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ADRENOCHROME INHIBITION OF OXIDATIVE PHOSPHORYLATION BY RAT BRAIN MITOCHONDRIA*

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Abstract—Adrenochrome at 10^{-4} M causes a 50 per cent inhibition of pyruvate oxidation and its associated phosphorylation in brain mitochondria. About three times as much inhibitor is required for the same effect with succinate as the substrate. Preincubation is necessary for most effective inhibition. The compound probably inhibits by binding free–SH groups on the enzymes.

ADRENOCHROME, the red quinone formed on oxidation of epinephrine, has been reported to relieve the cyanide inhibition of oxygen uptake by liver homogenates[1] and to uncouple oxidative phosphorylation at 5×10^{-5} M.[2] The compound is also said to cause psychotomimetic effects when amounts as low as 5 mg are injected into human experimental subjects.[3] Hoffer and Osmond state that it is present in human serum at about $0{\cdot}3 \times 10^{-6}$ M.[4] Any effects of the compound at these concentrations would therefore be of great interest.

This paper demonstrates that adrenochrome at 5×10^{-5} M to 10^{-4} M inhibits electron transport and oxidative phosphorylation by brain mitochondria to about the same extent. The relief of cyanide inhibition reported by Green and Richter[1] occurs only at very high adrenochrome levels and is apparently the result of a nonenzymatic oxidation catalyzed by adrenochrome.

PROCEDURES

Rat brain mitochondria were obtained from adult Sprague-Dawley rats by the procedure of Beer and Quastel[5] except that the homogenizing solution was 0·25 M sucrose, 0·05 M Tris buffer (pH 7·4), and 10^{-4} M EDTA.

Oxygen uptake was determined manometrically. The reaction mixtures used are shown in the figure legends. The vessels were incubated 10 min at 30° and the reactions initiated by adding inorganic phosphate from a side arm. Oxygen uptake was usually followed for 20 min. The composition of the reaction mixtures are shown under the figures. The reactions were stopped by the addition of a solution of 10% perchloric acid. We measured phosphate by assaying the amount of ^{32}P converted to organic phosphate esters, using a modification[6] of the method of Nielsen and Lehninger[7]. In several experiments where P/O values were determined by this method on six identical vessels, the standard deviation in the P/O ratio was no greater than 0·10.

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Adrenochrome was prepared by the method of Heacock *et al.*[8] and its concentration determined spectrophotometrically, with an extinction coefficient at 475 mμ of 3,900. This compound was quite pure as shown by the absence of absorption bands that could be attributed to either unreacted epinephrine or to adrenolutin. The absorption spectrum of our product was identical with that of commercially available adrenochrome obtained after the conclusion of this study. Our preparations were stable in air for over a year, showing that they were free from contaminating heavy metals.

RESULTS

The results of the experiment presented in Fig. 1 show that brain mitochondria, prepared as described above, behaved the same way with respect to increasing concentrations of 2,4-dinitrophenol as do other mitochondria.[9] Oxidative phosphorylation by our preparations was half inhibited by amobarbital (Amytal) at 5×10^{-4} M and by gramicidin at 0·03 μg per flask.

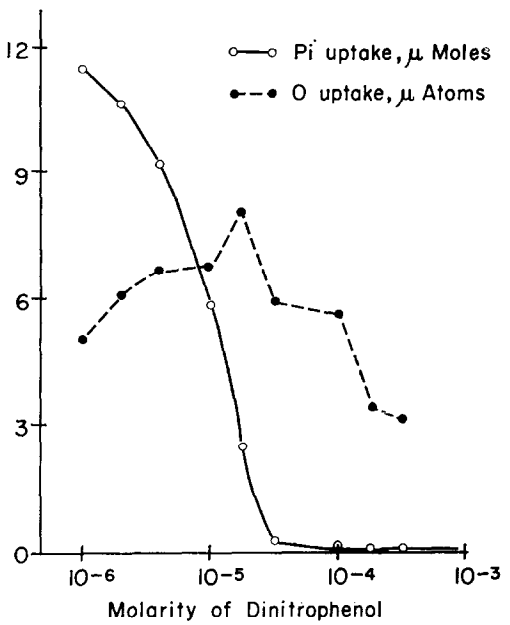

FIG. 1. Effect of 2,4-dinitrophenol on oxidative phosphorylation by rat brain mitochondria. Each flask contained, in μmoles: ATP, 3; $MgCl_2$, 20; NaF, 5; glucose, 80; pyruvate, 30; malate, 30; Tris (pH 7·4), 60; sucrose, 260 also 10 mg Sigma type III hexokinase, and once-washed mitochondria from about 0·5 g tissue in 2·0 ml. The side arm contained 30 μmoles PO_4 (pH 7·4) and 20 μc ^{32}P in 1·0 ml. The center well contained 0·2 ml 10% KOH. The vessels were equilibrated 10 min at 30° before adding side arm contents to initiate the reaction. Inhibitor was added to the main well.

The effects of adrenochrome on oxygen and phosphate uptake by brain mitochondria are shown in Figs. 2 and 3. Figure 2 shows the results with a mixture of malate and pyruvate as a substrate and Fig. 3 with succinate as the substrate. In these experiments the adrenochrome was added to the main compartment of the vessels when they were made up. The mitochondria were exposed to adrenochrome about 15 min before the reactions were initiated by addition of phosphate.

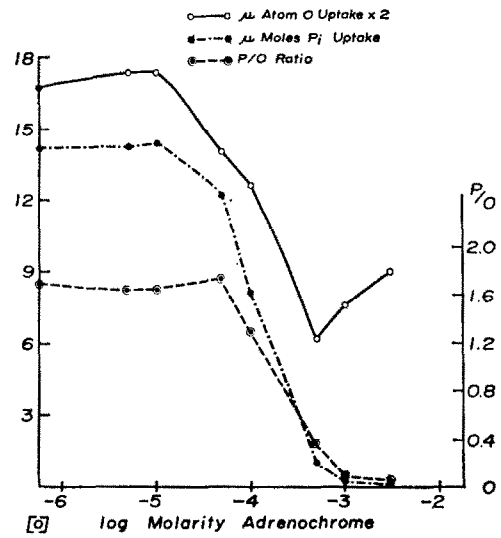

FIG. 2. Effect of adrenochrome on oxidative phosphorylation with malate and pyruvate as substrates. The reaction mixtures and conditions were the same as in Fig. 1.

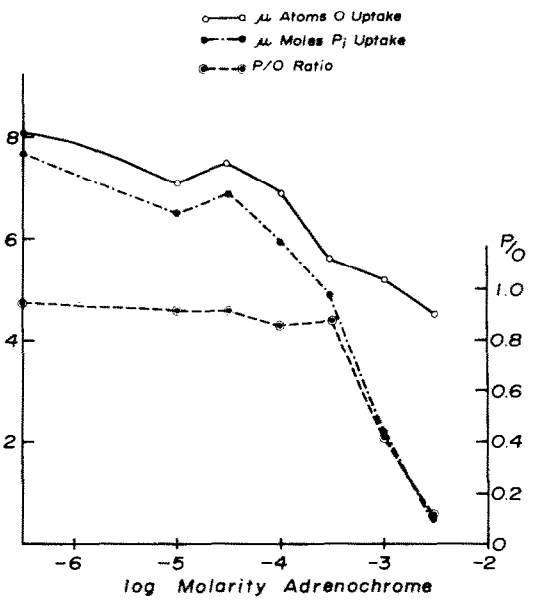

FIG. 3. Effect of adrenochrome on oxidative phosphorylation, with succinate as substrates. The reaction mixture was the same as in Fig. 1 except that 40 μmoles succinate was substituted for malate and pyruvate.

With malate–pyruvate as the substrate, phosphate esterification is inhibited 50% at about 2×10^{-4} M adrenochrome. At adrenochrome concentrations above 3×10^{-4}, the rate of oxygen uptake increases with increasing adrenochrome concentration. Figure 3 shows that, with succinate as the substrate, about three times as much

adrenochrome is required to give 50% inhibition of either oxygen uptake or phosphorylation.

Table 1 shows the effects of cyanide, adrenochrome, and a combination of the two inhibitors on oxidative phosphorylation. In the first three experiments malate and pyruvate were used as a substrate. In the fourth, succinate was the substrate. It is evident that adrenochrome is a somewhat better uncoupler in the presence of cyanide than when it is used alone. Adrenochrome caused a stimulation of oxygen uptake in the presence of cyanide at both levels of cyanide used.

TABLE 1. EFFECTS OF ADRENOCHROME AND CYANIDE ON OXIDATIVE PHOSPHORYLATION BY RAT BRAIN MITOCHONDRIA*

Expt.	Inhibitor	Uptake (P_i) (μmoles)	Uptake (O) (μmoles)	P/O
1	(a) None	15·9	7·7	2·06
	(b) 5×10^{-4} M adren.	8·6	6·1	1·42
	(c) 10^{-3} M CN^-	8·5	3·9	2·17
	(d) b + C	5·4	5·0	1·09
2	(a) None	19·4	10·0	1·94
	(b) 10^{-4} M adren.	13·3	7·8	1·70
	(c) 3×10^{-3} M CN^-	0·4	0·6	0·67
	(d) b + c	0·3	1·0	0·33
3	(a) None	21·2	7·6	2·7
	(b) 5×10^{-4} M adren.	9·3	5·1	1·8
	(c) 3×10^{-3} M CN^-	0·48	0·34	1·4
	(d) b + c	0·65	3·3	0·16
4	(a) None	12·7	9·5	1·34
	(b) 5×10^{-4} M adren.	8·4	9·7	0·85
	(c) 10^{-3} M CN^-	0·04	0·24	0·16
	(d) b + c	0·07	1·7	0·04

The reaction mixtures were the same as in Fig. 1. Adrenochrome and cyanide were added to side arms and tipped in at 0 time. Malate and pyruvate were the substrates in experiments 1, 2, and 3; succinate was used in 4. All values are the average of duplicates run at the same time. Adren. = adrenochrome.

Sodium amobarbital inhibits electron transport at both the NADH–flavin and the flavin–cytochrome b sites.[10] The results shown in Table 2 show that brain mitochondria behave the same as liver or heart mitochondria toward amobarbital. When adrenochrome and amobarbital are added together, a further drop in phosphate esterification occurs, but more oxygen is taken up than with amobarbital alone.

Abood[2] reported that mitochondria must be incubated with adrenochrome before carrying out oxidative phosphorylation in order to achieve maximal inhibition. Table 3 compares the inhibition obtained after preincubating the inhibitor with the mitochondria with that obtained on adding the adrenochrome at 0 time. Adrenochrome at 5×10^{-4} M has little effect on phosphorylation associated with succinate oxidation unless preincubated with the mitochondria. In Abood's experiments, the inhibitor was homogenized with the particles. Our results show that a 20-min preincubation is probably equally effective. Homogenization of already isolated

mitochondria would seem to be extremely deleterious to mitochondrial structure whether inhibitor was added or not.

Table 3 shows that the rate of oxidation of succinate was stimulated as much as 20% by addition of adrenochrome, whereas phosphate esterification was inhibited, the degree of inhibition depending on the length of preincubation with the inhibitor. This effect is similar to that of 2,4-dinitrophenol but requires forty times as much adrenochrome.

TABLE 2. EFFECTS OF AMYTAL AND ADRENOCHROME ON OXIDATIVE PHOSPHORYLATION BY RAT BRAIN MITOCHONDRIA

Inhibitor	P_i	O	P/O
(a) None	21·4	10·7	1·99
	20·1	10·4	1·93
(b) 5×10^{-4} M Amytal	7·7	4·1	1·85
	7·5	4·1	1·83
(c) 5×10^{-4} M adrenochrome	10·3	7·5	1·37
	9·9	7·4	1·35
(d) as b + c	3·31	5·28	0·63
	3·69	5·97	0·62

Reaction conditions the same as in Table 1. Inhibitors added at 0 time. Pyruvate–malate substrate.

TABLE 3. EFFECT OF PREINCUBATION OF ADRENOCHROME WITH MITOCHONDRIA ON INHIBITON OF OXIDATIVE PHOSPHORYLATION

Substrate	Adrenochrome concentration M	Time inhibitor added (min)	Uptake P_i	Uptake O	P/O
			(μmoles)		
Pyruvate/malate	0		7·8	4·8	1·62
	3×10^{-5}	0	7·3	4·7	1·56
	10^{-4}	0	6·3	4·9	1·28
	3×10^{-5}	—20	3·8	3·4	1·12
	10^{-4}	—20	2·5	3·5	0·71
Succinate	0		9·8	10·5	0·93
	5×10^{-4}	0	8·5	12·6	0·67
	5×10^{-4}	—20	5·3	12·5	0·42

Reaction conditions were the same as in Fig 1 except that the pre-equilibration period was 20 min. On half the inhibited vessels the adrenochrome was added from the side arm at the time the reaction was initiated (0 min). The values are the average of duplicate experiments.

The experiments reported in Table 4 demonstrate that adrenochrome, which nonenzymatically is oxidized only slowly by molecular oxygen, takes up oxygen when incubated with NADH. They also show that oxygen uptake by mitochondria without substrate is stimulated by adding adrenochrome. The rate of that uptake increases with increasing adrenochrome concentration. Although spectrophotometric experiments are difficult to run because of the color of adrenochrome, we have shown that NADH is oxidized nonenzymatically at about the same rate in the cuvet as oxygen was taken up in the manometric experiments reported here. In experiments with an NADH–cytochrome c reductase preparation made by grinding mitochondria with

alumina we have found that adrenochrome prevents electron transfer from NADH to cytochrome c by that preparation.

DISCUSSION

Preincubation of mitochondria with adrenochrome gave us essentially the same degree of inhibition of oxidative phosphorylation as that reported by Abood.[2] If the incubation time in the absence of phosphate is shortened or omitted, the results shown in the figures and in all but Table 3 are obtained. Uptake of oxygen in experiments

TABLE 4. OXYGEN UPTAKE BY ADRENOCHROME, MITOCHONDRIA AND NADH, IN THE ABSENCE OF SUBSTRATE

Additions	Rate of O_2 uptake (μl/min/flask)
Mitochondria	0·6
Mitochondria + 10^{-4} M adren.	1·00
Mitochondria + 5×10^{-4} M adren.	1·80
Mitochondria + 10^{-3} M adren.	2·80
10 μM NADH	0·0
5×10^{-4} M adren.	0·4
1 μM NADH + 5×10^{-4} M adren.	0·7
10 μM NADH + 5×10^{-4} M adren.	2·2

The reaction vessels contained all the reaction components listed in legend to Fig. 1 except the substrate and the mitochondria, which were added where indicated.

to which adrenochrome was added was linear with time as it was in control experiments. This showed that the degree of inhibition did not increase after oxidative phosphorylation began. Whether the degree of inhibition observed on long preincubation in the absence of ATP formation or that found without preincubation is more pertinent to the *in-vivo* situation is a debatable point.

The P/O values reported in this paper are lower than those reported by Abood. This may indicate that our mitochondria were not in the same physiological state as were his. Our mitochondria showed good respiratory control in other experiments in which oxygen uptake was followed polarographically. Magnesium was utilized in these experiments to activate the hexokinase–glucose trap for ATP. In other unpublished experiments we have observed that magnesium decreases P/O ratios, respiratory control, and maximal rates of ADP-stimulated oxygen uptake. Therefore the magnesium concentrations used in these experiments do not allow observation of optimal P/O ratios. The degree of inhibition observed with varying concentrations of adrenochrome was not related to the P/O ratio of the control experiments.

Adrenochrome apparently causes inhibition of oxidative phosphorylation at below 5×10^{-4} M and "uncoupling" at higher concentrations. The effect at the higher concentrations is a stimulation of oxygen uptake, which increases with increasing concentrations of adrenochrome. This is shown by the oxygen uptake curve in Fig. 2. It was also demonstrated by the increased oxygen uptake when 5×10^{-4} M adrenochrome was added to mitochondria already inhibited by amobarbital or cyanide (Tables 1 and 2). In these latter experiments the stimulation of oxygen uptake was greater when the degree of inhibition by the cyanide was greater. The stimulation

of oxygen uptake is also higher in the presence of the pyruvate–malate substrate than with succinate as substrate. All these conditions are those that would lead to a maximal level of reduction of bound mitochondrial pyridine nucleotides. Since adrenochrome and NADH together can take up oxygen in the absence of mitochondria, it is reasonable to explain the above stimulations of oxygen uptake as the result of nonenzymatic oxidation of intramitochondrial NADH. These effects probably account for the results observed by Green and Richter.[1]

Adrenochrome will complex free sulfhydryl groups.[11] Cohen and Hochstein have shown that several quinones, including adrenochrome, inhibit glycolysis by inhibiting the sulfhydryl-dependent enzyme, triose phosphate dehydrogenase.[12] Slater [13] has shown that mercury compounds will inhibit electron transport and oxidative phosphorylation and that a fairly long preincubation period with the inhibitor was required before the inhibition became evident. It seems likely that adrenochrome exerts its inhibitory effect at concentrations below 5×10^{-4} M by virtue of its ability to bind sulfhydryl groups.

In view of the relatively high concentrations of adrenochrome required to give the inhibitions reported here and the small amounts reported to induce psychological effects[3, 4] it seems unlikely, as has been previously postulated,[2, 4] that inhibition of oxidative phosphorylation in the brain is the basis for the reported psychological effects of adrenochrome.

REFERENCES

1. D. E. Green and D. Richter, *Biochem. J.* **31**, 596 (1937).
2. L. G. Abood, *Ass. Res. nerv. Dis. Proc.* **37**, 384 (1959).
3. A. Hoffer, H. Osmond and J. Smythies, *J. ment. Sci.* **100**, 24 (1954).
4. A. Hoffer and H. Osmond, in *The Chemical Basis of Clinical Psychiatry*, p. 46. Thomas, Springfield, Ill. (1960).
5. C. T. Beer and J. H. Quastel, *Canad. J. Biochem.* **36**, 543 (1958).
6. A. R. Krall, N. E. Good and B. C. Mayne, *Plant Physiol.* **36** 44 (1961).
7. S. O. Nielson and A. L. Lehninger, *J. biol. Chem.* **215** 555 (1955).
8. R. A. Heacock, C. Nerenberg and A. M. Payza, *Canad. J. Chem.* **36**, 853 (1958).
9. J. D. Judah, *Biochem. J.* **49**, 271 (1951).
10. B. Chance G. Hollunger and B. Hagihara, *Biochem. biophys. Res. Commun.* **8**, 180 (1962).
11. S. J. Roston, *J. biol. Chem.* **235**, 3315 (1960).
12. G. Cohen and P. Hochstein, *Dis. nerv. Syst. Suppl.* **24** 1–4 (1963).
13. E. C. Slater *Biochem. J.* **45**, 130 (1949).