rate as the single process in the "fast" organs. On the one hand we may consider a "free" magnesium with a turnover time of 1.2 hours and on the other hand, a "bound" magnesium with a turnover time of 25 hours.

In liver and kidney, all of the magnesium would be in the rapidly exchanging state and in skeletal muscle more than half the magnesium would be in the slowly exchanging state. The intimate association of magnesium with the contractile elements of muscle is also consistent with this proposal and it is especially noteworthy that a *stimulated* skeletal muscle shows a more rapid exchange than an unstimulated muscle. The possible storage of magnesium load in the liver, etc., is of interest but by analogy with other electrolytes the skeleton presumably contains the principal reserve of magnesium.

The decrease of specific activity in the plasma of immature rats is slightly faster than in the older rats. This is to be expected even if only from a consideration of their shorter circulation time. In the testes of immature rats initial rate of increase of r.s.a. is the same as in the older animals but it is maintained for longer so that after 7 hours the testes are 70% equilibrated rather than 50%. The implication is that the less mature testes contain more of the rapidly exchangeable magnesium. Otherwise there are no striking differences between mature and immature rats.

Summary. Mature and immature rats were injected with radioacitve magnesium and distribution of the isotope in various tissues was measured. The exchange with plasma magnesium was rapid and complete within 3 hours in liver, kidney, and heart muscle. In brain, testes, erythrocytes and skeletal muscle there was a rapidly exchanging component similar to that in liver, etc., but more than half the magnesium exchanged very slowly. The similarity between functionally disparate tissues in each of the 2 groups suggests that the magnesium is present in 2 or more physiologic states. In one state the turnover time is 1.2 hours and in the other it is 25 hours. The exchange was faster in muscles which were stimulated to contract repeatedly than in resting muscles. In immature rats uptake of radioactive magnesium by the testes was slightly faster than in the mature animals and net decay of plasma activity was faster but there were no other significant differences.

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Effect of Salicylates on Respiration and Phosphorylation in Heart Mitochondria. (24585)

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The observations that salicylate and dinitrophenol significantly increase basal metabolic rate in intact man(1-3) and animal(4-5) have been attributed to the ability of these phenols to uncouple oxidative phosphorylation(6-7). In view of the efficacy of salicylates in treatment of rheumatic fever and rheumatoid arthritis, the cardiovascular effects of salicylate-induced hypermetabolism have received some attention. Tenny and Miller(5) demonstrated in the dog that major cardiovascular adjustments to salicylates

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are increased in cardiac output and widening of arteriovenous oxygen difference. Because there was only a slight increase in pulse rate, it was assumed that the increase in cardiac output was predominantly the result of increase in stroke volume. This has recently been confirmed by Walton and Darby(8) who showed by more direct methods that salicylate injection in the dog produces a prompt increase in contractile force of heart and by Nayler(9) who observed that salicylate and dinitrophenol cause a positive ionotropic response in the isolated toad heart. Finally, studies in euthyroid(10) and myxedematous (11) human subjects have revealed that salicylate-induced hypermetabolism does not cause a significant increment in pulse rate unless toxicity is marked(12). In contrast, Brewster *et al.*(13) demonstrated that thyroid feeding in the dog produces significant increase in heart rate as well as increment in ventricular stroke work. Studies of Tenny and Miller(5) with salicylates and Brewster *et al.*(13) with desiccated thyroid are particularly noteworthy because both used dogs in which the metabolic rate was increased to a similar extent; yet only with thyroid feeding was there a significant increase in pulse rate. Moreover, Alexander and Johnson(11) found that patients with myxedema, restored to normal metabolic rate with triiodothyronine or salicylate, developed an increase in pulse rate and angina pectoris with the former but not with the latter. Thus, since thyroxine, like salicylate and dinitrophenol, is capable of uncoupling oxidative phosphorylation(14-15), the difference in cardiovascular effects of thyroxine and the 2 phenols have been interpreted to indicate that thyroid hormone increases reactivity of the cardiovascular system by some means other than or in addition to increased tissue oxygen consumption(10). It is possible, however, that salicylate is capable of uncoupling oxidative phosphorylation in peripheral tissues but not in the heart. Hence, a study of the effect of salicylate, various analogs of salicylate, and thyroxine on heart mitochondria was undertaken. It has recently been suggested(16) that uncoupling of oxidative phosphorylation is an important characteristic of agents possessing anti-inflammatory properties, it seemed important to confirm Brody's observation(6) that certain salicylate analogs with apparent anti-rheumatic properties do not uncouple oxidative phosphorylation. It was hoped that a study of uncoupling activity of substances chemically related to salicylate would indicate what characteristics were essential for uncoupling by this group of compounds.

Methods. Preparations. Heart muscle mitochondria were prepared from male rats of Sprague-Dawley strain by procedure of Cleland and Slater(17) using a sucrose (0.32 M)-versene (0.001 M) suspending medium at pH 7.5. The prepaartion was made with approximately 5 g cardiac tissue as starting material. Commercially available reagents were used as source for compounds examined. Thiosalicylic acid was recrystallized by sublimation procedures before use. Solutions of the compounds were neutralized with sodium hydroxide and then added to the reaction mixture in approximately 10 μl amounts. Oxygen consumption was measured polarographically with an oxygen electrode (Pt-Ag couple) of the "open type" described by Davies and Brink(18) using the apparatus and assay conditions previously described(19). The reaction mixture (1 ml) contained: 0.32 M sucrose, 9 mM KCl, 18 mM phosphate buffer, 1 mM versene, and 1-2 mg mitochondrial protein at pH 7.5. Other additions during experiments are indicated in the Figures. Time moves from left to right, and a downward deflection of curves indicates oxygen consumption. Numbers on curves refer to calculated rates of oxygen consumption in micromoles oxygen consumed/second/liter. Temperature was 26°C.

Results. Polarographic assay of oxidative phosphorylation and effct of DNP.‡ A control experiment with rat heart mitochondria illustrating the method for assay of respiration and oxidative phosphorylation is shown in Fig. 1. The experiment was begun by addition of aerobic suspending medium to the

‡ The following abbreviations are used: alpha-ketoglutarate, KG; adenosine diphosphate, ADP; adenosine triphosphate, ATP; 2,4-dinitrophenol, DNP.

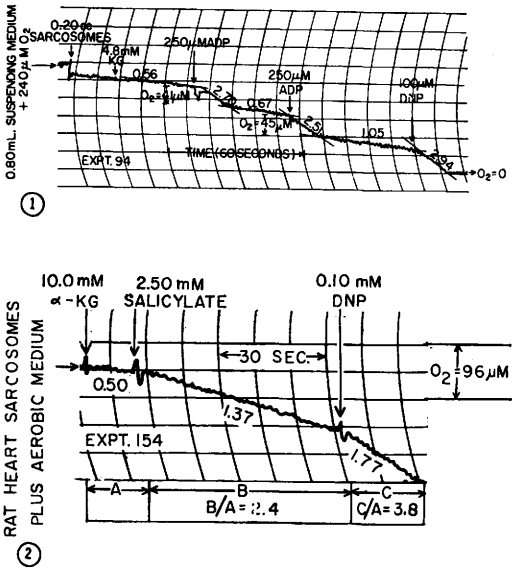

FIG. 1. Polarographic assay of respiration and oxidative phosphorylation in rat mitochondria.

FIG. 2. Respiratory stimulation caused by o-salicylate and DNP in rat mitochondria.

rotating cuvette in which the reaction was carried out. Initial oxygen concentration (240 μM) is indicated by level of recorded trace on the extreme left. An aliquot of mitochondrial suspension, sarcosomes, was then added, and the next portion of the trace shows slow rate of endogenous substrate respiration. The system was then saturated with substrate, KG, and after several seconds rate of oxygen consumption calculated from slope of curve was 0.56 μM/sec. Addition of phosphate acceptor, ADP, caused an approximately 5-fold increased rate of respiration, which proceeded linearly for about 10 seconds. After this, the original rate of KG respiration was approximated and it was presumed that the added ADP had been converted to ATP at the point where respiration declined. It can be calculated that 41 μM O_2 (or 82 micro-atoms/liter) were consumed during conversion of 250 μM ADP to ATP, giving an ADP/O ratio of 250/82 or 3.1. A second addition of ADP produced similar results, although a somewhat lower ADP/O ratio was obtained (2.8) due to small amount of ATPase activity. The presence of ATPase activity is suggested by the more rapid rate of KG respiration (1.05 μM/sec.) following second ADP addition(19). Adding multiple small doses of ADP in a single experiment permits many determinations of the efficiency as well as rate of phosphorylation. Uncoupling of oxidative phosphorylation also causes increased rate of respiration(14). The increased slope following addition of DNP in Fig. 1 indicates an approximate 5-fold increase over the initial rate of KG respiration. Exhaustion of oxygen in the reaction mixture is revealed by a horizontal trace. With this method it was possible to assess the effect of a suspected uncoupling agent on respiration several seconds after its addition to a mitochondrial suspension.

Effect of salicylates and related compounds on respiration. The procedure for testing these compounds is shown in Fig. 2. At start of experiment KG was added to the mitochondrial suspension in the reaction mixture. Respiration on KG proceeded several seconds until it was possible to calculate this initial rate of oxygen consumption (A). Salicylate was then added, and it can be seen that 2.50 mM salicylate increased the rate of linear respiration to 1.37 μM/sec. (B). Respiratory stimulation caused by salicylate, calculated from the B/A ratio, was a 2.4-fold increase. Addition of DNP caused further increase in respiration to 1.77 μM/sec. (C). Total respiratory stimulation caused by salicylate plus DNP (C/A) was 3.8-fold. Further stimulation caused by DNP in Fig. 2 indicates that the amount of salicylate added (2.50 mM) was insufficient to fully uncouple oxidative phosphorylation. Thus, by varying the concentration of a suspected uncoupler a sensitive assay is available for comparing its effect with a known uncoupling agent. With this procedure a variety of salicylates and related compounds were examined for their effect on KG respiration (Table I) in a phosphate-acceptor deficient system. The concentrations were less than that required for maximal stimulation (except for DNP). ADP was tested as control for full respiratory stimulation in the presence of phosphate-acceptor. Of monohydroxy benzoates tested, only the ortho compound stimulated respiration as shown by the B/A ratios; meta and para compounds were inactive (Table I). Acetylsalicylates produced approximately the same stimulation as sodium salicylate. Results with

TABLE I. Effect of Salicylates on Respiration of Alpha-ketoglutarate in Rat Heart Mitochondria.

(A) KG respiration, μM O_2/sec.	Second addition	Conc., mM	(B) Subsequent respiration, μM O_2/sec.	Respiratory stimulation (B/A)
.39	None		.39	1.0
.32	2-hydroxybenzoate (salicylate)	2.50	.83	2.6
.32	3- " "	"	.28	.9
.48	4- " "	"	.58	1.2
.45	2,4-dihydroxybenzoate (resorcylicate)	"	.45	1.0
.47	2,5-dihydroxybenzoate (gentisicate)	"	.47	"
.53	2,6-dihydroxybenzoate	"	.53	"
.28	3,5- "	"	.28	"
.56	2-mercaptobenzoate (thiosalicylate)	.30	4.11	7.4
.42	acetylsalicylate	1.75	.88	2.1
.39	2-hydroxybenzaldehyde (salicylaldehyde)	1.25	.56	1.4
.50	2-hydroxybenzamide (salicylamide)	2.50	.56	1.1
.35	methylsalicylate (wintergreen oil)	5.00	.26	.7
.45	salicyl ureate	2.50	.56	1.2
.34	5-sulfosalicylate	"	.32	.9
.34	benzene thiol	.10	.34	1.0
.71	phenol	.50	.87	1.2
.35	L-thyroxine	"	.88	2.5
.40	triiodothyronine	"	1.04	2.6
.70	2,4-dinitrophenol	.10	7.72	11.0
.60	adenosine diphosphate	.50	7.00	14.0
.65	benzoate	.50	.74	1.1

dihydroxy benzoates were uniformly negative; these compounds neither stimulated nor inhibited respiration at concentrations employed. Similarly salicylate derivatives such as sulfosalicylate, salicyl ureate, salicyl amide, and salicyl aldehyde showed very small or no stimulation. Methyl salicylate was inhibitory when tested at 5 mM concentration; lower concentrations were initially without effect, but as time of incubation increased, inhibition of respiration was observed. The response to thiosalicylate was considerably greater than that to salicylate. Table I also shows that some parent compounds of the salicylate and thiosalicylate, such as phenol, benzoate, and benzenethiol had no effect on respiration at concentrations used. Thyroxine and triiodothyronine readily accelerated respiration.

The effect of salicylate and thiosalicylate was investigated in more detail (Table II). With the thiol compound, a measurable stimulation of respiration can be observed with as little as a 5 μM concentration. In one case, the concentration required to give approximately the same respiratory stimulation (3.70 and 3.85 respectively) was 2.50 mM for the former and 0.10 mM for the latter. In parallel experiments it was shown that 0.25 mM thiosalicylate decreased ADP/O ratio of controls from 3.59 to 2.63, a 27% inhibition of phosphorylation. In these experiments thiosalicylate was added to ADP and the experiment performed as in Fig. 1 for ADP only. This result is in accord with the assumption that extent of respiratory stimulation by an uncoupling agent is inversely related to efficiency of phosphorylation.

Discussion. Salicylate, acetylsalicylate, and thiosalicylate increased respiration in rat

TABLE II. Effect of Salicylate and Thiosalicylate on Respiration of Alpha-ketoglutarate by Rat Heart Mitochondria.

	Respiratory stimulation (B/A)	
Conc., mM	Salicylate	Thiosalicylate
.001	1	1
.005	1	1.80
.01	1	2.10
.04	1	2.40
.10		3.85
.30		7.35
.50	1.65	11.90
1.50	2.20	
1.75	2.40	
2.50	3.70	
3.00	5.80	

heart sarcosomes whereas a variety of salicylate analogs were inactive. Hydrolysis of acetylsalicylate in alkaline solution accounts for similar activity of sodium salicylate and acetylsalicylate. Enhanced activity of thiol compound relative to phenol may be due to the more acid nature of the thiol compound. This would also explain why salicylate (o-hydroxy benzoic acid) was active and m- and p-hydroxy benzoic acids were not, since their pK's are 2.98, 4.08, and 4.58 respectively. Anthranilic acid, pK 11.3, was also inactive. On the other hand, such considerations do not adequately explain the inactivity of dihydroxy derivative resorcylic acid, which has a pK even lower than that of salicylate(20). Moreover, these conflicting results are not completely explained by studies of DeDeken(21) who showed that degree of uncoupling in yeast was correlated with several factors: mobility of the proton, compounds with low pK values being most active; a low extracellular pH, favoring undissociated form of the compound and better penetration; and high liposolubility serving to enhance penetration. Though the lesser liposolubility of the dihydroxy benzoic acids might be invoked to explain the inactivity of resorcylic acid, this seems unlikely, and additional factors such as steric hindrance, ability to activate ATPase activity, and many others may play a role.

Salicylate, like triiodothyronine, is capable of uncoupling oxidative phosphorylation in heart mitochondria, but unlike triiodothyronine, does not increase heart rate in the intact animal, suggests that thyroid hormone augments heart rate by some means other than increased tissue oxygen consumption. This is consistent with the evidence that thyroid hypermetabolism but not salicylate-induced hypermetabolism increases responsiveness of the cardiovascular system to endogenous epinephrine and norepinephrine(10).

As pointed out by Brody(6) the fact that gentisic and resorcylic acid does not uncouple oxidative phosphorylation but are apparently anti-rheumatic(22-23) is inconsistent with the suggestion that uncoupling is an essential property of an anti-inflammatory agent. Not only are there clinical studies suggesting that resorcylic acid is anti-rheumatic, but also there are animal experiments indicating that it is anti-inflammatory(22). Furthermore, dinitrophenol, a potent uncoupling agent, is not anti-inflammatory(16). Hence, the bulk of the available evidence does not support the contention that uncoupling is an essential property of an anti-inflammatory or anti-rheumatic drug.

Summary. 1) The polarographic method for measuring changes in O_2 concentration has been employed for assessing ability of salicylates and related compounds to uncouple phosphorylation. The extent of stimulation of respiration caused by addition of these compounds to rat heart mitochondria oxidizing alpha-ketoglutarate in a phosphate-acceptor deficient system was used as a criterion of uncoupling action. 2) Thiosalicylate was many times more active than salicylate and other salicylate-derivatives tested. Some chemical and physical properties possibly responsible for the uncoupling action of salicylate and certain of its congeners were considered. The *in vitro* action of salicylates was discussed in relation to their anti-rheumatic activity and cardiovascular effects.

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Effect of Prolonged Exercise on Atherogenesis in the Rabbit.* (24586)

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Morris(1) demonstrated that men who do physically active work have a lower incidence of coronary heart disease than men in sedentary jobs. The physiological basis for this finding has not been elucidated. Brown(2) found that 20 minutes of exercise/day did not inhibit atherosclerotic plaque formation in rabbits fed a high cholesterol diet, while Kobernick(3) noted that 5 minutes of exercise/day did inhibit cholesterol deposition as measured chemically in the aortae of rabbits fed cholesterol. Using young cockerels fed a high cholesterol diet, Orma(4) showed that mild exercise, possibly through its effect on the thyroid gland, inhibited atherogenesis and lowered serum cholesterol levels. The present study is designed to evaluate the effect of more prolonged exercise on cholesterol concentration in serum, aorta, muscle, liver and skin and on aortic plaque formation in rabbits fed a high cholesterol diet.

Method. Male American-Dutch rabbits 6 months old were fed a diet consisting of commercial rabbit pellets to which 0.3% of cholesterol by weight was added by dissolving in ether, mixing with the pellets, then allowing ether to evaporate. One-half of the animals were exercised daily by placing them in a revolving drum 3 feet diameter and 6 feet long. The drum was rotated by motor at a speed just sufficient to keep the animals moving with an automatic timing device which allowed alternating periods of rest and exercise every 15 minutes. All rabbits were fed high cholesterol diet and water *ad lib.* The exercise group received nothing by mouth during the 8 hours in the machine. After a few days of familiarization, the exercise group tolerated the forced activity well. Blood was drawn by cardiac puncture for cholesterol determination after 1 month, and at 2 months when experiment was terminated. The aortas were examined grossly and transverse sections through the entire circumference were taken from the proximal ascending aorta for tissue cholesterol determination. Tissue from liver, thigh muscle, and abdominal skin was similarly evaluated. Cholesterol levels of serum and tissue were determined by the method of Herrmann(5). The aortas were removed in continuity from aortic valve to the bifurcation and grossly graded for plaque formation.

Results. The results (Table I) revealed little difference between the 2 groups. Statistical analysis demonstrated a "p" value of less than 5% in only one comparative measurement—average serum cholesterol level at end of experiment. Average serum cholesterol of rabbits fed a plain diet was 59 mg %. The values of 903 mg % and 501 mg % for the exercise and non-exercise groups, respectively, on a high cholesterol diet were markedly elevated. The difference between means was significant ($p = .0001$).

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