Preferential Inhibition of Phosphorylation in Different Parts of the Respiratory Chain in Mitochondria Obtained from Animals Injected with Cobra Venom

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Previous studies have demonstrated that the phospholipase A from cobra venom can uncouple oxidative phosphorylation in brain and liver mitochondria (Aravindakshan & Braganca, 1959). These effects had been observed both in experiments *in vitro* as well as in animals injected with the material. Comparative studies carried out with animals injected with crude cobra venom and with heated venom (phospholipase A) had shown that heated venom could not lower the P:O ratio in the intact animal to less than 1.5, even if the animals were injected with large quantities of the material or killed after longer intervals of time. It is well established that three molecules of adenosine triphosphate are formed during the transfer of electrons from diphosphopyridine nucleotide-linked substrates to oxygen. The three phosphorylations are known to occur in steps in different regions of the respiratory chain (Chance & Williams, 1956). It was therefore of interest to explore whether the partial uncoupling of oxidative phosphorylation observed in animals injected with preparations containing phospholipase A was confined to a particular site. Experiments *in vitro* designed to localize the site of activity of some other uncoupling agents have shown that gramicidin (Lehninger, 1954-55) and salicylate (Penniall, 1958) preferentially inhibit the phosphorylation in the region of cytochrome *c*-oxygen. The effect of thyroxine has been investigated in the intact animal by Maley & Lardy (1955), and the data show that thyroxine is equally effective in uncoupling the various phosphorylation steps.

The evidence described in this paper demonstrates that phospholipase A (heated venom) can preferentially uncouple the phosphorylation coupled to cytochrome oxidase when added to isolated brain or liver mitochondria as well as in animals injected with the substance. Apparently in the intact animal the phospholipids associated with phosphorylation in the region of reduced diphosphopyridine nucleotide-cytochrome *c* are not easily attacked by phospholipase A.

EXPERIMENTAL

Inorganic phosphorus was determined according to Lowry & Lopez (1946). Hexokinase was prepared from

baker's yeast by the method of Berger, Slein, Colowick & Cori (1946). Heated cobra venom (*Naja naja*) was prepared by heating an aqueous solution (pH 6) of crude venom at 100° for 15 min. This was employed as the source of phospholipase A as recommended by Braganca & Quastel (1953). The quantity of heated venom employed is expressed in terms of the weight of crude venom from which it was obtained.

The P:O ratio in the reduced diphosphopyridine nucleotide (DPNH)-cytochrome *c* region was determined by using ferricyanide as the electron acceptor as recommended by Copenhaver & Lardy (1952). Phosphorylation coupled to cytochrome oxidase was studied by the method of Lehninger, ul Hassan & Sudduth (1954). In these experiments the mitochondria were pretreated with 0.075M-sucrose.

Mouse-brain and liver mitochondria were isolated according to Brody & Bain (1952) and Schneider (1948) respectively.

Experiments in vivo. Mice were injected with 50 µg. of crude venom or 75 µg. of heated venom. These doses were equivalent in so far as they were lethal in the same period of time (2.5 hr.). The animals injected with crude venom were killed after 15 min. since it had been observed that after longer intervals the phosphorylation process was completely inhibited. Mice injected with heated venom (phospholipase A) were killed after 30 min. Oxidative phosphorylations located in the regions of (a) DPNH-cytochrome *c* and (b) cytochrome *c*-oxygen were determined as in the experiments *in vitro*.

RESULTS

Effect of added heated cobra venom (phospholipase A) on phosphorylation in different parts of the respiratory chain. The esterification of inorganic phosphate in two parts of the respiratory chain was studied in mouse-brain and liver mitochondrial preparations. The system in which sodium pyruvate was used as substrate and potassium ferricyanide as the electron acceptor provided a measure of adenosine triphosphate (ATP) synthesis occurring in the region of DPNH-cytochrome *c* (Copenhaver & Lardy, 1952). ATP synthesized during the oxidation of cytochrome *c* was studied by the aerobic oxidation of ferrocytochrome *c* as recommended by Lehninger *et al.* (1954). The latter compound was produced *in situ* by the non-enzymic reduction of ferricytochrome *c* by ascorbate.

The results in Table 1 show the effect of varying the amounts of added heated cobra venom on

Table 1. *Effect of added heated cobra venom on oxidative phosphorylation in mouse-brain and liver mitochondria with ferricyanide as electron acceptor*

The system contained 15 mM-sodium glycyglycine, pH 7.4, 0.01 M-NaF, 0.01 M-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.4, 0.03 M-glucose, 0.15 M-KCl, 8 mM-MgSO₄, 2.5 mM-ATP, 13 mM-sodium pyruvate, 2 mM-sodium fumarate, 30 units of hexokinase, 200 equivalent mg. of brain mitochondria or 300 equivalent mg. of liver mitochondria (mitochondria obtained from mg. wet weight of tissue), 17 mM-NaHCO₃ and 17 mM-K₃Fe(CN)₆ in a total volume of 3.0 ml. Temp., 30°; gas phase, N₂ + CO₂ (95:5); period of incubation, 20 min. Venom was tipped from the side arm after equilibration for 5 min.

Venom added (μg./vessel)	Brain			Liver		
	ΔP (μmoles)	ΔK ₃ Fe(CN) ₆ (μmoles)	P:2K ₃ Fe(CN) ₆	ΔP (μmoles)	ΔK ₃ Fe(CN) ₆ (μmoles)	P:2K ₃ Fe(CN) ₆
—	11.7	13.7	1.8	16.2	19.0	1.7
1	13.6	15.7	1.8	19.2	23.1	1.7
5	11.1	14.8	1.5	15.5	22.2	1.4
20	7.7	14.0	1.1	11.9	19.9	1.2
50	2.0	12.4	0.3	13.5	17.9	0.4
—	11.4	13.4	1.7	18.3	21.3	1.7
1	14.0	16.5	1.7	20.2	23.9	1.7
5	10.3	14.7	1.4	15.7	22.4	1.4
20	7.3	13.2	1.1	10.8	19.6	1.1
50	2.1	10.2	0.4	2.0	17.6	0.1

Table 2. *Effect of added heated cobra venom on oxidative phosphorylation in mouse-brain and liver mitochondria with ascorbate as substrate*

The system contained 5 mM-Na₂HPO₄-NaH₂PO₄ buffer, pH 7.4, 5 mM-MgSO₄, 1 mM-sodium ethylenediamine-tetra-acetate, 5 mM-ATP, 0.01 M-NaF, 0.2 mM-cytochrome c, 0.05 M-sodium ascorbate, 0.03 M-glucose, 30 units of hexokinase and 200 equivalent mg. of brain mitochondria or 300 equivalent mg. liver mitochondria (mitochondria obtained from mg. wet weight of tissue) in a total volume of 3.0 ml. Temp., 30°; gas phase, air; period of incubation, 20 min. The centre well contained 0.2 ml. of 5 N-NaOH. Venom was tipped from the side arm after equilibration for 5 min.

Venom added (μg./vessel)	Brain			Liver		
	ΔP (μmoles)	ΔO (μg.atoms)	P:O	ΔP (μmoles)	ΔO (μg.atoms)	P:O
—	3.2	3.93	0.8	5.3	7.59	0.7
1	2.4	4.02	0.6	4.1	8.21	0.5
5	1.1	3.66	0.3	2.1	6.80	0.3
20	—	4.02	Nil	Nil	7.86	Nil
50	—	4.02	Nil	Nil	6.70	Nil
—	5.5	6.96	0.8	6.2	6.88	0.9
1	4.0	6.76	0.6	6.1	6.60	0.8
5	2.1	6.86	0.3	3.2	6.60	0.5
20	Nil	5.91	Nil	0.6	6.51	0.1
50	Nil	5.77	Nil	Nil	6.06	Nil

the reduction of ferricyanide and on the P:O [P:2K₃Fe(CN)₆] ratio. It is seen that 1–5 μg. of venom produced a 20% increase in the reduction of ferricyanide by brain and liver mitochondria. Addition of larger quantities of venom (50 μg.) only slightly inhibited the reduction of ferricyanide. The phosphorylating system was more susceptible to the action of heated venom. Thus the P:2K₃Fe(CN)₆ ratio in the absence of heated venom was generally within the range 1.5–1.9 [values which are in agreement with the results of others (Copenhagen & Lardy, 1952; Pressman, 1955)]. Amounts (5 and 20 μg.) of venom depressed the P:2K₃Fe(CN)₆ ratio to 1.4 and 1.1 respectively, whereas 50 μg. of venom produced almost complete uncoupling in this system.

The results show that in presence of small quantities of venom the uncoupling of phosphorylation is accompanied by stimulation of the oxidative process. Earlier experiments had shown that minute quantities of phospholipase A from cobra venom brought about a pronounced increase in the swelling properties of the mitochondria. It is therefore likely that the observed increase in the rate of electron transport is due to changes in the permeability of the mitochondria, which make the substrate, adenosine diphosphate and orthophosphate more easily accessible to the active centres in the mitochondria.

The results given in Table 2 show the effect of 1–50 μg. of heated venom on the respiration and P:O ratio of mitochondria in the presence of cyto-

chrome *c* and sodium ascorbate. These quantities of venom produced only a slight inhibition on the respiratory activity, but the phosphorylation was considerably inactivated. Thus 5 μ g. of heated venom reduced the P:O ratio from 0.8 to 0.3, and 20 μ g. caused complete uncoupling. On comparing the results in Tables 1 and 2 it is apparent that the phosphorylation coupled to the oxidation of cytochrome *c* was more susceptible to the action of heated cobra venom than the phosphorylation occurring in the DPNH-cytochrome *c* part of the respiratory chain. Thus 20 μ g. of heated venom, which completely uncoupled the phosphorylation in the cytochrome *c*-oxygen region, inhibited the phosphorylation in the DPNH-cytochrome *c* part of the chain by only 40%.

Phosphorylations in different parts of the respiratory chain in mitochondria of animals injected with cobra venom. The results of experiments designed to explore the manner in which cobra venom affected the various phosphorylations accompanying respiration in the injected animals are given in

Tables 3 and 4. Table 3 shows that the reduction of ferricyanide increased by about 20% in animals injected with crude venom or heated venom. Under these conditions the $P:2K_3Fe(CN)_6$ ratio was lowered from 1.8 to 1.4 in both instances, indicating that the phosphorylation process was inhibited by 20%. Injections of the same quantities of crude and heated venom produced 25% increase in the oxidation of cytochrome *c* but completely inhibited the phosphorylation in the region (Table 4).

On comparing the results given in Tables 3 and 4, it is seen that quantities of venom, which partially inhibit the phosphorylations in the region of DPNH-cytochrome *c*, completely uncoupled the terminal phosphorylation. There is thus complete agreement between the findings of experiments *in vitro* in which heated cobra venom (phospholipase A) was added to the isolated mitochondria and the findings of experiments with mitochondria of animals injected with the toxic material. These experiments demonstrate that the phosphoryl-

Table 3. *Oxidative phosphorylation with ferricyanide as electron acceptor in mitochondria of animals injected with cobra venom*

Conditions are as in Table 1. The results in each experiment represent values obtained from three different groups of animals.

Form of cobra venom injected	Venom injected (μ g.)	Time of killing after injection (min.)	Brain			Liver		
			ΔP (μ moles)	$\Delta K_3Fe(CN)_6$ (μ moles)	$P:2K_3Fe(CN)_6$	ΔP (μ moles)	$\Delta K_3Fe(CN)_6$ (μ moles)	$P:2K_3Fe(CN)_6$
None	—	—	10.5	12.4	1.7	13.8	15.3	1.8
			11.1	12.0	1.8	15.4	18.6	1.7
			15.8	20.6	1.5	14.9	18.1	1.6
Crude	50	15	12.2	17.5	1.4	13.4	20.6	1.3
			13.0	18.4	1.3	15.0	19.8	1.5
			11.0	16.6	1.3	14.5	21.8	1.3
Heated	75	30	12.2	17.5	1.4	13.4	20.6	1.3
			13.0	18.4	1.3	15.0	19.8	1.5
			11.0	16.6	1.3	14.5	21.8	1.3

Table 4. *Oxidative phosphorylation with ascorbate as substrate in mitochondria of animals injected with cobra venom*

Conditions are as in Table 2. The results in each experiment represent values obtained from three different groups of animals.

Form of cobra venom injected	Venom injected (μ g.)	Time of killing after injection (min.)	Brain			Liver		
			ΔP (μ moles)	ΔO (μ g.atoms)	P:O	ΔP (μ moles)	ΔO (μ g.atoms)	P:O
None	—	—	3.8	4.20	0.9	5.2	5.53	0.9
			4.5	4.65	0.9	5.8	6.78	0.9
			3.5	4.40	0.8	5.9	6.11	0.7
Crude	50	15	Nil	5.60	Nil	Nil	8.21	Nil
			Nil	5.57	Nil	Nil	8.33	Nil
			Nil	5.51	Nil	Nil	7.81	Nil
Heated	75	30	Nil	6.16	Nil	Nil	9.48	Nil
			Nil	4.81	Nil	Nil	7.40	Nil
			Nil	5.70	Nil	Nil	7.86	Nil

Table 5. *Effect of cobra venom on phosphorylations in different regions of respiratory chain*

Heated venom added (μg.)	Heated venom injected (μg.)	P:O ratios					
		DPNH-O ₂		DPNH-cytochrome c		Cytochrome c-O ₂	
		Brain	Liver	Brain	Liver	Brain	Liver
Nil	Nil	2.7	2.7	1.8	1.7	0.8	0.7
20	Nil	1.3	1.3	1.1	1.2	Nil	Nil
Nil	75	1.5	1.5	1.4	1.3	Nil	Nil

ation associated with the oxidation of cytochrome c in mitochondria is preferentially inactivated by phospholipase A.

The mitochondria employed for studying oxidative phosphorylation coupled to cytochrome oxidase were pretreated with hypo-osmotic sucrose as recommended by Lehninger *et al.* (1954). It was therefore necessary to know whether the preferential inhibition of phosphorylation in this part of the chain was due to the partial damage caused to the mitochondria by the pretreatment. The results of experiments in which the effects produced by cobra venom on the phosphorylation in the whole respiratory chain were compared with the changes brought about in the phosphorylations occurring in the different regions are summarized in Table 5.

The mitochondria employed for the determination of P:O ratio in the whole respiratory chain as well as in the region of DPNH-cytochrome c were prepared under standard conditions (0.25M-sucrose). Table 5 shows that under conditions in which the P:O ratio in the whole chain was partially lowered the amount of inorganic phosphate esterified could be accounted for entirely by the phosphorylations occurring in the region of DPNH-cytochrome c. It may therefore be concluded that no phosphorylation occurred in the region of cytochrome c-oxygen. Thus the greater susceptibility of the phosphorylation in the region of cytochrome c-oxygen to the action of cobra venom could not be due to the damage caused to the mitochondria by the pretreatment with hypo-osmotic sucrose.

DISCUSSION

The results obtained with brain and liver mitochondria have demonstrated that the three phosphorylations linked to the electron-transport chain are not equally susceptible to the destructive action of cobra venom. The enzyme complex concerned with the esterification of inorganic phosphate in the region of cytochrome c-oxygen portion of the electron-transport chain is considerably more susceptible to inactivation by the venom. This is apparent from the results of experiments *in vitro*, in which heated cobra venom (phospholipase A) was added to the separated mitochondria, as

well as the experiments with mitochondria prepared from animals injected with cobra venom. It is interesting to note that studies on the effect of phospholipase A on the respiration of mitochondria have also shown that cytochrome oxidase is more readily inactivated than some of the other respiratory enzyme systems (Aravindakshan, 1959).

Considerable evidence, including studies with crystalline phospholipase A, indicates that the inhibitory effects produced by cobra venom on oxidative phosphorylation in the mitochondria are due to the destruction of mitochondrial phospholipids by the phospholipase A (Aravindakshan & Braganca, 1961). Mitochondria are rich in phospholipids, and numerous studies have established the importance of phospholipids both in electron transport and in the accompanying phosphorylations (Green, 1959; Schneider, 1959). Recent studies by Green and associates (see Green & Lester, 1959) have resulted in the separation of several different lipoproteins which have been shown to be essential components of the respiratory chain. The nature of the phospholipids concerned with the various phosphorylation steps is less clear. The preferential inactivation produced by phospholipase A on the phosphorylation occurring in the cytochrome c-oxygen region could point to differences in the composition of phospholipids essential for the phosphorylation in the different parts of the respiratory chain.

Results obtained with animals injected with phospholipase A (heated venom) are of particular interest. Previous studies have shown that heated cobra venom when added in sufficient quantities to mitochondria from brain or liver can completely uncouple oxidative phosphorylation. Injections of heated venom (phospholipase A) have, however, been found not to lower the P:O ratio to a value less than 1.5. Greater uncoupling effect could not be observed even in animals injected with larger quantities of heated venom or in animals killed after longer intervals (Aravindakshan & Braganca, 1959). In the light of evidence described in this paper, a possible explanation for the above observations may be that in the intact animal the phospholipids concerned with the phosphorylation in the DPNH-cytochrome c region are considerably resistant to the action of phospholipase A.

SUMMARY

1. Experiments with brain and liver mitochondria have demonstrated that the three phosphorylations linked to the electron-transport chain are not equally susceptible to the uncoupling action of cobra venom.

2. The phosphorylation in the region of cytochrome oxidase is preferentially inactivated by heated venom. These observations have been made in direct experiments *in vitro* and with mitochondria obtained from animals injected with venom.

3. Evidence obtained with mitochondria of animals injected with heated venom (phospholipase A) suggests that the phospholipids concerned with phosphorylations in the reduced diphosphopyridine nucleotide-cytochrome c region are considerably resistant to the action of phospholipase A.

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Studies on Phospholipid Structures in Mitochondria of Animals Injected with Cobra Venom or Phospholipase A

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Considerable evidence has been obtained that venoms containing phospholipase A bring about inactivation of respiratory activity and uncouple oxidative phosphorylation when added to isolated tissue preparations (Braganca & Quastel, 1952, 1953; Nygaard, 1953*a, b*; Nygaard & Sumner, 1953; Petrushka, Quastel & Scholefield, 1957). It has been suggested that these effects are produced through the destructive action of phospholipase A on mitochondrial phospholipids. Nygaard (1953*a*) has shown that the inactivation of succinic oxidase by phospholipase A from *Crotalus terrificus* venom is dependent on the extent of phospholipid hydrolysed. Results of investigations on the changes produced on oxidative phosphorylation in the mitochondria of animals injected with cobra venom have shown remarkable agreement with findings of

experiments *in vitro*. The enzyme systems concerned with esterification of inorganic phosphate appear to be particularly sensitive to the action of cobra venom (Aravindakshan & Braganca, 1959). The present investigation was undertaken to study in greater detail the mechanism underlying the uncoupling action of cobra venom on oxidative phosphorylation in mitochondria. Data from studies on submitochondrial particles as well as on the swelling properties of mitochondria show that cobra venom brings about pronounced changes in mitochondrial structure. These effects have been observed *in vitro* as well as in animals injected with cobra venom or with crystalline phospholipase A.

Several reports have appeared on the uncoupling effect of lysolecithin on oxidative phosphorylation