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SOME EFFECTS OF RESERPINE AND HYDRALLAZINE UPON TISSUE RESPIRATION AND THE CONCENTRATION OF ADENOSINE NUCLEOTIDES IN CERTAIN TISSUES

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The effects of reserpine and hydrallazine upon the oxygen uptake of preparations of rat brain, liver, and kidney and rabbit aorta have been investigated using the Warburg " Direct " method. In these tissues reserpine did not significantly stimulate respiration at any of the doses used. Concentrations of 10 or 50 μg./ml. had no significant effect upon respiration, but higher doses (330 μg./ml.) depressed it in all the tissues studied. This effect was also seen in rat brain at a reserpine concentration of 100 μg./ml. which had no significant effect in other tissues. Hydrallazine (50 or 100 μg./ml.) depressed respiration in all tissues studied. Stimulation at lower doses was not seen.

After sublethal doses of reserpine, depletion of energy rich phosphate compounds occurred in brain and liver but not in skeletal muscle and heart. Hydrallazine also depleted brain of energy rich phosphate compounds, but did not affect liver, heart, or skeletal muscle. The bearing of these results on the mode of action of reserpine and hydrallazine is discussed.

Earlier studies by Gillis and Lewis (1957) have indicated that reserpine may inhibit carbohydrate metabolism at some point in the tricarboxylic acid cycle and that it depressed respiration in slices of rabbit intestinal smooth muscle. We have shown that inhibition by hydrallazine of drug induced contractions of arterial smooth muscle can be antagonized by certain intermediates of carbohydrate metabolism and that the effects of hydrallazine on arterial strips resembled those of anoxia (Kirpekar and Lewis, 1958b). Reserpine was also found to inhibit drug induced contractions of arterial strips, but the effect was virtually irreversible and it was therefore not possible to study the effects of intermediary metabolites.

Abood and Romanchek (1957) have shown that in oxidative phosphorylation reserpine acts as an uncoupling agent and at a concentration of 2×10^{-5}M produced a fall of more than 40% in the P/O ratio of rat brain mitochondria. This value compares favourably with 2:4-dinitrophenol, a strong uncoupling agent. It is a characteristic of uncoupling agents that, at lower concentrations, they stimulate tissue respiration and at higher concentrations depress it. We have therefore investigated the influence of reserpine upon tissue respiration and compared its effects with those of hydrallazine. A compound which interferes with cell metabolism may influence the high energy phosphate level, especially that of adenosine triphosphate. We have therefore supplemented our studies on tissues respiration with estimations of the content of adenosine nucleotides in rat brain, heart, skeletal muscle, and liver, following treatment *in vivo* with reserpine and hydrallazine.

METHODS

For the determination of tissue respiration a 2.5 mg./ml. solution of reserpine was used which was diluted as required. The *p*H was 3.6. A control solution without reserpine was also used. An aqueous solution of hydrallazine HCl (20 mg./ml.) was prepared and diluted as required before use with Krebs-Ringer-phosphate solution (Umbreit, Burris, and Stauffer, 1957) (" phosphate medium ") or Krebs medium IIA (Krebs, 1950) (" medium IIA "). Addition of 0.4 ml. of reserpine or hydrallazine solution to the side arm gave a final flask concentration after tipping of 10, 50, 100, or 330 μg./ml. of the drug.

Respiration in Isolated Rat Tissues

Rats of either sex weighing from 150 to 200 g. were used. To obtain preparations of brain, the animals were killed, bled out, and the brain rapidly removed

and weighed. A 10% suspension was prepared by grinding the brain in a glass pestle and mortar with ice-cold phosphate medium. The suspension was filtered through muslin; 1 ml. of this extract (which is equivalent to 100 mg. of brain tissue) was used in each flask. Liver and kidney were removed from the animals, washed with phosphate medium to remove excess blood, and cut on a tissue slicer to give sections of 0.364 mm. thickness. A sufficient number of liver or kidney slices to weigh 100 mg. wet weight was used in each flask.

Respiration in Isolated Rabbit Thoracic Aorta

Rabbits weighing between 2.0 kg. and 2.5 kg. were killed by a blow on the head and bled out. About 5 cm. of thoracic aorta was rapidly removed and washed with ice-cold phosphate or IIA medium. The excised aorta was opened by a longitudinal incision and sliced to give pieces of the same thickness as liver and kidney. A sufficient number of slices to weigh 100 mg. wet weight was used in each flask.

Experiments on oxygen consumption were carried out by the Warburg "Direct" method at 37° using an atmosphere of air. The main chamber of each Warburg flask contained 2.4 ml. of medium together with the appropriate tissue preparation. The centre well contained 0.2 ml. of 20% KOH. The side arm contained 0.4 ml. of drug solution or the control, which in the case of reserpine was the solution supplied by the makers and of hydrallazine the appropriate physiological saline. After equilibration, the contents of the side arm were tipped into the main chamber and readings were taken at 10 min. intervals for 1 hr.

Estimation of Adenosine Nucleotides

Estimations were carried out by the specific enzymatic method of Kalckar (1947). Female albino rats weighing between 180 and 200 g. were used for these estimations. Rats were given 10 g. of powdered diet No. 86 (Laboratory Animals Bureau) for at least 5 days before killing. The powder was made into a paste with water to avoid spilling. Water was given *ad libitum*. This regimen was strictly adhered to since it has been shown by Munro and Goodlad (personal communication) that the adenosine triphosphate content of the liver is very sensitive to variations in dietary energy intake. Immediately after the last feed, 1 mg. of reserpine was given by intraperitoneal injection or 10 mg. of hydrallazine by subcutaneous injection. The controls were given equivalent volumes of the control solution or physiological saline respectively. Only those rats which had eaten all their food were used. Reserpine treated animals and their controls were killed between 11 and 12 hr. after injection; hydrallazine treated animals and their controls were killed 3 to 4 hr. after injection.

To minimize struggling, since such movements cause the breakdown of energy-rich phosphate compounds, the rats were injected with 200 mg./kg. of mephenesin 5 to 10 min. before killing. This usually gave complete muscular relaxation. Liver, heart, and gastrocnemius muscle were rapidly removed from the same animals, but brain was obtained from separate animals because of the length of time required for the dissection. Since doses of the drugs used were sublethal, estimations were always made after killing the animals. It was found, however, that 10 mg./rat of hydrallazine was quite toxic and some of the animals were in a poor condition while a few had convulsions and died. Animals which died due to drug administration were not used for experiments.

The animals were killed by cutting the throat. The tissues were removed and washed with ice-cold water; the excess water was removed by lightly touching with tissue paper, weighed on a torsion balance and ground up in 1.5% perchloric acid solution.

Skeletal Muscle. — Gastrocnemius muscle was ground up in a glass pestle and mortar. For each g. of muscle, 5 ml. of 1.5% perchloric acid solution at 0° was used. The contents of the mortar were transferred to a glass centrifuge tube and the mortar and pestle washed with two 5 ml. portions of 1.5% perchloric acid solution. The washings were added to the centrifuge tube and the contents centrifuged for 10 min. at 2,000 rev./min. at 0°. The supernatant was decanted into a measuring cylinder, and the residue was re-extracted with 5 ml. of 1.5% perchloric acid solution and centrifuged. The supernatant was then added to the stoppered measuring cylinder and the total volume noted. 10 ml. of the extract was pipetted into a beaker containing 10 ml. of succinate buffer at *p*H 6.1. The contents of the beaker were adjusted to *p*H 6.1 by addition of 5N-NaOH using the glass electrode. The volume was adjusted to 25 ml. with water, giving an approximately 1 in 50 tissue extract.

Liver.—About 2 g. of liver was excised and washed with ice-cold distilled water to remove excess blood; this was then transferred to 20 ml. of 1.5% ice-cold perchloric acid in a "Nalco" breaker vessel and ground. The suspension was transferred to a cooled centrifuge tube, the breaker vessel and the grinder blades rinsed with two 5 ml. aliquots of ice-cold 1.5% perchloric acid and the washings added to the suspension. The contents of the tube were centrifuged at 2,000 rev./min. at 0° for 10 min. and the supernatant transferred to a stoppered measuring cylinder. The residue was re-extracted with 5 ml. of 1.5% ice-cold perchloric acid, centrifuged, and the supernatant added to the measuring cylinder. The total volume was noted and a 10 ml. aliquot transferred to a beaker containing 10 ml. succinate buffer at *p*H 6.1. The *p*H was adjusted to 6.1 with 5N-NaOH and the final volume adjusted to 50 ml. to give an approximately 1 in 75 extract.

Heart.—Hearts were removed, washed with ice-cold water and extracted in a manner similar to that used for liver to give an approximately 1 in 75 tissue extract.

Brain.—Brain was extracted in a manner similar to that for liver and heart to give an approximately 1 in 50 tissue extract.

All tissue extracts were stored in the frozen state until assayed.

RESULTS

Tissue Respiration

Concentrations of 10, 50, or 100 μg./ml. of reserpine had no significant effects on tissue respiration except in rat brain when 100 μg./ml. caused depression ($P<0.01$). Reserpine (10, 50, or 100 μg./ml.) did not stimulate the oxygen uptake of kidney slices. Hydrallazine (50 or 100 μg./ml.) depressed the oxygen uptake of brain homogenates ($P<0.02$), liver slices ($P<0.01$), kidney slices ($P<0.01$), and arterial smooth muscle ($P<0.01$). Reserpine (330 μg./ml.) depressed the oxygen uptake of brain homogenates ($P<0.01$), and liver and kidney slices ($P<0.01$). The results are shown in Tables I and II. The control solution had little or no effect upon tissue respiration in brain but caused stimulation in liver ($P<0.01$). Previous work

TABLE I

EFFECTS OF RESERPINE AND HYDRALLAZINE ON THE OXYGEN UPTAKE OF BRAIN SUSPENSIONS, LIVER AND KIDNEY SLICES

Oxygen uptake is given in μl. ± s.e./mg. wet tissue. Control solutions: see text for details.

Tissue Solution	Conc. (μg./ml.)	No. of Observations	Incubation Time (min.)		
			20	40	60
Brain					
Reserpine	10	12	0·158 (±0·0089)	0·320 (±0·0099)	0·457 (±0·01)
Control		12	0·163 (±0·005)	0·316 (±0·005)	0·46 (±0·0075)
Reserpine	50	27	0·188 (±0·006)	0·328 (±0·0095)	0·476 (±0·0107)
Control		24	0·185 (±0 0063)	0·348 (±0·0102)	0·498 (±0 013)
Reserpine	100	13	0·162 (±0·0119)	0·292 (±0·012)	0·397 (±0·07)
Control		13	0·166 (±0·008)	0·318 (±0·0097)	0·477 (±0·007)
Reserpine	330	9	0·156 (±0 0085)	0·188 (±0·0106)	0·232 (±0·010)
Control		9	0·151 (±0·005)	0·282 (±0·011)	0·386 (±0 012)
Hydrallazine	50	11	0·122 (±0·011)	0·209 (±0·015)	0·268 (±0·018)
,,	100	8	0·154 (±0·025)	0·278 (±0·017)	0·323 (±0·010)
Control		30	0·179 (±0·008)	0·362 (±0·013)	0·514 (±0·016)
Liver					
Reserpine	10	8	0·347 (±0·030)	0·550 (±0·032)	0·764 (±0·033)
Control		7	0·294 (±0·0407)	0·516 (±0·029)	0·710 (±0·035)
Reserpine	50	18	0·434 (±0·0105)	0·7947 (±0·0148)	1·10 (±0·0146)
Control		12	0·404 (±0·0081)	0·7609 (±0 0151)	1·06 (±0 019)
Reserpine	100	10	0·4938 (±0·0116)	0·898 (±0·025)	1·222 (±0·0124)
Control		10	0·5200 (±0·0119)	0·935 (±0 020)	1·316 (±0·031)
Reserpine	330	8	0·3935 (±0·0031)	0·768 (±0·0106)	1·152 (±0·001)
Control		9	0·4659 (±0 011)	0·934 (±0·009)	1·378 (±0·0086)
Hydrallazine	50	14	0·151 (±0·014)	0·263 (±0·022)	0·478 (±0·02)
,,	100	11	0·111 (±0·003)	0·216 (±0·014)	0·311 (±0·016)
Control		41	0·201 (±0·007)	0·388 (±0·012)	0·658 (±0·013)
Kidney					
Reserpine	10	12	0·657 (±0·016)	1·276 (±0·021)	1·913 (±0·0245)
Control		12	0·6212 (±0·0181)	1·219 (±0·027)	1·817 (±0 036)
Reserpine	50	9	0·6627 (±0 036)	1·271 (±0 053)	1·90 (±0·089)
Control		10	0·608 (±0·026)	1·23 (±0·0359)	1·861 (±0·0616)
Reserpine	100	12	0·639 (±0 0275)	1·24 (±0 036)	1·837 (±0·047)
Control		11	0·648 (±0·0176)	1·272 (±0·0297)	1·883 (±0 0334)
Reserpine	330	9	0·484 (±0·0087)	0·821 (±0 041)	1·389 (±0·0356)
Control		9	0·536 (±0·013)	1·05 (±0 021)	1·589 (±0·0303)
Hydrallazine	50	6	0·389 (±0·035)	0·823 (±0·040)	1·20 (±0·04)
,,	100	6	0·541 (±0·045)	1·02 (±0·045)	1·46 (±0·05)
Control		12	0·63 (±0·016)	1·18 (±0·022)	1·75 (±0·04)

TABLE II

EFFECTS OF HYDRALLAZINE ON THE OXYGEN UPTAKE OF ARTERIAL SMOOTH MUSCLE

Oxygen uptake is given in μl. ± s.e./mg. wet tissue.

Solution	Conc. (μg./ml.)	No. of Observations	Incubation Time (min.)		
			20	40	60
Phosphate medium		11	0·12 (±0·013)	0·243 (±0·026)	0·373 (±0·019)
Drug	50	6	0·059 (±0·008)	0·125 (±0·013)	0·217 (±0·014)
,,	100	8	0·078 (±0·0027)	0·117 (±0·020)	0·170 (±0 032)
Medium IIA		11	0·173 (±0·019)	0·318 (±0·020)	0·414 (±0 028)
Drug	50	6	0·185 (±0·018)	0·335 (±0·028)	0 451 (±0 041)
,,	100	5	0·164 (±0·015)	0·230 (±0·015)	0·292 (±0·016)

(Kirpekar and Lewis, 1958b) had indicated that hydrallazine inhibition of drug induced contractions of isolated artery strips could be antagonized by certain intermediates of carbohydrate metabolism. We therefore carried out some experiments using medium IIA which contains pyruvate, glutamate, fumarate, and dextrose. In the presence of this medium, hydrallazine (50 to 100 μg./ml.) only slightly inhibited the respiration of slices of arterial smooth muscle.

Adenosine Nucleotides

Tables III and IV summarize the effects of reserpine and hydrallazine upon the amounts of adenosine nucleotides in rat skeletal muscle, heart, brain, and liver. Mephenesin administration does not affect tissue adenosine triphosphate concentrations (Parker, 1954). Mephenesin-induced relaxation was partially antagonized by hydrallazine, and rats treated with hydrallazine required one and a half to twice the usual relaxant dose of mephenesin, while the time of onset of relaxation was 20 to 25 min. as against the more usual 5 to 10 min.

Neither reserpine nor hydrallazine had a significant effect upon the adenosine nucleotide content of gastrocnemius muscle or heart. In the brain, reserpine caused a significant decrease in adenosine triphosphate (ATP) ($P<0.01$) and a corresponding increase in adenosine diphosphate (ADP) ($P<0.05$). The ATP/ADP ratio which may be taken as a measure of the synthetic potential of the cell is also significantly reduced ($P<0.01$). In liver reserpine caused a significant fall in adenosine triphosphate content ($P<0.01$) and there was a corresponding increase in adenosine diphosphate ($P<0.05$); the ATP/ADP ratio and the total adenosine nucleotides were also significantly reduced ($P<0.05$). Hydrallazine had no significant effect upon liver adenosine triphosphate: on the other hand, adenosine diphosphate was significantly increased ($P<0.05$) and the ATP/ADP ratio reduced ($P<0.01$). In brain, hydrallazine reduced the adenosine triphospate level significantly ($P<0.01$), adenosine diphospate was significantly increased ($P<0.01$) and the ATP/ADP ratio was also significantly reduced ($P<0.05$).

TABLE III

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT SKELETAL MUSCLE AND HEART

All values are expressed in μmole/g. wet wt. ± s.e. ATP, ADP and AMP refer to adenosine tri-, di-, and mono-phosphate.

Group and No. of Rats	Solution and Dose (mg./100 g.)	ATP	ADP	AMP	ATP+ADP+AMP
Muscle					
1 (4)	Reserpine, 0·5	7·21 (±0·17)	0·35 (±0·19)	0·14 (±0·015)	7·52 (±0·36)
2 (4)	Hydrallazine, 5·0	6·88 (±0·33)	0·17 (±0·06)	0·05 (±0·02)	7·09 (±0·36)
3 (8)	Controls	7·57 (±0·31)	0·31 (±0·08)	0.12 (±0 05)	7·97 (±0·35)
Heart					
1 (4)	Reserpine, 0·5	3·27 (±0·35)	0·78 (±0·15)	0·60 (±0·12)	4·65 (±0·32)
2 (4)	Hydrallazine, 5·0	4·21 (±0·35)	0·79 (±0·15)	0·70 (±0·17)	5·71 (±0·17)
3 (8)	Controls	3·67 (±0·20)	1·18 (±0·17)	0·94 (±0·11)	5·79 (±0·23)

TABLE IV

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT BRAIN AND LIVER

All values are expressed in μmole/g. wet wt. ± s.e. ATP, ADP and AMP, see Table III.

Group and No. of Rats	Solution and Dcse (mg./100 g.)	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP
Liver						
1 (4)	Reserpine, 0·5 ..	0·49 (±0·05)	1·16 (±0·07)	1·18 (±0·08)	2·82 (±0·19)	0·42 (±0·03)
2 (4)	Hydrallazine, 5·0 ..	1·15 (±0·15)	1·47 (±0·19)	1·33 (±0·15)	3·95 (±0·18)	0·77 (±0·07)
3 (8)	Controls	0·91 (±0·03)	0·94 (±0·06)	1·48 (±0·09)	3·33 (±0·07)	1·00 (±0·061)
Brain						
1 (6)	Reserpine, 0·5 ..	0·68 (±0·06)	0·50 (±0·08)	0·87 (±0·05)	2·02 (±0·10)	1·40 (±0·30)
2 (5)	Hydrallazine, 5·0 ..	0·52 (±0 09)	0·55 (±0·09)	0·89 (±0·09)	1·96 (±0·07)	1·08 (±0·28)
3 (9)	Controls	1·10 (±0·06)	0·26 (±0·03)	0·90 (±0·07)	2·30 (±0·08)	4·74 (±0·88)

Discussion

Under normal conditions the oxidative metabolism of living cells and of certain isolated systems leads to the synthesis of compounds containing high energy phosphate bonds. These compounds are essential for growth, muscle contraction, nerve conduction, etc. Uncoupling agents can disturb metabolism by depressing the formation of high energy phosphate bonds without depressing or even slightly stimulating the oxygen consumption of the system (Brody, 1955).

Reserpine depresses the P/O ratio (Abood and Romanchek, 1957) in brain mitochondria—a property it shares with other uncoupling agents. On the other hand, hydrallazine depresses tissue respiration at all concentrations used, thereby indicating a general depressant effect upon the oxidative metabolism of living cells. Hydrallazine therefore appears to act differently from reserpine, which at lower doses (10 μg./ml. and 50 μg./ml.) has no significant depressant or stimulant effects.

Douglass, Dillaha, Dillaha, Janis, and Kountz (1957) have shown that hydrallazine inhibits the acetylation of sulphanilamide and glucosamine in pigeon liver extracts and have concluded that this inhibition is of a competitive nature and appears to be the result of the drug being preferentially acetylated by acetyl-coenzyme A. The acetylation of hydrallazine by acetyl - coenzyme A may ultimately mean interference in the functioning of the tricarboxylic acid cycle. Our previous finding (Kirpekar and Lewis, 1958b) that some intermediates of the tricarboxylic acid cycle antagonize the effects of hydrallazine supports this view. Our observation, in experiments on the respiration of arterial smooth muscle, that the inhibition of oxidation by hydrallazine can be overcome to a certain extent by supplying the tissue with pyruvate, glutamate, fumarate, and dextrose, also points in the same direction.

The effects of reserpine on the brain and liver adenosine nucleotides suggest interference with oxidative phosphorylation. No effect could be demonstrated in skeletal muscle or heart. It is possible that in these tissues phosphocreatine levels are affected. The accepted role of phosphocreatine is to maintain the level of adenosine triphosphate through the Lohmann reaction. Therefore, in the absence of the major synthetic source of energy-rich phosphate bonds, the loss of phosphocreatine will be the first indication of a disturbed phosphate metabolism. We are investigating this. Hydrallazine causes a decrease in the adenosine triphosphate level of the brain which is accompanied by an increase in the adenosine diphosphate level. This suggests that at any rate, in brain *in vivo*, hydrallazine also interferes with normal oxidative processes. This drug, however, had no effect on the adenosine nucleotide content in skeletal muscle, heart, or liver.

Many substances which uncouple phosphorylation from oxidation have been employed in experimental pharmacology and therapeutics. A number of antipsychotic drugs and central nervous system depressants inhibit oxidative phosphorylation: chlorpromazine and barbiturates are examples. Whether the effectiveness of these substances as drugs is dependent upon their effectiveness as uncoupling agents is not yet clear. The fact that a substance uncouples phosphorylation from oxidation does not mean that it is devoid of other actions. The relative importance of uncoupling as a mechanism of drug action differs with different drugs. Brody (1955) concludes that uncoupling is of major importance in the action of certain drugs and is at present the most attractive hypothesis, which explains, at least in part, the mechanism of action of barbiturates, antibiotics (such as gramicidin and the tetracyclines), salicylates, and thyroid hormones.

Reserpine has been shown to cause depletion of noradrenaline and 5-hydroxytryptamine from brain and other tissues (Holzbauer and Vogt, 1956; Pletscher, Shore, and Brodie, 1955). Carlsson and Hillarp (1956) have shown that the depletion of the adrenal medulla of catechol amines by morphine or insulin is accompanied by a parallel depletion of adenosine triphosphate. Depletion of this substance in rat brain may be linked with depletion of brain catechol amines. The observations of Kirpekar, Goodlad, and Lewis (1959) have recently shown that reserpine depletes adrenal medullary catechol amines and adenosine nucleotides in roughly the same proportions. There appears, however, to be a fundamental difference between the effects of reserpine on the adrenal medulla and brain since, in the former, depletion of adenosine triphosphate is not accompanied by a corresponding rise in the di- and mono-phosphates, and in the latter the fall in the triphosphate concentration is accompanied by a corresponding and expected rise in that of the diphosphate. In the case of hydrallazine, *in vitro* studies (Kirpekar and Lewis, 1957, 1958a, 1958b) have indicated a mainly peripheral site of action and have led us to

conclude that this drug is a general depressant of cell metabolism. The effect of this compound on brain adenosine nucleotide level was therefore not unexpected: but we cannot yet explain the lack of effect on skeletal muscle, heart, and liver.

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