82. The Effect of Salicylate and Cinchophen on Enzymes and Metabolic Processes

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The value in rheumatism therapy of salicylic acid was recognized as early as 1876 and cinchophen (phenylcinchoninic acid; atophan) was introduced in gout treatment in 1908. Several of the many clinical observations are of great interest to the biochemist, e.g. the effect of these substances on the excretion of uric acid, their ability to promote the secretion of bile etc. More recent chemical studies were mainly concerned with attempts to isolate from urine products of salicylate detoxication such as salicyluric acid and salicylglucuronide. Chemical work on cinchophen dates back to the time of Skorczewski & Sohn [1911] and Dohrn [1912], who isolated various oxidation products of cinchophen from human urine.

However, information is far from complete with regard to the actual mechanism in which salicylate and cinchophen exert their beneficial influence on the diseases of the joint. Continuing the work on problems connected with the tissues which make up the joint [Lutwak-Mann, 1940; 1941], a study was made of the effect of salicylate and cinchophen, first on the *in vitro* activity of isolated tissues and enzyme systems, and secondly on certain metabolic processes in the intact animal (rat).

A. EXPERIMENTS IN VITRO

Respiration and anaerobic acid production

Rat liver and kidney cortex, in the form of slices, extracts and pulp, were used in experiments which were carried out at 38° in Barcroft differential manometers for the measurement of the O₂-uptake and the anaerobic acid production. The tissue slices were suspended in Ringer-phosphate pH 7.2 for the measurement of respiration and in Ringer-bicarbonate for the CO₂-evolution due to anaerobic acid production. The results recorded below give values (μl./0.2 g. fresh tissue) for the first 20–30 min. when the reactions proceeded at a steady rate; but readings were always continued for another 30–60 min. Salicylate and cinchophen (Na salt), as well as certain benzoic acid derivatives other than salicylic acid, were added in various concentrations to tissue slices respiring either alone or in the presence of certain substrates. Table 1 shows that *M*/100 salicylate had little effect on the O₂-uptake of the liver slices; *M*/100 cinchophen had a slight inhibitory effect which increased markedly at *M*/50 concentration. In *M*/20 salicylate the respiration was well below the control and *M*/10 salicylate caused a considerable inhibition. This effect, however, varied from one animal to another. Table 1 gives also results obtained with *M*/10 benzoate, *o*-aminobenzoate (anthranilate) and *o*-cresotinate. Of these, only *o*-cresotinate inhibited the O₂-uptake to a degree comparable with that of salicylate, while the others had little effect. On the other hand, *M*/12 cinchophen almost completely stopped the respiration of liver slices.

Experiments of this kind were repeated with livers from starved rats and also from rats which received an excess of glucose for one or more days before the experiment, in addition to their ration. With *M*/10 salicylate it was found that the respiration in the starved tissue slices was more affected than either in the normal or the glycogen-rich slices (Table 1).

The addition to the respiring slices of glucose, lactate, dihydroxyacetone or acetaldehyde (all of which were oxidized by the liver slices), did not alter the degree of inhibition

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Table 1. *Effect on the respiration of liver slices*

	O ₂ -uptake μl. O ₂ /30 min.		O ₂ -uptake μl. O ₂ /30 min.
Control	240	Na cinchophen <i>M</i> /100	190
Na salicylate <i>M</i> /100	248	Na cinchophen <i>M</i> /50	120
Na salicylate <i>M</i> /60	198	Na cinchophen <i>M</i> /12	36
Na salicylate <i>M</i> /20	162	Na benzoate <i>M</i> /10	200
Na salicylate <i>M</i> /10	120 (normal rat)	Na anthranilate <i>M</i> /10	210
Na salicylate <i>M</i> /10	99 (starved rat)	Na <i>o</i> -cresotinate <i>M</i> /10	110
Na salicylate <i>M</i> /10	136 (glucose-fed rat)	Na <i>p</i> -aminobenzoate <i>M</i> /10	268

due to *M*/10 salicylate or *M*/20 cinchophen; when, however, pyruvate was added to the slices, then the inhibition caused by salicylate was somewhat smaller than in the absence of pyruvate.

Kidney cortex slices were on the whole more sensitive than liver to salicylate and cinchophen, but not to benzoate, anthranilate or *p*-aminobenzoate. It may be added that several experiments were carried out with anthranilate and *p*-aminobenzoate to see if they were oxidized and/or deaminated by the rat liver or kidney, with negative results.

Extracts from liver were made by grinding the tissue at 0° with Ringer-Na₂HPO₄ solution (1 part tissue : 2 parts Ringer-phosphate); the residue was removed by centrifugation and the supernatant was used for the experiments. The O₂-uptake of these extracts decreased rapidly after the initial 20–30 min. unless cozymase and nicotinic acid amide were added. Salicylate *M*/60–*M*/30 had relatively little effect, but cinchophen decreased the O₂-uptake at *M*/80–*M*/50 concentration; higher concentrations of salicylate and cinchophen caused a considerable decrease in the O₂-uptake of the extracts. The degree of inhibition was the same whether cozymase and nicotinic acid amide were added or not. The addition of various substrates to the respiring extract made no difference to the inhibitory effect of strong salicylate or cinchophen, except for pyruvate, the presence of which slightly diminished the extent of the salicylate inhibition. The addition of strong methylene blue increased the initial stage of respiration in the liver extracts, and in the presence of this carrier *M*/10 salicylate had a slightly less pronounced effect. When *M*/100 KCN was added to the extract together with methylene blue, then the small 'KCN-resistant' O₂-uptake was completely unaffected by *M*/10 salicylate but was still further depressed by *M*/20 cinchophen. Experiments with liver extracts are given in Table 2.

Table 2. *Effect on the O₂-uptake in liver extract and pulp*

Extract (cozymase and nicotinic acid amide added)	O ₂ -uptake μl. O ₂ /30 min.
Control	292
Na salicylate <i>M</i> /30	200
Na salicylate <i>M</i> /10	108
Cinchophen <i>M</i> /20	95
Control with 1.5 mg. methylene blue	318
Na salicylate <i>M</i> /10 with 1.5 mg. methylene blue	272
Control with 1 mg. methylene blue and KCN <i>M</i> /100	78
Na salicylate <i>M</i> /10 with 1 mg. methylene blue and KCN <i>M</i> /100	70
Na cinchophen <i>M</i> /20 with 1 mg. methylene blue and KCN <i>M</i> /100	32
Pulp	
Control	174
Na salicylate <i>M</i> /100	78
Na salicylate <i>M</i> /20	52
Na cinchophen <i>M</i> /100	53
Na cinchophen <i>M</i> /20	32

A preparation from rat liver which was much more sensitive to salicylate and to cinchophen than either the slices or the extracts, was made by grinding the liver rapidly

(1–2 min., 0°) with one volume of phosphate pH 7.4 containing equal molar amounts of K^+ and Na^+ . The pulp obtained in this way respired fairly vigorously during the first 30 min. and its O_2 -uptake was considerably diminished in the presence of $M/100$ salicylate and still more so, in the presence of $M/100$ cinchophen (Table 2).

Extracts and pulp from rat kidneys behaved towards salicylate and cinchophen like the liver preparations except that their O_2 -uptake was lower.

On several occasions Na acetylsalicylate (Na aspirin) was used instead of salicylate. However, with this substance added to either liver or kidney slices or other preparations, the pH fell within a short time to less than 5.0. This was found to be due to a very rapid hydrolysis of acetylsalicylate by these tissues. Table 3 gives results of the enzymic hydrolysis of Na aspirin by liver and kidney as well as by other animal tissues and fluids and also by yeast juice.

Table 3. *Enzymic hydrolysis of Na acetylsalicylate (Na aspirin)*

N_2 95 % + CO_2 5 %; $NaHCO_3$ 0.5 %. 38°. Acetylsalicylate $M/10$.

	Acid production $\mu l. CO_2/15$ min.			Acid production $\mu l. CO_2/15$ min.	
	Control	Acetyl-salicylate		Control	Acetyl-salicylate
Liver slices	22	198	Stomach mucosa	4	36
Liver extract	15	256	Serum	0	12
Kidney cortex slices	18	167	Milk (cow)	0	28
Brain slices	12	88	Yeast juice	28	48

The effect of acetylsalicylate upon the initial stages of the respiration in liver slices was usually less than with a corresponding amount of salicylate; however, in the later stages, when the control respiration began to decrease, the tissue sample containing acetylsalicylate showed a gradual increase in O_2 -uptake while the inhibition due to salicylate continued (Table 4). It was thought at first that the phenomenon observed with acetylsalicylate was due to an oxidation of acetic acid, but it was found that acetate added to liver slices was rather poorly oxidized.

Table 4. *Effect of Na salicylate and acetylsalicylate on the O_2 -uptake of liver slices and extract*

	O_2 -uptake, $\mu l.$			
	0–20 min.		50–70 min.	
	Slices	Extract	Slices	Extract
Control	178	120	110	60
Salicylate $M/10$	90	45	45	25
Acetylsalicylate $M/10$	100	77	126	98
Cinchophen $M/20$	78	30	36	15

The anaerobic acid production in liver slices was tested manometrically; it was not affected by $M/10$ salicylate, and with $M/20$ cinchophen there was some inhibition (Table 5). The tissues of starved and glucose-fed animals were also examined, but the results as regards anaerobic acid production were the same as with the tissue of normally fed animals.

Table 5. *Effect of Na-salicylate and cinchophen on the anaerobic acid production in liver slices*

N_2 95 % + CO_2 5 %; 0.5 % $NaHCO_3$.

	Acid production $\mu l. CO_2/30$ min.
Control	138
Salicylate $M/10$	126
Cinchophen $M/20$	102

The formation of conjugated glucuronides

It was of considerable interest to test the ability of the rat liver to form in vitro, conjugated glucuronides from salicylate and from cinchophen as well as from certain benzoic acid derivatives other than salicylic acid. Following the technique of Lipschitz & Bueding [1939] for the quantitative estimation of ether-soluble glucuronides, it was first established that rat-liver slices readily formed a glucuronide from *d*-borneol, provided that a very low concentration of this alcohol was used; *l*-menthol was found to be conjugated to a much smaller degree, and occasionally negative results were obtained with menthol, even in the presence of pyruvate, lactate and dihydroxyacetone, all of which, according to Lipschitz & Bueding, enhance the conjugation.

No data exist for the properties of salicylglucuronide, but it may be assumed that it is ether-insoluble like the glucuronide of benzoic acid [Quick, 1926]. Using the method of the authors quoted above, the glucuronic acid estimations were done both in the ether extract from the tissue slices which respired in the presence of the examined substances, as well as in the ether-extracted residue. In later experiments the time-consuming ether extraction was given up and the Tollens test was carried out either (1) directly in the trichloroacetic or tungstic acid extract from the liver slices, or else (2) the protein-free acid extract was brought to pH 6.5 and neutral Pb acetate was added avoiding excess; the precipitate was discarded, the supernatant made slightly alkaline with NaOH, and basic Pb acetate was added to complete precipitation; after the removal of Pb from the precipitate, this fraction was used for the estimation of glucuronic acid by means of the Tollens test [Mozołowski, 1940].

Negative results for glucuronic acid were obtained from liver slices which respired in various concentrations of salicylate; in a few experiments, however, with *M*/50 and *M*/80 salicylate, a feeble and unstable positive Tollens test was obtained. Much time was spent in attempting to establish optimum conditions for the conjugation of salicylate, but in the end the conclusion seemed unavoidable that the ability of the rat liver to form a conjugated glucuronide from salicylate is negligible. Negative results were also obtained when using livers from rats which were fed for several days on small amounts of salicylate.

Negative results were also obtained with cinchophen. On the other hand, however, a positive Tollens test was invariably obtained when liver slices respired in the presence of small amounts of benzoate, anthranilate, *m*- and *p*-hydroxybenzoate and *p*-amino-benzoate; however, no satisfactory quantitative measurements could be made by the Tollens test.

Isolated enzyme systems

Indophenol oxidase was prepared from washed and ground heart muscle (pig), and its activity was tested manometrically by measuring the O₂-uptake in presence of *p*-phenylenediamine and cytochrome *c*. The addition of salicylate weaker than *M*/20 had little effect. *M*/10 salicylate caused an appreciable diminution of the initial O₂-uptake, but during the next 10–20 min., when the activity of the control began to decrease, the O₂-uptake gradually rose in the salicylate sample till it reached the control value, and finally, some 40 min. from the onset of the experiment, the O₂-uptake in presence of salicylate was more than twice the control value (Table 6).

With Na cinchophen the indophenol oxidase activity was inhibited at *M*/50 and still more at *M*/20 concentration, both in the early and in the late stage of the reaction (Table 6). It should be added that salicylate and cinchophen were by themselves not oxidized by minced heart or skeletal muscle.

Dehydrogenases: succinic, lactic, malic and α -glycerophosphoric dehydrogenases were tested by means of the Thunberg technique using Latapie-minced and thoroughly washed skeletal muscle as the source of enzymes. With salicylate weaker than *M*/20 little effect was obtained on any of these dehydrogenases; *M*/50 cinchophen caused a pronounced

Table 6. *Effect of Na salicylate and cinchophen on the activity of indophenol oxidase and succinic oxidase*

	O ₂ -uptake, μ l. O ₂				O ₂ -uptake, μ l. O ₂		
	0-10 min.	10-20 min.	40-50 min.		0-10 min.	10-20 min.	40-50 min.
Indophenol oxidase				Succinic oxidase			
Control	292	198	28	Control	221	144	56
Salicylate <i>M</i> /10	132	152	78	Salicylate <i>M</i> /10	119	132	99
Cinchophen <i>M</i> /20	100	78	20	Cinchophen <i>M</i> /20	93	36	17

inhibition, and *M*/20 almost completely prevented the activity especially of the α -glycerophosphoric dehydrogenase (Table 7).

The activity of the succinic oxidase system was also tested manometrically in the presence of cytochrome *c*. The initial O₂-uptake was fairly strongly inhibited by *M*/10 salicylate; in the later stages of the reaction the degree of inhibition diminished, the O₂-uptake gradually increased and in the end it surpassed the control value. No such 'late' effect was observed with cinchophen which inhibited the oxidation of succinate throughout the whole experimental period (Table 6). It is hoped to find an explanation for the effect of salicylate on respiring minced muscle, in the course of future research.

Table 7. *Effect on the activity of muscle dehydrogenases*

1 ml. methylene blue *M*/1000; 38°.

	Time of reduction, min.				
	Control	Salicylate <i>M</i> /10	Cinchophen <i>M</i> /20	Benzoate <i>M</i> /10	Anthranilate <i>M</i> /10
No substrate	120	∞	∞	120	120
No substrate + cozymase	45	180	∞	60	48
Lactate + cozymase	3	7	60	3	4
Malate + cozymase	7	13	75	8	10
Succinate	8	19	28	10	10
α -Glycerophosphate	18	60	∞	22	30

Xanthine oxidase activity was tested by means of the Thunberg technique using a preparation from milk [Dixon & Thurlow, 1924]. It was found that *M*/10 salicylate caused considerable inhibition, but a much weaker effect was achieved with equally strong cinchophen; benzoic and anthranilic acid had scarcely any effect, *o*-cresotic acid inhibited the enzyme. Even with *M*/100 salicylate there was a decrease in the xanthine oxidase activity (Table 8). When *M*/10 salicylate was allowed to act on the enzyme preparation for 1 hr. and was then removed by dialysis, the enzyme activity was fully restored. Cinchophen inhibition was also found to be reversible.

Table 8. *Effect on the activity of milk xanthine oxidase*

2 ml. 4% 'casein' preparation; 1 ml. methylene blue *M*/1500; 38°.

	Time of reduction min.		Time of reduction min.
Control	3	Cinchophen <i>M</i> /10	12
Salicylate <i>M</i> /100	7	Benzoate <i>M</i> /10	3
Salicylate <i>M</i> /60	9	Anthranilate <i>M</i> /10	5
Salicylate <i>M</i> /10	32	<i>o</i> -Cresotinate <i>M</i> /10	29
Cinchophen <i>M</i> /60	6		

Several attempts were made to test (manometrically) the oxidation of xanthine in rat-liver slices, but with little success. When, however, liver pulp was used instead of slices, it was possible to obtain a small positive O₂-uptake with xanthine as substrate. Salicylate inhibited the uptake due to xanthine to the same extent as the control respiration.

The *dismutation between hexosediphosphate and pyruvate* was examined manometrically as described by Green, Needham & Dewan [1937], using a muscle acetone powder or

fresh skeletal muscle extract as the source of enzymes. Cinchophen, $M/50$ to $M/12$, had comparatively little effect, but salicylate decreased the extent of dismutation even at $M/100$ and stopped the reaction at $M/10$ concentration. Benzoate and anthranilate also showed an inhibitory effect but only when used in high concentration (Table 9). When Embden ester was used as substrate for the acid production instead of hexosediphosphate + pyruvate, salicylate seriously inhibited the reaction even in low concentration. The results with salicylate were the same whether muscle acetone powder or an extract from fresh skeletal muscles served as the source of enzymes. Experiments were also carried out using muscle extract prepared from rats injected with salicylate; they are described below (Section B).

Table 9. *The dismutation between hexosediphosphate and pyruvate in presence of salicylate and cinchophen*

Extract from muscle acetone powder. N_2 95 % + CO_2 5 %; $NaHCO_3$ 0.5 %; 38°.

	Acid production $\mu l.$ $CO_2/20$ min.		Acid production $\mu l.$ $CO_2/20$ min.
Control	192	Cinchophen $M/12$	120
Salicylate $M/100$	87	Benzoate $M/10$	92
Salicylate $M/10$	19	Anthranilate $M/10$	76
Cinchophen $M/50$	172		

Bone phosphatase activity was examined using aqueous-toluene extracts from the bones of young rats. The following substrates were used and their dephosphorylation tested in the presence of salicylate and cinchophen: adenylic acid, inosinic acid, hexosediphosphoric acid, Cori ester, Neuberger ester, Embden ester, β -glycerophosphoric acid. All of them were very rapidly dephosphorylated by the rat bone extract, but the course of the reactions was in no way affected by either salicylate or cinchophen. Similar results were obtained by Lutwak-Mann [1940] with calf articular cartilage as the source of phosphatase. Experiments with phosphatase from the bones of rats injected with salicylate are described below (Section B).

Other enzymes examined and found not to be much affected by salicylate and cinchophen were: choline esterase (serum), tyramine oxidase (liver), methylglyoxalase (liver), adenylic deaminase (muscle), carbonic anhydrase (laked blood corpuscles). Two plant enzymes, the horse-radish peroxidase and catechol oxidase from mushrooms, were found to be resistant to both salicylate and cinchophen. Enzymes in this group were also tested in the presence of benzoate, anthranilate, *p*-aminobenzoate, *o*-cresotinate, *m*- and *p*-hydroxybenzoate, but their activity was not influenced by these substances.

Yeast enzymes. Some experiments were made with fresh bakers' yeast, dried brewers' yeast and a purified carboxylase preparation from yeast-juice [Green, Herbert & Subrahmanyam, 1941]. The fermentation and oxidation of glucose by fresh bakers' yeast was found to be only very slightly inhibited by $M/10$ salicylate when the reaction was carried out at pH 7.0–7.2. But at pH 6.5 both processes were quite considerably inhibited by $M/10$ salicylate (Table 10). Weaker salicylate had little effect regardless of pH . Acetyl-salicylate was deacetylated by fresh yeast as well as by yeast extracts. $M/10$ cinchophen

Table 10. *Oxidation and fermentation of glucose by bakers' yeast in presence of salicylate and cinchophen*

	O_2 -uptake $\mu l.$ $O_2/20$ min.		CO_2 -production in N_2 $\mu l.$ $CO_2/20$ min.	
	pH 6.5	pH 7	pH 6.5	pH 7
Control	258	298	297	212
Salicylate $M/10$	94	212	106	190
Cinchophen $M/10$	12	28	0	0

completely prevented the fermentation and oxidation of glucose by bakers' yeast (Table 10), and weaker solutions caused a large inhibition. The effect of both salicylate and cinchophen on fresh yeast was reversible: when they were removed from the yeast by washing, its activity was found to be normal.

Decarboxylation of pyruvate was found to be considerably inhibited by $M/10$ salicylate and cinchophen; benzoate and *o*-aminobenzoate, $M/10$, also depressed the reaction though to a smaller extent. Low concentration of salicylate still caused a slight inhibition (Table 11). The results with carboxylase and salicylate were the same, using as the source of enzyme either dried brewers' yeast, cell-free juice from bakers' yeast or a purified enzyme preparation. In the presence of citrate instead of phosphate buffer the inhibitory effect was somewhat less. When the rates of glucose fermentation and pyruvate decarboxylation were compared using the same dried brewers' yeast, it was found that carboxylase activity was more affected by $M/10$ salicylate than the CO_2 production from glucose.

Table 11. *Effect on the activity of yeast carboxylase*

Carboxylase preparation from bakers' yeast juice, 2 mg. dry weight. 30° .

	CO_2 -production $\mu\text{l. CO}_2/5 \text{ min.}$		CO_2 -production $\mu\text{l. CO}_2/5 \text{ min.}$
Control	308	Cinchophen $M/10$	172
Salicylate $M/100$	248	Benzoate $M/10$	256
Salicylate $M/10$	38	Anthranilate $M/10$	222

B. EXPERIMENTS ON THE WHOLE ANIMAL

Young male rats (150–250 g.) received subcutaneous injections of salicylate, Na cinchophen and also of benzoic acid derivatives other than salicylate, in doses varying from 1.0 to 0.1 mg./g. body weight. The following observations were made concerning the behaviour of the injected animals. Salicylate: the dose 1.0 mg./g. was tolerated only if the animals were given an excess of glucose (5–10 % solution substituted for water) in addition to their normal diet during one or more days preceding the experiment; otherwise this dose caused a 50 % mortality. Although there were individual variations in the response of the rats to large amounts of salicylate, yet their behaviour after the injection was very typical. About 4 hr. after the injection, but seldom before, the rats showed various degrees of distress, from drowsiness and muscular limpness to vomiting, muscular twitches and severe convulsions. The bladder sphincter was frequently paralysed; this resulted in a continuous drip of urine and a brown staining of the moist abdominal coat (*vide infra*). This was the most critical period and in fatal cases death occurred at that time. The glucose-fed animals survived this stage but showed little improvement in the next few hours. Usually from the 7th hr. onwards the condition gradually improved and 24 hr. later most animals appeared fully recovered. Occasionally the recovery was delayed and did not take place till 30–40 hr. after the injection.

Doses of salicylate below 1.0 mg./g. were comfortably tolerated. Among the benzoic acids used for injection experiments, *o*-cresotinate was found to be too toxic when injected (1.0 mg./g.) and invariably caused death from convulsions within 1 or 2 hr.; *p*-cresotinate was decidedly less toxic. Benzoate, anthranilate, *m*- and *p*-hydroxybenzoate, did not affect the animals when injected 1.0 mg./g.

Na cinchophen, 1.0 mg./g., was tolerated by most rats and these showed sleepiness and dragging of hind legs; but there were some animals which died from violent convulsions 4–6 hr. after the injection. In these infrequent cases the urine excreted was almost colourless instead of showing the 'claret' colour typical for urine after well-tolerated cinchophen treatment.

The following routine was adopted for the injection experiments. Several rats of similar weight and age were given extra glucose in addition to their food for a day or two

before the experiment proper. In some experiments glucose was discontinued after the injections, but usually the animals had 3% glucose as their drink in addition to their usual food. The injected animals were placed in metabolism cages for urine collection. At 4, 7 and 24 hr. intervals after the injection the animals were killed and various organs were removed for analysis.

Feeding, instead of injections, of salicylate and cinchophen was attempted but was abandoned because the animals refused food and drink which contained more than 20–40 mg. per day of these substances. On the other hand, they did not object to the addition in their food of benzoate or anthranilate up to 0.3 g. per day.

The enzymic activity of tissues after injections

(a) *The respiration of liver slices* was measured manometrically at various intervals after the injection of salicylate and compared with the activity of liver slices from untreated animals. No striking changes were observed with doses ranging from 0.1 to 1.0 mg./g., regardless of whether the O_2 -uptake was measured in the presence or absence of glucose, lactate, dihydroxyacetone or xanthine. In some of these experiments, however, a small increase in respiration was noticeable in the liver slices obtained 4 hr. after the injection of salicylate; inhibition was not observed at any stage. A similar slight rise was occasionally registered 4 hr. after the injection of Na cinchophen (1.0 mg./g.). Otherwise there were no changes in the O_2 -uptake even in the livers of very distressed animals. Table 12 gives the results of these experiments. Benzoate 1.0 mg./g. had no effect at 4 hr., but in some experiments there was a small increase in the respiration 7 hr. after the injection.

Table 12. O_2 -uptake in liver slices from rats injected with salicylate and cinchophen (1 mg./g.)

Substrate	Untreated rat	$\mu l. O_2/20 \text{ min.}$		
		Time after injection hr.	Rats injected	
None	211	4	With salicylate	With cinchophen
		7	239	222
		24	220	258
Lactate	240	4	218	220
		7	278	256
		24	246	260
Dihydroxyacetone	312	4	237	230
		7	320	
		24	325	
Glucose	300	4	310	
		7	336	
		24	316	
			293	

Several experiments of this kind were done with rats maintained on a B_1 -deficient diet (in the early stage of deficiency). The control liver slices of these rats oxidized dihydroxyacetone rather poorly in contrast to normal animals; after the injection of salicylate the liver slices failed altogether to oxidize dihydroxyacetone. The slight increase in respiration 4 hr. after salicylate injection was noticeable in most B_1 -deficient rat livers; in addition there was often a similar rise in the livers from rats killed 24 hr. after the injection.

(b) *The dismutation of hexosediphosphate and pyruvate.* Extracts were made from the skeletal muscles, 4, 7 and 24 hr. after the injections and their activity compared with that of extracts from untreated animals. These experiments were repeated several times but no difference was found between the activity of the 'salicylate' and the 'control' muscle extracts.

(c) *The dephosphorylation of various substances by bone extract.* Four young rats (90 g.) were injected with salicylate (1.0 mg./g.) and killed 6 hr. later, when their bones were removed and an aqueous extract was prepared from them; the bone extract from four litter mates served as control. The bone phosphatase from the salicylate-treated rats was slightly but distinctly less active than the control towards adenylic acid, hexosediphosphoric acid, Neuberg ester and β -glycerophosphoric acid, but not towards Cori ester or inosinic acid.

In other experiments older animals were used and the activity of the bone extract was examined at 4, 7 and 24 hr. intervals after salicylate injection; however, in these experiments the phosphatase activity showed no change as compared with the control.

Liver glycogen

The measurement of respiration in liver slices after the injections was accompanied by estimations of liver glycogen by Pflüger's method (2-3 g. tissue). To obtain control values for liver glycogen an untreated rat's liver was analysed at the onset of the experiment while a second control animal was killed simultaneously with the last (24 hr.) injected rat. While the respiration of liver slices after salicylate and other substances differed but slightly from the control, liver glycogen showed striking behaviour in response to the various substances examined.

Salicylate caused an almost complete disappearance of glycogen from the liver 4-7 hr. after the injections; but 24 hr. later the glycogen was found to be fully restored to control level and moreover, in many experiments, it surpassed the control values by 30-50%. Results such as these were best obtained with full doses of salicylate, but they were also observed with 0.5 and 0.2 mg./g. and, also, regardless of whether glucose was fed or discontinued during the actual experiment. There were, however, animals the livers of which had low glycogen values 24 hr. after the injection of salicylate; this happened invariably in rats which continued to show signs of distress for more than 24 hr. after the injection (Table 13).

Owing to the fairly high mortality rate it was not practicable to carry out salicylate injection experiments on starving animals, but data were collected for rats which did not receive additional glucose before the experiment. Here, the 4 hr. values for glycogen were low and they did not improve 24 hr. later (Table 13).

Table 13. *Behaviour of liver glycogen after injection of salicylate and cinchophen.*
Normal and B₁-deficient rats

		% glycogen in liver				
		Injected rats, time after injection				
	Exp. no.	Dose injected per g. body wt.	4 hr.	7 hr.	24 hr.	Untreated rats*
Normal rats						
Glucose added to diet	1	—				2.98
		1 mg. salicylate	0.38	0.11	4.42	
	2	—				3.82
		1 mg. salicylate	0.08	0.04	1.22	
No glucose added	3	—				3.86
		0.3 mg. salicylate	0.19	0.46	5.80	
	4	—				1.52
		0.8 mg. salicylate	0.12	0.26	0.66	
B ₁ -deficient rats						
Glucose added to diet	5	—				2.12
		0.7 mg. salicylate	0.10	0.26	0.98	
Normal rats						
Glucose added to diet	6	—				4.92
		0.8 mg. Na cinchophen	0.92	1.58	3.33	

* Killed 24 hr. after the beginning of the experiment.

B₁-deficient rats which prior to the experiment consumed large amounts of glucose, showed a nearly complete disappearance of liver glycogen soon after the salicylate injection, and although glucose was given all the time, not much more glycogen was found in the livers at the 24 hr. interval (Table 13).*

The behaviour of liver glycogen after injection of 1.0 mg./g. of Na cinchophen was as follows: a fall in the glycogen content after 4 hr. but seldom as drastic as after salicylate, no change or a slight increase after 7 hr. and a return to almost control level 24 hr. later. Never was the control level at this stage exceeded by the glycogen content of the cinchophen-treated animal. In a few cases cinchophen caused a substantial loss of liver glycogen soon after the injection and the corresponding urine sample contained glucose (see later).

To compare the effect of salicylate with that of other benzoic acids, experiments were carried out with aspirin, benzoate, anthranilate, *o*-cresotinate and *m*-hydroxybenzoate. Acetylsalicylate, 0.8 and 0.2 mg./g., was injected to rats fed on glucose; the injection was well tolerated. The loss of glycogen after the larger dose was slow and reached its maximum 24 hr. after the injection; the smaller dose resulted in the disappearance of liver glycogen 7 hr. after the injection, but on the next day there was some increase (Table 14). *o*-Cresotinate, owing to its toxicity, could only be injected in small doses (0.6 mg./g.); very little glycogen was found in the livers during 24 hr. after the injections. After 1.0 mg./g. of anthranilate comparatively little glycogen was lost from the liver after 4 hr., more disappeared during the next few hours, but never as much as after a similar dose of salicylate; on the next day the glycogen was largely, but not fully, restored to control level. With *m*-hydroxybenzoate there was a gradual and progressive loss of glycogen throughout the 24 hr. period. Benzoate caused a fall in glycogen up to 7 hr. after the injection; 24 hr. later there was an increase, but the values were still below the control (Table 14).

Table 14. *The effect of various derivatives of benzoic acid on liver glycogen*

Exp. no.	Dose injected per g. body wt.		% glycogen in liver			
			Injected rats, time after injection			Untreated rats
			4 hr.	7 hr.	24 hr.	
1	Acetylsalicylate	0.8 mg.	1.77	1.40	0.35	2.70
		0.3 mg.	0.76	0.09	0.68	
2	Benzoate	1 mg.	1.23	0.28	2.66	3.85
3	Anthranilate	1 mg.	3.00	0.82	3.12	5.00
4	<i>o</i> -Cresotinate	0.6 mg.	0.72	0.04	0.27	3.70
5	<i>m</i> -Hydroxybenzoate	0.8 mg.	1.40	0.92	0.63	5.00

At this stage the behaviour of glycogen in the presence of salicylate was examined in liver slices. However, no significant changes were obtained with the slices when glycogen and ferricyanide-reducing sugar were estimated at various intervals of incubation with different concentrations of salicylate.

Blood

The above-described behaviour of liver glycogen in the rat led one to expect changes in the *blood-sugar* level. Estimations were carried out, using the method of Hagedorn & Jensen [1923], and the results of one of these experiments are in Table 15. No marked changes were found at any time after the injection of salicylate which would reflect those

* The KOH-hydrolysates from B₁-deficient rat livers frequently gave a green-coloured glycogen precipitate on addition of alcohol. This was not due to salicylate treatment, as it occurred also in the livers of the untreated deficient animals.

Table 15. *Glucose and lactic acid content in blood after injection of salicylate (0.8 mg./g.)*

	mg. % glucose normal rats	mg. % lactic acid	
		Normal rats	B ₁ -deficient rats
Untreated rats	115	20	25
Rats injected with salicylate			
Time after injection { 4 hr.	110	18	27
7 hr.	124	18	22
24 hr.	120	23	21

which occurred under such conditions in the liver glycogen. *Lactic acid* was estimated by the method of Friedemann, Cotonio & Shaffer [1927]; no striking differences were found either in normal or in B₁-deficient rats in response to salicylate (Table 15). *Pyruvate* was estimated by the method of Bueding & Wortis [1940] in rats injected with acetyl-salicylate. The 4 and 7 hr. values in the injected animals were lower than the control blood pyruvate, but the 24 hr. values were equal to the control. The colorimetric estimation of the 2:4-dinitrophenylhydrazone was made inaccurate by the appearance of a yellowish colour in the acetylsalicylate samples. *Blood phenol* estimations were carried out after the injections of salicylate, benzoate and cinchophen using the method of Theis & Benedict [1924] but were rendered difficult owing to the small amount of blood available from single rats. It was, however, possible to observe a considerable rise in the phenol values in blood, which persisted beyond 24 hr. after benzoate and salicylate injection. No increase was seen after cinchophen treatment.

Urine

General properties. Samples of urine from injected and untreated rats were collected for analysis at the 4, 7 and 24 hr. intervals. The volume, pH, colour and presence of albumen in the urine were recorded. Salicylate in doses 1.0–0.8 mg./g. invariably caused the excretion of large amounts of pale, almost colourless urine; polyuria was sometimes observed also after much smaller doses, but the colour remained normal. The increased urine excretion continued for a day or two after the injection. The 0–4 hr. samples after large doses of salicylate were alkaline, pH 7.6–8.0, and were found to contain bicarbonate. Small doses did not cause bicarbonate excretion. Between 4 and 7 hr. a much more acid urine was excreted irrespective of the dose, and from then onwards the pH varied between 6.5 and 6.9. Salicylate always caused albuminuria regardless of the amount injected; albumen was present for several days after a big dose. The test for salicylic acid with FeCl₃ was positive after the injection from the earliest moment. It was still positive 5–6 days following a large dose and 2–3 days after smaller doses.

Cinchophen injections were followed by the excretion for 2–3 days of 'claret'-coloured urine, there were no changes in the pH, and only sometimes the volume was increased. Albuminuria was rare. The urine gave the following reactions: it turned yellow on addition of conc. HCl, gave a precipitate with phosphotungstic acid, and turned green on addition of ammonium sulphate + NH₃.

Benzoate (1.0 mg./g.) caused the excretion of copious urine of normal colour; early samples were alkaline and contained some bicarbonate; there was no albuminuria. Cresotates, *o*- and *p*-, were followed by polyuria and very slight albuminuria. There were no changes in volume, colour or pH after *p*- and *m*-hydroxybenzoate. Urine excreted after *p*-aminobenzoate showed a distinct yellow colour in the early samples.

Urine excreted after the injection of anthranilate was mostly of normal colour; the pH of the early samples varied from 7.0 to 7.8. On standing, this urine sooner or later turned red-brown. The colour first developed on the air-exposed surface and gradually pervaded the whole sample; this change occurred also below pH 7.0 though not very readily.

Only the early samples after anthranilate injection showed this spontaneous darkening in contact with air. The length of excretion of anthranilate itself was followed by observing the fluorescence it gave in ultraviolet light. Its presence in urine could be detected in this manner several days after the injection of 1.0 mg./g.

Reduction of Benedict's reagent and ammoniacal Ag. Behaviour towards alkalis. The behaviour of liver glycogen after the injection of salicylate and cinchophen and the other substances used in these experiments, suggested the possibility of sugar excretion in urine. Accordingly, all urines were tested using Benedict's qualitative reagent, and the following observations were made. Positive reduction results were obtained after large doses of salicylate in the 0-7 hr. samples; 7-24 hr. urine showed only a trace of reduction, and later samples did not reduce the reagent at all. Polarimetric readings in strongly reducing samples revealed dextrorotation. After the incubation of such urine with fresh bakers' yeast at pH 6.5 the reduction was very largely diminished, but it did not disappear completely (Table 16). Only in one case was laevorotation recorded in salicylate urine; in this sample the reduction after fermentation with yeast remained unchanged. Reduction results in urine excreted after small doses of salicylate are also given in Table 16.

Cinchophen urine very seldom reduced Benedict's reagent. In a few experiments there was reduction in the early sample. The sugar, presumably glucose, showed dextrorotation and was fermentable. The excretion of urine such as this always coincided with a serious loss of liver glycogen.

Early urine samples after the injection of benzoate, *m*- and *p*-hydroxybenzoate, or *o*- and *p*-aminobenzoate, all strongly reduced the copper reagent. All of them showed laevorotation which was particularly conspicuous in the anthranilate urine. The reduction remained unchanged after fermentation with yeast. Doses of *o*-cresotinate of 0.5-0.7 mg./g. caused glycosuria (Table 16). Similar reduction results were obtained both in normal and B₁-deficient rats.

Table 16. *Reduction of Benedict's reagent. Polarimetric readings*

α_D values are for $l = 1$ dm.

Injection	Time of urine excretion								
	0-4 hr.			4-7 hr.			7-24 hr.		
	Reduction		Rotation α_D	Reduction		Rotation α_D	Reduction		Rotation α_D
	Before ferment.	After ferment.		Before ferment.	After ferment.		Before ferment.	After ferment.	
Salicylate, 1 mg./g.	+++	Trace	+1.09°	+	Trace	0	Trace	Trace	0
Salicylate, 0.1 mg./g.	Trace	Trace	0	+	Trace	0	0	0	0
Cinchophen, 1 mg./g.	+++	0	+0.22°	0	0	0	0	0	0
Benzoate, 1 mg./g.	+++	++	-0.28°	++	++	-0.08°	Trace	Trace	0
Anthranilate, 1 mg./g.	++++	+++	-1.42°	+++	++	-0.78°	+	Trace	Trace
<i>o</i> -Cresotinate, 0.7 mg./g.	++	Trace	+0.08°	+	+		Trace	Trace	0
<i>m</i> -Hydroxybenzoate, 1 mg./g.	+++	+++	-0.29°	++	++		0	0	0
<i>p</i> -Hydroxybenzoate, 1 mg./g.	+++	+++	-0.13°	+	+		0	0	0
<i>p</i> -Aminobenzoate, 1 mg./g.	+++	++	-0.15°	Trace	Trace		0	0	0

When anthranilate urine was added to Benedict's reagent at room temperature the colour changed to a dark blue which gradually deepened to a blackish brown and on further standing Cu₂O was formed. This spontaneous reduction occurred only with early samples, but its extent varied greatly from one experiment to another; it bore no relation to the colour of the urine itself. The test suggested the presence of a strongly reducing substance and, indeed, it was found that this urine was capable of reducing ammoniacal Ag in the cold. It was then noticed that the fluid above the AgO precipitate became pink to purple on standing. Subsequently it was established that all early samples of urine, after the injection of anthranilate, gave a pink to purple colour on addition of NH₃ (or pyridine) in the presence of air. The colour developed and vanished with varying rapidity in

different samples. The test was positive in urine up to 10 hr. after the injection, but some rats excreted the substance responsible for the alkali test for a longer time.

Other urines were then tested both as regards reduction of Ag and the behaviour towards alkalis. It was found that in addition to anthranilate, salicylate, benzoate, *m*-hydroxybenzoate and *o*-cresotinate, all caused the excretion of a strongly Ag-reducing urine. But only salicylate, and to a much smaller extent, *o*-cresotinate, caused the presence in urine of substances which gave a dark colour on addition of alkali. In the presence of NH_3 (not more than 5 %) salicylate urine slowly turned a light red-brown colour which was stable for hours. A more typical test was obtained if *N* NaOH was added (0.5 ml./2 ml. of urine) and the sample was allowed to stand without shaking; a dark brown colour began to show on the air-exposed surface within 20–30 min., and after a few hours the whole sample turned dark brown. This colour was stable for days, then it began to fade from the surface downwards, and after a further few days the urine was again almost its original colour. The urine after injection of anthranilate did not give a colour with *N* NaOH, but only with NH_3 or pyridine. Also alkapton urine (obtained by feeding of tyrosine to rats) did not behave towards NaOH in the manner described for salicylate urine. Except for *o*-cresotinate, which was sometimes followed by the excretion of traces of the alkali-susceptible substance, other benzoic acids used in this study, as well as cinchophen, failed to show a positive test with alkalis.

Attempts are being made to isolate and identify the substances responsible for the tests described above. As yet it has been established that they are not precipitated with Hg or Pb acetates and that they are resistant to mild acid hydrolysis at 100°. The dark pigment formed was found to be readily soluble in acid amyl alcohol (but not in ether, benzene or ethyl alcohol), whence it could be brought into aqueous solution by successive washings with dilute Na_2CO_3 . The pigment solution showed no absorption band in the visible spectrum; it was not fluorescent. Traces of hyposulphite caused a reversible change of colour from red-brown to yellow, weak H_2O_2 completely destroyed the colour.

When describing the behaviour of the rats after the injection of large doses of salicylate it was mentioned that the abdominal coat, continually moistened with urine owing to incontinence, showed a brown staining. It seems highly probable that this colour was due to the same substance in urine which in vitro turned dark on addition of alkali.

Quantitative estimation of reduction in urine. To follow in a quantitative manner the excretion of the reducing substances in the various urines, use was made of the 2:6-dichlorophenol indophenol titration as applied to ascorbic acid [Birch, Harris & Ray 1933]. The urine was diluted (1 : 4) and acidified with trichloroacetic acid, and the amount of dye per titration was 0.05 ml. (1.0 mg. dye/ml.) which corresponded to 0.02 mg. of ascorbic acid. Care was taken to carry out the titration as soon as enough urine was collected at a given time, but it was found that when an estimation was repeated several hours later, the titration values hardly differed from those obtained at first. It was also found that while with salicylate urine the results were the same whether the titration was carried out in acid or at the pH of the urine itself, it was necessary to acidify the anthranilate urine as otherwise very low titration values were obtained.

It is by no means certain that the same reducing substance is excreted in urine after the injection of the various substances studied. The substance in the urine after salicylate injection behaves like ascorbic acid, but the same cannot be said about, for example, cinchophen urine, which is also strongly reducing (see below). However, until the reducing substances are finally isolated and identified it is proposed, for the sake of simplicity, to express the quantitative results obtained as mg. ascorbic acid.

Table 17 gives the results for the reduction in urine in response to various doses of salicylate. It can be seen that all three doses caused a considerable increase in the titratable reduction, but the figures for the whole experimental period (24 hr.) clearly show that there is proportionality between the amount of reducing substances excreted and

the dose injected. In addition, it can be seen that the large dose was followed by polyuria. The high *pH* in the early sample after 1.0 mg./g. salicylate was due to the presence of bicarbonate.

Table 17. *Estimation of reduction in urine by means of 2:6-dichlorophenol indophenol titration, after various doses of salicylate*

Salicylate injected per g. body wt.	Time of urine excretion												Ascorbic acid excreted during 24 hr. mg.
	0-4 hr.				4-7 hr.				7-24 hr.				
	Vol. ml.	pH	Ascorbic acid, mg.*	NaOH-test	Vol. ml.	pH	Ascorbic acid, mg.*	NaOH-test	Vol. ml.	pH	Ascorbic acid, mg.*	NaOH-test	
—	0.5	6.0	0.016	—	1.0	6.0	0.019	—	5.5	6.5	0.049	—	0.084
1 mg.	8.0	7.9	1.900	+	4.0	6.7	0.433	+++	16.0	6.5	1.092	++	3.425
0.6 mg.	6.0	7.7	0.624	+	1.8	6.5	0.240	++	4.5	6.5	0.420	+	1.284
0.1 mg.	4.0	6.9	0.520	Trace	1.6	6.7	0.110	+	7.0	6.2	0.200	+	0.830

* Total ascorbic acid in volume excreted (also in other Tables).

More experiments of this kind are presented in Table 18. Here, rats were used which did not receive extra glucose, as well as animals which were starved for 20 hr. before the injections. In addition, there are results obtained with B_1 -deficient rats fed on glucose before and during the experiment. The data illustrate well the large individual variations in response to the same dose of salicylate, both as regards the reduction and the strength of the alkali test. The deficient animals did not differ essentially from the normal ones, except that the *pH* of the early sample failed to show the rise typical for these samples in normal animals. The same is true of rats which were starved before the experiment.

Table 18. *Reduction in urine after injection of salicylate (0.5 mg./g.) in normal and B_1 -deficient rats*

	Time of urine excretion											
	0-4 hr.				4-7 hr.				7-24 hr.			
	Urine ml.	<i>pH</i>	Ascorbic acid, mg.	NaOH-test	Urine ml.	<i>pH</i>	Ascorbic acid, mg.	NaOH-test	Urine ml.	<i>pH</i>	Ascorbic acid, mg.	NaOH-test
Normal rats												
Ordinary diet:												
Not injected	2.0	6.8	0.042	—	1.0	6.8	0.008	—	6.0	6.7	0.102	—
Injected	3.5	7.8	0.123	Trace	2.0	6.9	0.100	++	12.0	6.6	1.062	+
Injected	2.0	7.7	0.224	+	1.0	6.6	0.320	+	8.0	6.4	0.910	Trace
Starved 20 hr.:												
Not injected	1.5	6.8	0.060	—	1.5	6.8	0.021	—	7.0	6.6	0.190	—
Injected	4.0	7.0	0.200	Trace	2.0	6.4	0.200	+	10.0	6.6	1.000	+
Injected	4.0	6.8	1.022	+	2.0	6.2	0.321	++	6.0	6.3	0.320	Trace
B_1 -deficient rats												
Glucose added to diet:												
Not injected	1.0	5.6	0.011	—	1.0	5.6	0.009	—	4.5	5.5	0.044	—
Injected	2.0	7.0	0.400	+	1.0	6.8	0.110	+	6.5	5.8	0.300	+
Injected	1.5	6.7	0.360	+	1.0	6.8	0.360	++	9.0	6.8	0.210	+
Injected	1.5	6.8	0.170	+	3.0	6.5	0.720	+++	4.5	6.5	0.610	+

Several experiments were carried out injecting rats with benzoate, *m*- and *p*-hydroxybenzoate, *o*- and *p*-aminobenzoate. Table 19 illustrates the results obtained in urine after the injection of 0.65 mg./g. of these substances to rats fed on glucose. A rise in *pH* followed the treatment with benzoate and anthranilate. There was a distinct increase in the reducing power of urine in all, except the urine excreted after the injection of *p*-aminobenzoate. Individual variations as regards reduction were found in response to anthranilate. One may add that the three anthranilate urines differed also in colour, only urines 1 and 2 were brownish; the third one turned brown only after 2 days' standing at 0°.

Table 19. *Reduction in urine after the injection of benzoate, anthranilate, m-hydroxybenzoate, p-hydroxybenzoate, p-aminobenzoate (0.65 mg./g.)*

Injection	Time of urine excretion											
	0-4 hr.				4-7 hr.				7-24 hr.			
	Urine ml.	pH	Ascorbic acid, mg.	NH ₃ -test	Urine ml.	pH	Ascorbic acid, mg.	NH ₃ -test	Urine ml.	pH	Ascorbic acid, mg.	NH ₃ -test
None	1.0	6.0	0.016	—	0.8	6.0	0.019	—	5.5	6.5	0.019	—
Benzoate	3.0	7.5	0.180	—	3.5	6.4	0.350	—	17.0	6.6	0.204	—
Anthranilate 1	2.0	7.6	0.250	++	1.5	6.8	0.150	++	4.0	5.8	0.180	—
Anthranilate 2	1.5	7.5	0.270	+	2.0	6.5	0.684	+	8.0	6.0	0.382	Trace
Anthranilate 3	3.0	7.0	0.362	Trace	1.5	6.5	0.490	+	3.0	6.0	0.150	—
m-Hydroxybenzoate	4.0	6.6	0.640	—	1.0	6.5	0.024	—	3.0	6.7	0.102	—
p-Hydroxybenzoate	6.0	6.7	0.102	—	1.0	6.9	0.020	—	7.5	6.9	0.099	—
p-Aminobenzoate	2.0	7.0	0.056	—	1.0	6.6	0.040	—	2.5	6.6	0.032	—

Results obtained with Na cinchophen as well as with *o*- and *p*-cresotinate are given in Table 20. The large doses of cinchophen and *o*-cresotinate proved too toxic and the animals died 5 hr. after the injections. The smaller dose of cinchophen was followed by the excretion of 'claret'-coloured urine which was strongly reducing not only in the early but also in the later stages of the experiment. Exceptionally high titration values were obtained in this experiment after the injection of 0.6 mg./g. of *o*-cresotinate. *p*-Cresotinate urine also reduced the dye much more strongly than normal urine.

Table 20. *Reduction in urine after the injection of Na cinchophen, o-cresotinate and p-cresotinate*

Dose injected per g. body wt.	Time of urine excretion											
	0-4 hr.				4-7 hr.				7-24 hr.			
	Urine ml.	pH	Ascorbic acid, mg.	NaOH-test	Urine ml.	pH	Ascorbic acid, mg.	NaOH-test	Urine ml.	pH	Ascorbic acid, mg.	NaOH-test
None	3.0	6.9	0.095	—	2.0	6.8	0.098	—	9.0	6.7	0.072	—
Cinchophen 1 mg.	6.0	7.1	1.140	—	Rat died							
Cinchophen 0.6 mg.	2.0	6.0	0.420	—	4.0	6.0	0.620	—	22.0	6.3	1.720	—
<i>o</i> -Cresotinate 1 mg.	5.0	6.7	0.475	—	Rat died							
<i>o</i> -Cresotinate 0.6 mg.	4.0	7.0	2.00	Trace	3.0	6.6	0.928	+	28.0	6.4	2.00	Trace
<i>p</i> -Cresotinate 0.8 mg.	2.0	7.0	0.90	—	8.0	6.6	0.442	—	19.0	6.6	0.679	—

While the work on the identification of the reducing substances in the various urines is being continued, it seems worth while to report briefly certain experiments which may help to throw light on the nature of these substances. Two urines were examined, one obtained after the injection of salicylate, the other after anthranilate treatment. To both the procedure of Emmerie & Eekelen [1934] was applied. When the urines were separated with Hg acetate into two fractions, one corresponding to the Hg precipitate, the other to the Hg filtrate, it was found that the filtrate fraction from salicylate urine was invariably more strongly reducing than the insoluble fraction. Exactly the opposite was found in anthranilate urine where the insoluble Hg fraction reduced more strongly than the soluble one.

Other experiments designed to yield more information as to the character of the reducing substances were set up in the following manner. The urine examined (1 ml.) was shaken in air at 20° in Barcroft manometers in the presence of 20 μ g. Cu⁺⁺. Urine without Cu⁺⁺ served as control. A small O₂-uptake was observed in the presence of Cu in salicylate and *o*-cresotinate urine. Table 21 contains the titration results obtained with fresh and Cu-treated urines, expressed in ml. diluted and acidified urine required to reduce 0.05 mg. 2:6-dichlorophenol indophenol. The control and the salicylate urine showed a fall in their reducing power; cinchophen and *o*-cresotinate urine remained unchanged after the exposure to air in presence of Cu.

Table 21. *Effect of air-exposure in presence of Cu⁺⁺ on reducing power of urine*

1 ml. urine (acidified and diluted 1:4) required to reduce 0.05 mg. 2:6-dichlorophenol indophenol.

	Before Cu treatment	After Cu treatment		Before Cu treatment	After Cu treatment
Normal urine	2.2	2.9	Cinchophen urine	1.3	1.4
Salicylate urine	1.1	2.2	<i>o</i> -Cresotinate urine	0.3	0.36

Behaviour of the alkali test. Data on the behaviour of the salicylate, anthranilate and *o*-cresotinate urines towards NaOH and NH₃ have been included in the tables together with the reduction results. After the injection of salicylate the test was positive from a very early moment regardless of the dose injected. It was still positive 24 and 32 hr. later. On the whole, the test with NaOH appeared in the urine for nearly as long as the FeCl₃ test for salicylic acid. In anthranilate urine it was positive only during the first 4–10 hr. and seldom later. After the injection of *o*-cresotinate the test was weakly positive or entirely negative.

An attempt was made to measure manometrically the amount of O₂ taken up by salicylate urine in the presence of *N* NaOH. To 1 ml. urine 0.2, 0.5 and 1 ml. *N* NaOH were added. There was a slow O₂-uptake which continued for 20 hr. at 20° when 72 μl. O₂ were used up by the sample with most NaOH and a little less by the other two. There was no O₂-uptake in the presence of NaOH by normal rat urine.

The behaviour of reducing substances in liver and other tissues. The increased reduction by the urine excreted after the injection of salicylate suggested the possibility of changes in the reducing substances in the tissues of the injected animal. An experiment was carried out using three rats injected with salicylate (1.0 mg./g.) and a control animal, all of whom had glucose before and during the experiment. The livers of these rats were examined at the 4, 7 and 24 hr. intervals for their glycogen content, and the rest of the tissue was used for an estimation of the reducing substances following the procedure of Hopkins & Morgan [1936]. The tissue was ground with sand and trichloroacetic (8%) + metaphosphoric acid (2%), 20 ml. acid mixture per 10 g. tissue, then water was added to make a 2.5% final acid concentration. One part of the acid protein-free filtrate (corresponding to half the weight of tissue) was titrated with *M*/100 I₂ in the presence of KI till a distinct yellow colour was reached; the end-point was established by means of a back-titration with *M*/100 Na₂S₂O₃. The rest of the acid filtrate was used for titration with 2:6-dichlorophenol indophenol in the usual way. The results in Tables 22 and 23 are given as mg. glutathione (GSH) and mg. ascorbic acid per g. tissue wet weight, and in Table 23 also in ml. *M*/100 I₂ per g. tissue. The GSH values were arrived at by converting the ascorbic acid titration into the iodine value and deducting it from the total iodine figure obtained by the titration with *M*/100 I₂ (1 ml. *M*/100 I₂ = 3.07 mg. GSH).

The results obtained in this way for liver are given in Table 22. In comparison with the control, there was a slight rise in the GSH content 4 and 7 hr. after the injection of salicylate and an almost twofold increase in the GSH content after 24 hr. The level of ascorbic acid remained unchanged throughout the experiment. The liver glycogen showed the usual behaviour, a fall during the first 4–7 hr. and an increase beyond the control value, 24 hr. after the injection.

Table 22. *Estimation of reducing substances and glycogen in liver after salicylate injection (1 mg./g.)*

	Time after injection of salicylate	GSH mg./g.	Ascorbic acid mg./g.	Glycogen g./100 g.	Time after injection of salicylate	GSH mg./g.	Ascorbic acid mg./g.	Glycogen g./100 g.
Control	—	1.20	0.23	3.10	7 hr.	1.53	0.20	0.04
	4 hr.	1.50	0.24	0.12	24 hr.	2.11	0.23	4.90

Several more experiments of this kind were carried out with remarkably similar results as regards the behaviour of the GSH fraction in liver. In other experiments the reducing substances were also determined in the kidneys and in the intestine, not only after salicylate, but also after *o*-cresotinate injection (0.6 mg./g.). The results are given in Table 23. The liver showed the notable rise in the GSH content 24 hr. after both salicylate and *o*-cresotinate. In kidney and intestine there was a distinct rise in the GSH values 4 and 7 hr. after the salicylate injection; the ascorbic acid values at these intervals also exceeded the control, but after 24 hr. there was no further increase and all values were almost completely equal to those found in the untreated rat. The amounts of the reducing substance excreted by each animal are also recorded in Table 23.

Table 23. *Reducing substances in liver, kidney, intestine and urine, after salicylate and o-cresotinate injections (0.6 mg./g.)*

	Liver			Kidney			Intestine			Urine mg. ascorbic acid excreted during exp. period
	M/100 I ₂ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	M/100 I ₂ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	M/100 I ₂ ml./g.	Ascorbic acid mg./g.	GSH mg./g.	
Control	0.71	0.30	1.50	0.40	0.19	0.71	0.37	0.20	0.66	0.38/10 ml.
After injection of salicylate										
4 hr.	0.85	0.30	1.77	0.55	0.22	0.88	0.60	0.33	0.98	1.76/16 ml.
6 hr.	6.76	0.22	1.49	0.51	0.23	0.78	0.58	0.35	0.83	3.42/18 ml.
24 hr.	1.10	0.23	2.46	0.44	0.19	0.78	0.36	0.18	0.64	2.8/24 ml.
After injection of <i>o</i> -cresotinate										
5 hr.	0.76	0.27	1.32	0.43	0.22	0.85	0.36	0.28	0.74	0.805/7 ml.
24 hr.	1.80	0.32	2.02	0.44	0.23	0.69	0.44	0.29	0.63	2.22/30 ml.

The effect of ascorbic acid on the excretion of the alkali-susceptible substance after the injection of salicylate and on the excretion of homogentisic acid in artificial alkaptonuria

The behaviour of salicylate urine towards alkalis bore a certain resemblance to that of homogentisic acid in alkaptonuria. Since no real alkapton urine was available an attempt was made to induce alkaptonuria artificially by feeding rats with an excess of tyrosine (1–2 g. daily). The experiment was successful, and shortly after the feeding of tyrosine was started the urine gave distinctly positive tests for homogentisic acid (transient blue in acid ether extract with FeCl₃, red-brown colour on standing with NH₃, reduction of alkaline copper reagents; in addition, homogentisic acid was identified as dibenzoyl homogentisamide [Papageorge & Lewis, 1938].

The alkapton urine differed from the salicylate urine in that unlike the latter it did not darken in the presence of *N* NaOH but gave a dark colour only with NH₃. Without, however, attaching too much weight to purely qualitative tests, a further difference between these two urines was shown to exist when the effect was studied of treatment with ascorbic acid, (a) on the excretion of the 'alkapton'-like substance in salicylate urine, and (b) on the excretion of homogentisic acid in alkaptonuria.

Table 24 gives the results of the following experiment: rats (140 g.) were given injections of (1) ascorbate 0.6 mg./g., (2) ascorbate 0.4 mg. + salicylate 0.6 mg./g., and

Table 24. *Effect of ascorbic acid on urine excretion after salicylate injection*

Dose injected per g. body wt.	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
	Urine ml.	pH	Ascorbic acid, mg.	NaOH- test	Urine ml.	pH	Ascorbic acid, mg.	NaOH- test	Urine ml.	pH	Ascorbic acid, mg.	NaOH- test
None	1.0	6.5	0.068	—	1.0	6.3	0.080	—	4.0	6.3	0.079	—
Ascorbate 0.6 mg.	5.0	5.2	49.0	—	2.0	5.5	5.70	—	8.0	6.3	0.490	—
Ascorbate 0.4 mg. + salicylate 0.6 mg.	3.0	7.0	26.0	Trace	8.0	6.8	2.40	+	30.0	6.9	2.85	++
Salicylate 0.6 mg.	5.0	7.1	1.10	+	11.0	6.6	0.44	+	20.0	6.3	0.60	++

(3) salicylate 0.6 mg./g. Urine collection was started 20 hr. before the injections so as to establish the reducing power of each urine (values ranged from 0.008 to 0.015 mg. ascorbic acid, the volumes from 9 to 11 ml., pH was 6.9). Glucose was given only the night before the experiment. It follows clearly from the results presented in Table 24 that in spite of the large dose of ascorbic acid injected, the substance which causes the NaOH test to appear continued to be excreted in response to salicylate. In complete contrast to this all tests for homogentisic acid were negative when ascorbic acid was added to the tyrosine diet of an alkaptonuric rat. The full details of this experiment are given below.

The properties of urine after tyrosine feeding. In addition to the qualitative tests for homogentisic acid, quantitative estimations of reducing power were carried out on the urine of a rat receiving daily 1–2 g. tyrosine with his diet. The titration against 2:6-dichlorophenol indophenol was done in exactly the same manner as with other urines in this study. The results obtained during a 14 days' feeding experiment are given in Table 25. The reduction values are expressed as ml. diluted and acidified urine required to reduce 0.05 mg. dye. The estimations were run twice daily, on the overnight urine and again in the evening.

Traces of alkapton were noticeable already $1\frac{1}{2}$ days after the start of the experiment. During the 3rd day alkaptonuria was fully developed, and in the course of the next few days the qualitative tests became gradually stronger. At the same time, however, the general condition of the rat deteriorated, it developed light-shyness due to keratitis and its paws and tail were remarkably pink. The appetite was not affected. A large amount of glucose solution was substituted for water on the 8th day, but the only effect it had was polyuria, while the tests for alkapton continued positive. On the 11th day the rat received 40 mg. of ascorbic acid in addition to the usual tyrosine dose. The urine tested 14 hr. later gave negative results with FeCl_3 and NH_3 . The feeding of ascorbic acid was continued for 2 more days, and although during this time the rat consumed 4 g. of tyrosine, there was no alkaptonuria.

The titration results with 2:6-dichlorophenol indophenol were of considerable interest. From the moment when the first traces of alkapton appeared in the urine, the titratable reduction began to rise until it reached a fairly high and steady level. When, however, the large amounts of ascorbic acid were given, there was no further increase in urine reducing power as would be the case with a normal animal. It looked as if the rat retained

Table 25. *Effect of tyrosine feeding on urine excretion in rat.*
Effect of ascorbic acid on alkaptonuria

Tyrosine feeding duration	ml. urine (acidified and diluted 1:4) to reduce 0.05 mg. indophenol	FeCl_3 -test	NH_3 -test	Reduction of Benedict's reagent
0–18 hr.	3.80	—	—	—
18–24 hr.	3.50	—	—	—
24–48 hr.	3.00	Trace	Trace	Trace
3rd day	1.10	+	+	+
4th day	0.45	++	+	+
5th day	0.30	+++	+	++
6th day	0.60	+++	+	++
7th day	0.66	++	+	++
8th day	0.40	+++	+	++
Rat given glucose (7 g./40 ml.)				
9th day	0.72	+++	+	+
10th day	0.28	+++	+	+
11th day	0.63	+++	+	++
Rat given ascorbic acid (2 × 20 mg.)				
12th day	0.42	—	—	—
13th day	0.80	—	—	—
14th day	0.26	—	—	—

the huge doses of ascorbic acid, perhaps owing to the loss of reducing substance from the body caused by the feeding of tyrosine.

Further interesting results were obtained when an attempt was made to estimate colorimetrically, by the method of Briggs [1922], homogentisic acid in the tyrosine urine, and the alkapton-like substance in salicylate urine. For colorimetric estimation 0.5 ml. samples were used of (a) two salicylate urines, and (b) tyrosine urine. Their titratable reduction was 0.65 and 1.00 ml. for the salicylate urines, and 0.72 ml. for the tyrosine urine (diluted and acidified), against 0.05 mg. dye. To all three samples 2 ml. molybdate and 2 ml. KH_2PO_4 were added; the final volume was made up with water to 25 ml. The standard solution containing hydroquinone (1 mg./ml.) was treated in the same manner and standards ranging from 0.2–20 mg./25 ml. hydroquinone were prepared (1 mg. corresponds to 0.79 mg. homogentisic acid). The blue-green colour which developed in a few min. in the two salicylate urines matched 0.2 and 0.4 mg. hydroquinone/25 ml. The tyrosine urine, however, on addition of molybdate turned a deep yellow which quickly changed into olive green, and when phosphate was added a dark blue colour was obtained which exceeded in intensity 20 mg. hydroquinone/25 ml. It must be pointed out that the strength of the qualitative tests for homogentisic acid in this urine was not such as to suggest a very high content of homogentisic acid, and it seems probable that yet another reducing substance in this urine was responsible for the results obtained with molybdate and phosphate.

To gain information about the character of the reducing substance in tyrosine urine as compared with salicylate urine and also with urine which contained added ascorbic acid, an experiment was set up similar to that described above. The following urine samples were titrated before and after exposure to air, in the presence of Cu-salicylate urine, alkapton urine, urine from a rat which was fed excess of ascorbic acid, and normal rat urine to which ascorbic acid had been added. Table 26 gives the titration results expressed as ml. diluted and acidified urine required to reduce 0.05 mg. dye. All urines, except the alkapton urine, showed a substantial fall in the reducing power after the treatment with Cu.

Table 26. *Effect of air-exposure in presence of Cu^{++} on reducing power of urine*

Sample	ml. urine (acidified and diluted 1:4) to reduce 0.05 mg. indophenol	
	Before treatment	After treatment
Salicylate urine	1.00	2.80
'Alkapton' urine	0.30	0.50
Urine after feeding excess ascorbic acid	1.00	3.20
Normal urine + added ascorbic acid	0.42	2.20

Experiments with salicylic aldehyde

It was shown by Dixon & Lutwak-Mann [1937] that liver contains two enzymes capable of converting in vitro an aldehyde into an acid; the aldehyde oxidase which was found to act equally well on aliphatic and aromatic aldehydes, and the mutase which acted readily on aliphatic but only very poorly on aromatic aldehydes. It was interesting to examine the effect in vivo of salicylic aldehyde and to compare the results obtained with those established for salicylic acid.

In the preliminary experiment two rats fed on glucose were given 0.8 mg./g. of salicylic aldehyde (aqueous emulsion pH 7.0). In spite of the comparatively large dose there were no signs of distress, and the animals looked decidedly better than after a similar dose of salicylate. Table 27 contains the results on urine excretion. The earliest sample was alkaline and of yellow colour (due to the presence of salicylic aldehyde which turns yellow

above pH 7.0), there was no albuminuria, no increase in the reducing power, and the test with *N* NaOH was negative in one rat but weakly positive in the other. However, the urine excreted later showed an increase in reduction and a fully developed alkali test in both rats. There was a further rise in reduction in the 10–24 hr. urine samples, but the test with NaOH was weaker. Albumen was present only in the later samples.

Table 27. *Urine excretion after the injection of salicylic aldehyde (0.8 mg./g.)*

Injection	Time of urine excretion											
	0–4 hr.				4–7 hr.				7–24 hr.			
	Urine ml.	pH	Ascorbic acid, mg.	NaOH-test	Urine ml.	pH	Ascorbic acid, mg.	NaOH-test	Urine ml.	pH	Ascorbic acid, mg.	NaOH test
None	2.5	6.9	0.069	—	0.5	7.0	0.085	—	2.0	7.0	0.092	—
Salicylic aldehyde 1	4.0	8.0	0.092	—	3.0	6.9	0.300	++	15.0	6.9	0.750	++
Salicylic aldehyde 2	4.0	8.0	0.098	Trace	4.0	7.0	0.600	+++	17.0	6.9	1.360	++

In another experiment three rats received salicylic aldehyde (0.8 mg./g.) and three others salicylate (0.8 mg./g.), while two of the same group were kept as controls. All had extra glucose before the start of the experiment. Liver glycogen was estimated 4, 7 and 24 hr. after the injections. Table 28 gives the results obtained for liver glycogen and urine excretion. It illustrates the difference in the response of the rat towards salicylate and salicylic aldehyde as regards the reducing power of urine, albuminuria and the strength of the NaOH test. The usual results were obtained for liver glycogen after the injections of salicylate, i.e. exhaustion of glycogen in the 4–7 hr. period followed by recovery on the next day. The change due to the aldehyde after 4–7 hr. was less pronounced, but the recovery after 24 hr. was almost as good as after salicylate itself.

Table 28. *Comparison between the effect of salicylate and salicylic aldehyde (0.8 mg./g.) on urine excretion and on liver glycogen*

Exp.	Substance injected	Time of urine excretion hr.	Urine					Liver glycogen %
			Vol. ml.	pH	Ascorbic acid, mg.	NaOH-test	Albumen	
1	Salicylate	0–4	5.0	8.0	1.120	+	+	0.40
	Salicylic aldehyde	0–4	2.0	8.0	0.035	Trace	—	1.60
2	Salicylate	0–7	8.0	7.8	1.760	+	+	0.80
	Salicylic aldehyde	0–7	4.0	7.0	0.320	+	—	0.80
3	None	0						5.00
	None	0–3½	2.0	6.9	0.092	—	—	
	None	3½–18	5.0	5.9	0.109	—	—	
	None	18–24	3.0	6.9	0.088	—	—	4.10
	Salicylate	0–3½	5.0	7.9	0.650	+	+	
	Salicylate	3½–18	12.0	6.8	1.20	++	+	
	Salicylate	18–24	4.0	5.2	0.160	++	+	4.70
	Salicylic aldehyde	0–3½	6.0	8.0	0.130	+	—	
	Salicylic aldehyde	3½–18	12.0	5.7	1.400	++	Trace	
	Salicylic aldehyde	18–24	2.0	5.8	0.210	Trace	Trace	3.90

DISCUSSION

The object of this work was to gain information about the mechanism of action of salicylate and phenylcinchoninate. To this end two types of experiments were carried out: (a) on tissues separated from the body and on isolated and partially purified enzyme systems, and (b) on the whole animal, by introducing these substances parenterally and studying their effect at intervals chosen so as to coincide with marked changes in the behaviour of the injected animals. On the whole, there is not unsatisfactory agreement in the results obtained by means of these two experimental methods. For instance, it was found in experiments on liver slices *in vitro* that only relatively high concentrations of salicylate were able to depress the respiration. Correspondingly, the respiration of

liver slices from injected animals was found not to be much affected even after a relatively high dose of salicylate. The inability of rat liver to form *in vitro* salicylglucuronide was reflected in the findings on urine excretion, which proved the absence of appreciable amounts of glucuronide in response to salicylate treatment. On the other hand, glucuronides were found to be excreted after, for example, benzoate or *o*-aminobenzoate, both of which gave rise to glucuronide formation *in vitro*. With regard to the effect of salicylate on the dismutation between hexosediphosphate and pyruvate, one would have expected from *in vitro* results this reaction to be more sensitive to the injection of salicylate than was actually found.

The proof that profound changes in metabolism are provoked by the injection of salicylate and cinchophen, as well as by some of the benzoic acid derivatives examined, is provided by the results obtained with liver glycogen and the changes in the composition of reducing substances in the tissues. The disappearance soon after the salicylate injection of nearly all the liver glycogen and its full restoration 24 hr. later, clearly indicate an effect on several as yet unidentified enzymic processes. The differences in the behaviour of liver glycogen in response to the various substances used in this work suggest that the fall in glycogen may be brought about in a different way in every case. In B_1 -deficient rats, while the results for urine excretion differed but little from those obtained with normal rats, there were differences in the behaviour of liver glycogen, and also it was found that the oxidation of dihydroxyacetone by liver slices of the deficient animals was diminished by salicylate.

The study of urine excretion brought out several facts as regards the effect on the animal organism of salicylate in comparison with other derivatives of benzoic acid. Certain features, such as the increase in the titratable reducing power of urine, were found common to most of them, but bicarbonate was excreted only after salicylate, benzoate and anthranilate. Albuminuria was typical after salicylic acid, irrespective of the dose. Yet more specific were the tests obtained in the presence of alkalis: only *ortho*-substituted acids such as salicylic, anthranilic and *o*-cresotic, caused the excretion of substances which formed dark pigments with alkalis in the presence of O_2 . The specificity of salicylate was further underlined by the findings with salicylic aldehyde. It was obvious that the aldehyde itself did not cause an increase in the reducing power of the urine, or albuminuria. As soon, however, as some of it had been transformed in the body into the acid, a matter of a few hours in the rat, the urine became strongly reducing, contained albumen, and showed a strongly positive test with NaOH.

The injection of Na cinchophen also increased greatly the titratable reducing power of urine, which, however, was not affected in the least by exposure to air in the presence of catalytic amounts of Cu, as was the substance responsible for the increased reduction in salicylate urine. Resistance of the reducing substance to oxidation was also shown by urine excreted after the injection of *o*-cresotinate and after feeding with tyrosine. On the other hand, ascorbic acid added to normal urine was sensitive to air exposure in the same manner as salicylate urine. This similarity suggests, but by no means proves, that ascorbic acid may be the substance excreted by the rat in response to salicylate treatment.

There is no doubt that the reducing substances estimated by titration in the salicylate and anthranilate urines are not identical with those which give dark-coloured pigments with alkalis. This is borne out by results in which the alkali test was strongly positive and the reduction slight, e.g. samples excreted late after the injection of salicylate; vice versa, there were strongly reducing urines with a very weak alkali test, like the *o*-cresotinate urine. While it remains for future work to identify the substances responsible for the behaviour towards alkalis, one can claim with some certainty based on the striking difference in the behaviour towards ascorbic acid treatment, that the alkapton-like substance which appears in urine during salicylate treatment is not identical with homogentisic acid.

The findings made with regard to reducing substances in the urine and in the tissues (particularly liver) are of interest in connexion with the observations of Abbassy, Gray-Hill & Harris [1936], who stated that in acute rheumatism as well as in rheumatic arthritis the excretion of ascorbic acid in response to large doses of it is much smaller than in normal subjects. Possibly in rheumatic disease it is the metabolism of the reducing substances which is affected in some hitherto unknown way, and the beneficial influence of the anti-rheumatic drugs may be linked with their ability to influence this phase of metabolism.

There are other recent indications that a strongly reducing substance, such as ascorbic acid, may be involved in the pathology of the joint tissues. It has been shown by Robertson, Ropes & Bauer [1941; 1942] that the mucin of synovial fluid, as well as collagen from tendons, undergo far-reaching changes in the presence of ascorbic acid and small amounts of H_2O_2 . It is hoped that further research on the anti-rheumatic drugs will help to bring into a logical whole the accumulating facts and observations, and thus accelerate the solution of the clinical problems of rheumatic disease.

SUMMARY

The effect of salicylate and cinchophen was investigated on isolated enzyme systems and in the whole animal. Normal as well as B_1 -deficient rats were used. The action of salicylate was compared with that of other benzoic acid derivatives.

The respiration of slices and extracts from rat liver and kidney cortex was not much affected by salicylate up to $M/20$ concentration, but was inhibited to an appreciable degree by $M/10$ salicylate. Cinchophen restricted the O_2 -uptake even at $M/50$ concentration. Anaerobic acid production remained unchanged in the presence of $M/10$ salicylate, but was diminished by $M/20$ cinchophen. Acetylsalicylate was rapidly hydrolysed by rat liver and kidney and to a smaller extent also by other animal tissues and fluids as well as by yeast.

The ability of the rat liver to form conjugated glucuronides in vitro was negligible with salicylate and negative with cinchophen, but positive results were obtained with benzoate, *o*- and *p*-aminobenzoate, *p*- and *m*-hydroxybenzoate.

The activity of various animal, plant and yeast enzyme systems was studied. Those affected more by salicylate than by cinchophen were xanthine oxidase, dismutation between hexosediphosphate and pyruvate, carboxylase: cinchophen acted more strongly than salicylate on indophenol oxidase, certain dehydrogenases, glucose fermentation in yeast.

Experiments on the intact animal were done by injecting various doses of salicylate and cinchophen as well as certain benzoic acid derivatives other than salicylate. The behaviour of the injected animals is described. Tissues, blood and urine were examined at 4, 7 and 24 hr. intervals after the injections.

The respiration of liver slices and the dismutation of hexosediphosphate + pyruvate in muscle were not affected by injections of salicylate and cinchophen. There were remarkable changes in liver glycogen. It disappeared almost completely 4–7 hr. after salicylate injections, but was found fully restored, or even above the control value, 24 hr. later. The behaviour of liver glycogen in response to the injection of cinchophen as well as to benzoic acid derivatives other than salicylate, is also described.

Quantitative estimation of reduction in urine showed a considerable increase in the reducing power of urine after the injection of salicylate, several other derivatives of benzoic acid, or cinchophen. There are indications that the reducing substance in salicylate, but not in cinchophen urine, is ascorbic acid. Bicarbonate, albumen and glucose, but not glucuronide, were found in urine after large doses of salicylate; appreciable amounts of glucuronide were present in urine after the injection of benzoate, anthranilate, *p*-aminobenzoate, *m*- and *p*-hydroxybenzoate.

Substances, which on addition of alkalis (NH_3 , NaOH , pyridine) and in presence of O_2 give dark-coloured pigments, were found in urine after treatment with salicylate, anthranilate and *o*-cresotinate, but not after cinchophen or any other of the benzoic acid derivatives examined. It is thought unlikely that the alkali-susceptible substance found in urine during salicylate treatment is alkapton, because its excretion was not suppressed by large doses of ascorbic acid, which, however, abolished completely the excretion of homogentisic acid in alkaptonuria.

The estimation of reducing substances in tissues (liver, kidney, intestine) revealed a nearly twofold increase in liver glutathione content 24 hr. after the injection of salicylate.

Salicylic aldehyde did not cause an increase in the reducing power of urine or the excretion of the alkali-susceptible substance until it was transformed by the organism into salicylic acid.

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