EFFECTS OF RESERPINE, CHLORPROMAZINE AND SODIUM SALICYLATE ON THE ENZYMIC ACTIVITY OF RAT LIVER

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The effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation, stimulation of glutamic acid oxidation and adenosinetriphosphatase activity have been compared *in vitro* with those of 2,4-dinitrophenol using rat liver preparations. All three drugs inhibit oxidative phosphorylation, slightly stimulate glutamic acid oxidation and stimulate adenosinetriphosphatase activity at high concentrations. Reserpine has no effect on adenosinetriphosphatase activity. It is suggested that the characteristic depressant effects of reserpine on isolated tissues *in vitro* may be due to interference with production of energy by oxidative phosphorylation.

Substances which depress the formation of high energy phosphate bonds without markedly affecting oxygen consumption are known as uncoupling agents. 2,4-Dinitrophenol is a typical uncoupling agent and has been extensively used in studies on the mechanism of oxidative phosphorylation. A number of compounds chemically unrelated to one another and to dinitrophenol dissociate oxidation from phosphorylation and have been claimed to exert their biological actions through this property (Brody, 1955). Thus the antipsychotic agents, chlorpromazine and reserpine, inhibit oxidative phosphorylation in rat brain mitochondria (Abood and Romanchek, 1957) and chlorpromazine exerts a similar action in rat liver mitochondria (Berger, Strecker, and Waelsch, 1957). It has also been shown (Brody, 1956) that salicylates act as uncoupling agents, and reserpine, after *in vivo* administration, causes a significant fall in the concentrations of high energy phosphate in rat liver (Kirpekar and Lewis, 1959a).

Dinitrophenol inhibits oxidative phosphorylation (Loomis and Lipmann, 1948), stimulates the oxidation of various substrates such as glutamate, α -ketoglutarate, succinate and pyruvate + malate (Potter and Recknagel, 1951), and stimulates adenosinetriphosphatase activity (Lardy and Wellman, 1953; Myers and Slater, 1957). In the present experiments a comparison has been made

between the effects of reserpine, chlorpromazine, sodium salicylate and the effects of dinitrophenol on these three systems in rat liver preparations *in vitro*.

METHODS

A solution of reserpine (2.5 mg./ml.) and the corresponding control solvent solution (Ciba) were used and diluted as required. Aqueous solutions of chlorpromazine hydrochloride (Smith, Kline and French), sodium salicylate and 2,4-dinitrophenol were used, and diluted to give the required concentrations.

Preparation of Mitochondria

All operations were carried out at 0°. The method used for the isolation of mitochondria was essentially that described by Schneider (1948) and Schneider and Hogeboom (1950). The animals were fasted overnight to reduce the glycogen content of the liver. The liver from a freshly killed albino rat was quickly excised, washed in ice-cold 0.25 M-sucrose, blotted gently with filter paper and weighed on a torsion balance. The liver was disintegrated for 2 min. at 1,500 rev./min. in 9 volumes of cold 0.25 M-sucrose in a Potter-Elvehjem homogenizer using a plastic pestle. The mitochondrial pellet was finally suspended in 0.25 M-sucrose, so that 2.5 ml. of suspension contained the mitochondria derived from 1 g. of fresh liver. Mitochondria obtained in this manner showed a large increase in oxygen uptake if exposed to dinitrophenol or with glucose and hexokinase (Aldridge, 1957).

Measurement of Oxidative Phosphorylation

All incubations were carried out in standard Warburg flasks using the medium described by Parker

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(1958) to suspend the mitochondria. Each flask contained 0.5 ml. of mitochondrial suspension, representing 200 mg. wet wt. of liver, 0.00115 mole sodium adenosine triphosphate, 0.00115 mole adenosine monophosphate, 0.125 mole potassium chloride, 0.014 mole magnesium chloride hexahydrate, 0.001 mole disodium ethylenediamine tetra-acetic acid, 0.0154 mole potassium phosphate, 0.0166 mole glycylglycine (pH 6.75), 0.01 mole glutamic acid as substrate, 0.06 mole glucose, 400 units of hexokinase (a preparation of type II hexokinase [Sigma Chemical Company] containing 70,000 units/g. was used) and 0.1 ml. of aqueous drug solution: for reserpine controls, 0.1 ml. of the diluted control solution was used. In the absence of drug 0.1 ml. of distilled water was used. The final flask volume was 3.0 ml., the centre well containing 0.2 ml. of 20% (w/v) KOH and a filter paper strip. The pH of the medium was 6.8 and the incubations were carried out at 37° in an atmosphere of air. In these experiments, glucose and hexokinase were not added to the medium in the main chamber but placed separately in the side arm. After equilibration for 10 min., the contents of the side arm were tipped and the oxygen uptake recorded for a further period of 10 min. The flasks were then immediately removed from the manometers and the reaction terminated by addition of 0.5 ml. of 60% (w/w) perchloric acid. In each experiment, the initial inorganic phosphorus content was determined in a blank flask at zero-time by deproteinizing with 0.5 ml. of 60% (w/w) perchloric acid before adding the mitochondria. The blank flask was taken through the procedure in the same manner as the others. After deproteinizing with 60% (w/w) perchloric acid the solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. In this way both oxygen and phosphorus uptake were measured over a period of 10 min.

Measurement of Stimulation of Oxidation

The oxidation of glutamic acid was measured using the Warburg method. The contents and the final concentration of each flask were as described in the previous section, but glucose and hexokinase were omitted. The mitochondrial suspension (0.25 ml.) added to each flask was equivalent to 100 mg. wet wt. of liver. The final volume was 3.0 ml., the pH 6.8 and the bath temperature 37°. The contents of the flasks were equilibrated for 10 min. and readings taken at 10 min. intervals for 1 hr.

Measurement of Adenosinetriphosphatase Activity

Adenosinetriphosphatase activity of whole liver homogenates was measured using the method of Lardy and Wellman (1953). The amount of inorganic phosphorus liberated in 10 min. from adenosine triphosphate in the presence of a liver suspension was taken as a measure of activity. Experiments were carried out in Warburg flasks at 25° in an atmosphere of air. Each flask contained 0.06 mole sodium adenosine triphosphate, 0.15 mole potassium chloride,

0.1 ml. water, reserpine control solution or the drug solution, and 0.3 ml. of liver homogenate in 0.25 M sucrose, equivalent to 100 mg. wet wt. of liver in the side arm. The total volume after tipping was 1.0 ml., the pH of the solution 7.2. The flasks were placed in the Warburg bath and shaken for 5 min. for temperature equilibration and the reaction started by tipping in the enzyme source. After shaking for 10 min. the flasks were immediately taken out and the reaction stopped by adding 1.0 ml. of 10% (w/w) perchloric acid. In each experiment the inorganic phosphate formed in a blank flask was estimated by deproteinizing with 1.0 ml. of 10% (w/w) perchloric acid and this value subtracted from all readings. The solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. Phosphate determinations were made by the method of Allen (1940).

RESULTS

Table I shows the effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation in rat liver mitochondria. In the presence of glucose and hexokinase the rate of oxygen uptake was about 2.5 times that without the acceptors. All three drugs inhibited phosphorylation without simultaneously affecting oxygen uptake. At 10^{-6} M dinitrophenol had very little effect on oxidative phosphorylation: it caused almost 50% inhibition at 10^{-3} M and 100% at 10^{-4} M. Chlorpromazine at 10^{-5} M had practically no effect on the P/O ratio (uncoupling only 5%) but began to exert its effect at 10^{-4} M, when uncoupling was about 30%, while at 5×10^{-4} M the phosphorus uptake was completely inhibited. At this same concentration chlorpromazine depressed oxygen uptake. Sodium salicylate had no uncoupling effect at 10^{-5} M, but caused 35% depression at 10^{-3} M and there was complete inhibition at 10^{-2} M. At 0.8×10^{-4} M reserpine caused 36% inhibition of phosphorylation while at 1.5 times this concentration the depression was 61%. A volume of the reserpine control solution equivalent to higher concentrations of reserpine slightly depressed the P/O ratio. Reserpine had a smaller effect on oxygen uptake, but a greater effect on phosphorus uptake than chlorpromazine. When compared with dinitrophenol, neither reserpine nor chlorpromazine nor sodium salicylate was a strong uncoupling agent. Chlorpromazine and reserpine appeared to be equipotent, but salicylate was much less potent than either.

Table II shows stimulation of the oxidation of glutamic acid in an acceptor-deficient medium. Dinitrophenol slightly stimulated it at 10^{-6} M: the effect was doubled at 10^{-5} M and increased more

TABLE I

THE EFFECT OF DINITROPHENOL, CHLORPROMAZINE, SODIUM SALICYLATE, AND RESERPINE ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

All concentrations are molar.

Drug	Conc.	$\Delta P\mu$ Mole ($\pm$ S.E.)	$\Delta O\mu$ Atoms ($\pm$ S.E.)	P/O ($\pm$ S.E.)	% Uncoupling
Control	—	25.82 ($\pm$ 0.52)	9.5 ($\pm$ 0.28)	2.76 ($\pm$ 0.05)	—
Dinitrophenol ..	1×10^{-6}	19.39 ($\pm$ 0.16)	7.94 ($\pm$ 0.10)	2.43 ($\pm$ 0.05)	12
	1×10^{-5}	12.28 ($\pm$ 0.46)	8.80 ($\pm$ 0.47)	1.40 ($\pm$ 0.11)	49
	1×10^{-4}	0.00	6.90 ($\pm$ 0.12)	0.00	100
Chlorpromazine ..	1×10^{-5}	20.25 ($\pm$ 0.27)	7.74 ($\pm$ 0.11)	2.61 ($\pm$ 0.00)	5
	1×10^{-4}	17.22 ($\pm$ 1.60)	8.67 ($\pm$ 0.60)	1.96 ($\pm$ 0.06)	29
	5×10^{-4}	0.00	2.14 ($\pm$ 0.05)	0.00	100
Sodium salicylate ..	1×10^{-5}	27.52 ($\pm$ 1.10)	10.05 ($\pm$ 0.02)	2.71 ($\pm$ 0.10)	2
	1×10^{-4}	16.44 ($\pm$ 0.86)	7.63 ($\pm$ 0.20)	2.16 ($\pm$ 0.17)	22
	1×10^{-3}	12.46 ($\pm$ 1.40)	6.87 ($\pm$ 0.14)	1.79 ($\pm$ 0.17)	35
	1×10^{-2}	0.00	5.96 ($\pm$ 0.07)	0.00	100
Reserpine control ..	—	17.91 ($\pm$ 1.6)	7.94 ($\pm$ 0.58)	2.25 ($\pm$ 0.10)	—
Reserpine	1.6×10^{-5}	22.51 ($\pm$ 0.16)	11.25 ($\pm$ 0.18)	2.00 ($\pm$ 0.04)	11
	0.8×10^{-4}	12.44 ($\pm$ 0.29)	8.68 ($\pm$ 0.58)	1.44 ($\pm$ 0.09)	36
	1.2×10^{-4}	4.51 ($\pm$ 1.27)	4.88 ($\pm$ 0.31)	0.87 ($\pm$ 0.23)	61

TABLE II

EFFECTS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE, AND SODIUM SALICYLATE ON OXYGEN UPTAKE IN AN ACCEPTOR-DEFICIENT MEDIUM

Control values (1 hr.) are taken as 100%. All concentrations are molar.

Drug	Conc.	% Oxygen Uptake
Dinitrophenol ..	1×10^{-6}	115
	1×10^{-5}	200
	1×10^{-4}	265
Reserpine ..	1.6×10^{-5}	115
	0.8×10^{-4}	117
	1.2×10^{-4}	83
Chlorpromazine	1×10^{-5}	107
	5×10^{-5}	113
	1×10^{-4}	124
	5×10^{-4}	6
Sodium salicylate	1×10^{-5}	104
	1×10^{-4}	119
	1×10^{-3}	108
	5×10^{-3}	100
	1×10^{-2}	100

than 2.5 times at 10^{-4} M. Reserpine at 1.6×10^{-5} M and 0.8×10^{-4} M stimulated oxygen uptake 15%. The reserpine control solution had virtually no effect on glutamic acid oxidation. When used with liver slices, the control solution significantly stimulated oxygen uptake (Kirpekar and Lewis, 1959a). Chlorpromazine had no effect at 10^{-5} M, caused approximately 25% stimulation at 10^{-4} M and almost complete inhibition at 5×10^{-4} M. Sodium salicylate caused slight stimulation at 10^{-4} M, but even at very high concentrations (10^{-3} to 10^{-2} M) it had very little effect. None of the drugs tested had so marked an effect on glutamic acid oxidation as dinitrophenol. Dinitrophenol apparently compensates for the deficiency of glucose and hexokinase, since in its presence oxygen uptake was even greater than in the presence of such an acceptor.

Table III shows the effects of chlorpromazine, reserpine, sodium salicylate and dinitrophenol on liver adenosinetriphosphatase activity. Dinitrophenol had a very high stimulant activity at 3×10^{-4} M, but at lower concentrations it was less effective and at 10^{-6} M inactive. Chlorpromazine had no effect at 10^{-5} M and 10^{-4} M, but at 10^{-3} M had about half the effect of dinitrophenol (3×10^{-4} M). Reserpine at the concentrations tested had no stimulant effect upon adenosine-

TABLE III
EFFECTS OF DINITROPHENOL CHLORPROM-
AZINE, RESERPINE, AND SODIUM SALICYLATE
ON LIVER ADENOSINETRIPHOSPHATASE
ACTIVITY

All concentrations are molar.

Drug	Conc.	μ mole Inorganic Phosphate Liberated $\pm$ (S.E.)
Control ..	—	0.87 ($\pm$ 0.05)
Dinitrophenol ..	3×10^{-6}	1.10 ($\pm$ 0.07)
„ ..	3×10^{-5}	2.59 ($\pm$ 0.12)
„ ..	3×10^{-4}	8.35 ($\pm$ 0.10)
Chlorpromazine	1×10^{-5}	1.04 ($\pm$ 0.16)
„ ..	1×10^{-4}	1.04 ($\pm$ 0.08)
„ ..	1×10^{-3}	3.67 ($\pm$ 0.02)
Reserpine ..	1.6×10^{-5}	1.04 ($\pm$ 0.14)
„ ..	0.8×10^{-4}	0.37 ($\pm$ 0.13)
„ ..	1.2×10^{-4}	0.22 ($\pm$ 0.09)
Sodium salicylate	1×10^{-4}	1.32 ($\pm$ 0.00)
„ „	1×10^{-3}	2.15 ($\pm$ 0.21)
„ „	1×10^{-2}	5.97 ($\pm$ 0.19)

triphosphatase activity, but caused slight inhibition at higher concentrations. Sodium salicylate enhanced adenosinetriphosphatase activity (6.00 μ mole of phosphate at 10^{-2} M); had little stimulant effect at 10^{-3} M and none at 10^{-4} M. It was interesting to note that the concentrations of dinitrophenol which produced maximum stimulation of oxygen uptake and of adenosine-triphosphatase action were similar. This does not,

however, appear to be true of the other compounds investigated.

Table IV summarizes the results and gives comparative data on the four compounds studied.

DISCUSSION

Dinitrophenol, which is a typical uncoupling agent, powerfully stimulates adenosinetriphosphatase activity and glutamic acid oxidation, and causes marked inhibition of phosphorylation without affecting oxygen uptake. Reserpine, chlorpromazine and sodium salicylate all inhibit oxidative phosphorylation, and with higher concentrations both oxygen and phosphorus uptake are depressed. There is some difference of opinion regarding the action of chlorpromazine on rat brain mitochondria. Abood and Romanchek (1957) have shown that chlorpromazine depresses oxidative phosphorylation, while Berger, Strecker and Waelsch (1957) did not confirm this but reported depression of P/O ratios in rat liver mitochondria and thereby stressed the difference in the metabolic behaviour of mitochondria from the brain and liver. Our observations support their finding that chlorpromazine inhibits phosphorylation in rat liver mitochondria. Reserpine has been reported by Abood and Romanchek (1957) to be an effective uncoupling agent in rat brain mitochondria, and our results show that it is an uncoupling reagent in rat liver mitochondria. It appears therefore that, as far as reserpine is concerned, brain and liver mitochondria behave similarly. It is also possible that reserpine, which has marked effects on the central nervous system, may possess a preferentially higher affinity for brain than for liver cells. Reserpine has also been reported by the same authors in *in vivo* studies to have an uncoupling effect in preparations of mitochondria

TABLE IV
COMPARATIVE CONCENTRATIONS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE,
AND SODIUM SALICYLATE AFFECTING THE P/O RATIO, OXIDATION AND ADENOSINE-
TRIPHOSPHATASE ACTIVITY IN RAT LIVER

All concentrations are molar.

Experiment	Dinitrophenol	Reserpine	Chlorpromazine	Sodium Salicylate
50% inhibition of P/O ratio	1×10^{-5}	1×10^{-4}	2×10^{-4}	2.5×10^{-3}
Maximum stimulation of oxidation ..	1×10^{-4}	0.8×10^{-4}	1×10^{-4}	1×10^{-4}
Maximum stimulation of adenosine- triphosphatase activity	3×10^{-4}	None	1×10^{-3}	1×10^{-2}

from the region of the basal ganglia. It has been shown recently by Desci and Mehes (1959) that the tranquillizing agents benactyzine and meprobamate also have an uncoupling effect which is exerted more effectively on preparations from the same regions.

At a concentration of 10^{-4} M dinitrophenol markedly stimulates the oxidation of glutamic acid. The other drugs are inactive, and although they inhibit oxidative phosphorylation in certain concentrations they do not possess the property of stimulating oxidation. Dinitrophenol stimulates oxidation markedly and at the same time inhibits phosphorylation. Chlorpromazine (10^{-3} M) and sodium salicylate (10^{-2} M) stimulate adenosinetriphosphatase activity considerably, but there appears to be no relation between the dose causing maximum stimulation of adenosinetriphosphatase activity and the effect on oxygen uptake. Reserpine, on the other hand, has little stimulant effect on oxidation (10^{-4} M) but at the same concentration has a slight inhibitory effect on adenosinetriphosphatase activity.

The effects of chlorpromazine and reserpine, like those of thyroxine, may be due to intracellular physical processes such as the swelling of the mitochondria. Thyroxine causes the mitochondria to swell (Dickens and Salmony, 1956; Tapley, Cooper, and Lehninger, 1955) and the uncoupling effect that it produces is attributed to this property rather than to its interaction with the enzymes concerned with oxidative phosphorylation. It would be interesting to see whether chlorpromazine or reserpine cause swelling of mitochondria although the general pharmacological properties of reserpine and chlorpromazine are quite different from those of thyroxine.

Chlorpromazine and salicylates react with ferric salts. Chlorpromazine when mixed with ferric chloride solution is oxidized and produces a brilliant red colour. Our recent observations (Kirpekar and Lewis, 1959b) of the apparent formation of a hydrallazine-iron complex and the observation of Fels and Kaufmann (1959) that chlorpromazine possesses a specific affinity for iron make it possible that chlorpromazine influences phosphorylation at the cytochrome-cytochrome oxidase level. But a few experiments carried out with crystalline catalase (an iron containing enzyme) have not shown chlorpromazine to have an inhibiting effect even when used at very high concentrations (1.8×10^{-2} M). Salicylates are chelating agents, but reserpine has not so far been reported to form chelation complexes.

In intact animals reserpine is known to exert its characteristic effects rather slowly. Gillis and Lewis (1956) and Kirpekar and Lewis (1958) have shown that reserpine depresses skeletal muscle, the smooth muscles of intestine, uterus and arteries, and that it also depresses heart muscle. This effect may be due to its uncoupling action (Kirpekar and Lewis, 1959a). Born and Bülbring (1955) have shown that dinitrophenol in high concentrations decreases the tension and spontaneous movements of the taenia coli muscle. Moreover, when the dinitrophenol-treated muscle is stimulated with histamine, the tension increases but is not maintained although histamine is still present. Anoxia also depresses tissue activity. There is thus some evidence that, under the conditions of our experiments, reserpine and chlorpromazine act like dinitrophenol, but the absence of a reserpine effect on adenosinetriphosphatase activity shows that chlorpromazine and reserpine have different modes of action from one another and from dinitrophenol, a view supported by pharmacological observations. Sodium salicylate has no antipsychotic action of the type exerted by reserpine and chlorpromazine, but it has some marked effects on the central nervous system: it can, for example, cause nausea and vomiting, analgesia, antipyresis and respiratory stimulation. It has also been shown to inhibit catalase. The effects of sodium salicylate on the metabolic rate (Cochran, 1952) and in causing depletion of liver glycogen (Sproull, 1954) and hyperglycaemia (Bornstein, Meade, and Smith, 1952) may have some bearing on its effect on oxidative phosphorylation, but in man even large doses of salicylate do not produce the effects typical of an uncoupling agent.

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