward in this paper lead to the conclusion that the enzyme responsible for the oxidation of cytochrome in adrenal medulla, as in all other animal tissues so far studied, is a haem-protein cytochrome oxidase. This view is supported by the facts that: (1) at the temperature of liquid air a combined band of cytochromes *a* and *a₃* can clearly be seen; (2) the enzyme is affected to the same degree as cytochrome oxidase of other animal tissues by all known inhibitors of cytochrome oxidase; (3) the enzyme functions as well at low oxygen partial pressure as in air; and (4) it is inhibited by carbon monoxide and this inhibition is reversed by light.

Other cytochrome components. That the adrenal medulla contains very little, if any, cytochrome *c* is revealed by spectroscopic observations at the temperature of liquid air. The most prominent band of the medulla cytochrome system is that of cytochrome *b* at 561 m μ . (Bierich & Rosenbohm, 1928; Huszak, 1942), although Cohen & Elvehjem (1934) reported that the adrenal medulla contained cytochrome *c* but no cytochrome *b*. The presence in this tissue of an enzyme capable of catalysing the oxidation of ferrocytochrome *c* seems to suggest that cytochrome *c* is also present, but at such a low concentra-

tion that it cannot be detected by spectroscopic observation even at the temperature of liquid air. A point of some interest is that although this tissue shows a strong cytochrome *b* band, it has very little succinic dehydrogenase activity. This is in agreement with the view (Tsou, 1951) that succinic dehydrogenase is not identical with cytochrome *b* as was suggested by some workers.

SUMMARY

1. Spectroscopic observation showed the presence in adrenal medulla preparation of a strong cytochrome *b* band at 561 m μ . Cooling to the temperature of liquid air, which shifts all absorption bands towards the shorter wavelengths, further revealed a weak band at 595 m μ . corresponding to the combined band of cytochromes *a* and *a₃*.

2. Adrenal medulla preparations catalysed the oxidation of ferrocytochrome *c* and the oxidation of ascorbic acid and *p*-phenylenediamine in presence of added cytochrome *c*.

3. This catalytic oxidation of ascorbic acid was inhibited by cyanide, azide, sulphide, hydroxylamine and carbon monoxide, the latter inhibition being light reversible.

4. It is concluded that the adrenal medulla, like all the other animal tissues, contains the usual cytochrome system, though the cytochrome *c* content is very low.

5. The adrenal medulla contained very little, if any, peroxidase activity with pyrogallol as substrate.

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