THE APPLICATION OF PAPER PARTITION CHROMATOGRAPHY TO THE STUDY OF THE METABOLISM OF SALICYLATE IN THE RAT

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SALICYLATES have been found to cause a reduction in the glycosuria and hyperglycæmia of the alloxan-diabetic rat.[1] Conjugation of the salicylates with glucuronic acid may have contributed to depletion of available glucose in these animals and was considered as a possible mechanism to explain the observed effects. Although glucuronic acid conjugates form a high proportion of salicylate metabolites in man[2] and the dog[3] the evidence for their occurrence in the rat is conflicting. Lutwak-Mann[4] reported that the ability of the rat's liver to form conjugated glucuronides from salicylate was negligible and could not detect such glucuronides in the urine of rats receiving salicylates. Schayer[5] studied the metabolism of C^{14} carboxyl salicylic acid in the rat by paper chromatography and in addition to identifying salicylic, salicyluric and gentisic acids obtained two unidentified substances, of which one of low R_F value could have been a glucuronide. In the present work the metabolism of salicylate in the rat has been investigated by a paper chromatographic method which gave a complete separation of the known metabolites which were quantitatively estimated. Previous observations in this species have only been qualitative in nature.

The presence of glucose in the urine of rats receiving salicylates was reported by Lutwak-Mann[4] and this observation is of great interest because of the reduction of glycosuria caused by salicylates in rats made diabetic either by partial pancreatectomy[6] or alloxan.[1] The urines of the rats which had been given salicylates were therefore examined for glucose by a paper chromatographic technique capable of detecting 1 μg. of the sugar.

METHODS

Paper chromatography of salicylate metabolites. A descending method using one-dimension strips of Whatman No. 4 filter paper and a mixture of *n*-butanol 40, glacial acetic acid 4 and water 56 (all per cent. v/v) in an atmosphere of ammonia (0·2 per cent. w/v aqueous ammonia) was used. The solvents were based on those employed by Bray, Thorpe and White[7] and Consden and Stanier.[8] Each chromatogram was run for 16 hours and the salicylate compounds visualised by means of their fluorescence in ultra-violet light[8]; a Hanovia Chromalite lamp was used as the light source. For the qualitative identification of the metabolites in the urine of rats receiving salicylate, 5 μml. of each urine specimen was chromatographed and run in conjunction with a solution containing salicylic, salicyluric and gentisic acids as marker substances. Blood

specimens were collected in oxalated tubes and the plasma separated after centrifuging. To 1 ml. of plasma was added 2 ml. of 20 per cent. aqueous trichloracetic acid and the mixture shaken and centrifuged. The supernatant liquid was neutralised to *p*H 7 with 10 per cent. sodium hydroxide solution and 50 μml. used for each run. The total salicylate in each blood sample was estimated by the method of Smith and Talbot.[9]

The ultra-violet absorption spectra of the salicylate metabolites in distilled water show maxima as follows: salicylic acid, 295 mμ; salicyluric acid, 320 mμ; and gentisic acid, 320 mμ. For the quantitative estimation of these metabolites in urine 50 μml. of urine were used in each run and the areas of paper containing the substances, as determined by their fluorescence in ultra-violet light, were cut out and placed in ground glass-stoppered test tubes together with 3 ml. of distilled water. The tubes were shaken for 15 minutes in a microid flask shaker (Griffin and Tatlock), the mixture filtered through a small sintered glass filter and the optical densities of the filtrates measured at the appropriate wavelengths in a Uvispek ultra-violet spectrophotometer. A portion of the chromatogram not containing any salicylate metabolites was treated in the same way to provide the control solution. This paper blank had a negligible optical density at 320 mμ but gave much higher values at 295 mμ which however were uniform enough to enable measurements of salicylic acid to be made. Recoveries of 95 to 100 per cent. of salicylic acid and 90 to 95 per cent. of gentisic acid were obtained by elution of chromatograms of known amounts of these substances dissolved in the urine of rats not receiving salicylates.

Paper chromatography of glucose. A descending method using one dimension strips of Whatman No. 1 filter paper and a *n*-butanol-acetic acid-water mixture[10] was employed. Each chromatogram was run for 24 hours and the spraying reagent was aniline hydrogen phthalate (0·9 ml. of redistilled aniline dissolved in 100 ml. of 0·1M phthalic acid) which gave a yellow-brown spot with glucose having an intense greenish-blue fluorescence in ultra-violet light.[11] 50 μml. of urine was used for each chromatogram.

Experimental

4 male rats of the Wistar strain, weighing approximately 300 g. and maintained on a diet of commercial rat cubes (Thompson), were placed in metabolism cages and 24-hour collections of urine made. The rats were given, by stomach tube, 117 mg. of sodium salicylate ($\equiv$100 mg. of salicylate ion) dissolved in 5 ml. of water. Successive 24-hour urine specimens were chromatographed for salicylate compounds and glucose for 2 days before and 3 days after the injections.

2 male rats of the same colony were given a solution of 117 mg. of sodium salicylate in 1 ml. of distilled water by intraperitoneal injection and killed 2 hours later by the intraperitoneal injection of 2 ml. of 10 per cent. w/v thiopentone solution. Blood was obtained by decapitation and collected in oxalated bottles.

RESULTS

Identification of salicylate metabolites in urine. The R_F values of a number of the possible metabolites of salicylate and their fluorescence in ultra-violet light are given in Table I.

TABLE I

R_F VALUES AND FLUORESCENCE IN ULTRA-VIOLET LIGHT OF SALICYLATE COMPOUNDS

Substance	R_F value (not corrected for temperature)	Fluorescence in ultra-violet light
Salicylic acid	0·76	Violet
Salicyluric acid	0·65	Blue-violet
Gentisic acid	0·57	Intense blue
Salicylamide	0·88	Blue-violet
Gentisamide	0·75	Turquoise

Good separations on the chromatogram of the first 3 substances were made from a mixture and they were easily distinguished by their fluorescence. The fluorescence of gentisic acid in ultra-violet light is especially intense and as little as 0·1 μg can be detected by this means.[8]

The pattern found in the urine of rats receiving sodium salicylate is given in Table II; an estimate of the intensity of the fluorescence is indicated.

TABLE II

SALICYLATE METABOLITES IN THE URINE OF RATS RECEIVING SODIUM SALICYLATE

Collection	Identified substances			Other fluorescent spots
	Salicylic	Salicyluric	Gentisic	
1st 24 hours	+++	trace	+++	Blue-violet fluorescent spot R_F value 0·08 observed on days 1 and 2
2nd ,, ,,	++	none	++	
3rd ,, ,,	none	none	none	Absent

TABLE III

URINARY EXCRETION OF SALICYLIC AND GENTISIC ACIDS AFTER THE ADMINISTRATION OF 117 MG. OF SODIUM SALICYLATE (≡100 MG. OF SALICYLATE ION) TO 4 RATS. (THE FIGURES REPRESENT THE RANGE OF VALUES FOUND)

24-hour urine collection	Salicylic acid mg./24hours (≡ per cent. of ingested dose)	Gentisic acid	
		mg./24 hr.	per cent. of ingested dose
1st	24 to 35	11·0 to 15·5	12 to 18
2nd	17 to 28	5·1 to 14·0	6 to 15·5
Combined 1st and 2nd ..	41 to 63	16 to 30	18 to 33·5

No fluorescent spot with a larger R_F value than that of salicylic acid (0·76) was observed in any chromatogram although Schayer[5] had reported an unidentified compound of high R_F value.

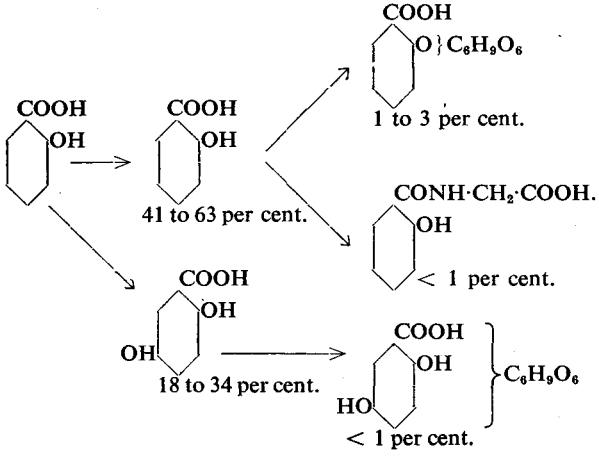
Identification of spot with R_F *value of* 0·08. 3·6 ml. of a 24-hour urine collection from a rat receiving salicylate was applied to paper, with intermediate drying, as a series of 50 μml. spots. The development of the chromatogram and the visualisation of the unknown spot were carried out as before and the areas containing the unknown substance were cut out and eluted with water in the apparatus described by Consden,

Gordon and Martin[12] the eluate being run into test tubes and the completeness of elution being checked by screening with ultra-violet light. The combined eluates (60 ml.) were concentrated by distillation under reduced pressure to a volume of 2 ml. 1 ml. of the concentrate was added to 1 ml. of N sulphuric acid and the mixture refluxed for 1 hour on a boiling water bath. The mixture was then neutralised to *p*H 7 with N sodium hydroxide and 50 μml. quantities chromatographed as before. Examination of the chromatograms under ultra-violet light showed the presence of salicylic acid and a trace of gentisic acid. The acid hydrolysate gave a strong positive naphtha-resorcinol test for glucuronic acid.[13]

The spot with an R_F value of 0·08 therefore consisted of acid labile salicyl glucuronide with a small amount of an acid labile conjugate of gentisic acid, probably a glucuronide. Two types of salicylic glucuronides occur in human urine after salicylate administration, in one of these the glucuronic acid is conjugated in an ester linkage with the carboxyl group of the salicylic acid and in the other the conjugation is an ether linkage with the hydroxyl group of the salicylic acid. The first type contains a free phenolic group and the salicylic glucuronide spots from the rats' urine were therefore sprayed with ferric chloride solution. No blue colour developed and therefore the glucuronide must have been an ether linked type.

Quantitative estimation of salicylic and gentisic acids in urine. The chromatograms showed only a trace of salicyluric acid in the first 24-hour urine collection in each rat and quantitative estimation of this substance was therefore not attempted. The figures for the excretion of salicylic and gentisic acids for the 4 rats are shown in Table III.

It is seen that the ratio of salicylic to gentisic acid is approximately 2:1. Only traces of salicyluric acid were seen in the first 24-hour collection and a rough assessment from the area and degree of fluorescence of the salicyl and gentisyl glucuronide spots was that about 1 to 3 per cent. of the ingested dose of salicylic acid was excreted on each day as these substances. The metabolites of salicylic acid occurring in the urine of the rat may be summarised as follows:



Identification of salicylate metabolites in blood. Chromatograms of the plasma filtrates showed only the presence of a salicylic acid spot, the total plasma salicylate of the rats being 69 and 62 mg./100 ml. respectively. The spot of high R_F value reported to occur in the plasma by Schayer[5] was not observed in any of the chromatograms.

Examination of the urine for glucose. The 24-hour urine collections when tested with Benedict's reagent showed only traces of reducing substances in 3 of the specimens. Examination under ultra-violet light of the chromatograms sprayed with aniline hydrogen phthalate revealed the presence of glucose. Comparison of the intensity of the fluorescence with that given by chromatograms from a series of glucose solutions of known strengths showed that less than 5 μg. was present in 0·05 ml. of the urine collections. The volumes of the 24-hour urines were between 16 and 25 ml. and this means that the rats excreted between 1·6 and 2·5 mg. of glucose per day. Lutwak-Mann[4] reported that stronger reduction occurred in urine collections made 0 to 4 hours and 4 to 7 hours than in the 7- to 24-hour collection after the administration of salicylate. 4 male rats were therefore given an intraperitoneal injection of 117 mg. of sodium salicylate in 1 ml. of water and urine collections made at similar time intervals. Testing with Benedict's and chromatographic analysis did not show any excess of glucose in the early specimens and the total 24-hour excretion was of the same order as the 24-hour specimens previously examined.

Discussion

Lutwak-Mann[14] isolated gentisic acid from the urine of rats receiving salicylate and Schayer identified salicylic, salicyluric and gentisic acids in the urine and salicylic acid in the plasma.[5] In addition the latter worker reported two unidentified compounds in the urine and one in the plasma. The results of the present work confirm the presence of salicylic, salicyluric and gentisic acids in the urine and salicylic acid in the plasma and also show that the rat excretes small amounts of an ether linked glucuronide of salicylic acid and a conjugated gentisic acid, probably a glucuronide, after the administration of salicylate. Schayer's unidentified substance of low R_F value may well be identical with this glucuronide mixture; no substances of high R_F values corresponding to his unknown substances in urine and plasma were observed. The rat is therefore similar to the dog in excreting an ether-linked glucuronide of salicylic acid in the urine and differs from man and the rabbit which excrete two types of salicyl glucuronides in one of which glucuronic acid is linked to the carboxyl group of salicylic acid and in the other it is linked to the hydroxyl group. The quantities of metabolites excreted in the urine of the rat were measured and Table IV summarises the metabolites of salicylate found in the urine of various species.

The rat has been found to excrete only 1 to 3 per cent. of the ingested dose of salicylate as glucuronide and this means that a 100 mg. dose of salicylic acid requires only a few mg. of glucose to supply the necessary glucuronic acid. The daily injection of 100 mg. of sodium salicylate to

TABLE IV

METABOLITES OF SALICYLIC ACID IN THE URINE OF VARIOUS SPECIES

Species	Metabolites (figures represent per cent. of ingested dose)						Reference
	Salicylic acid	Salicyluric acid	Gentisic acid	Salicylglucuronides		Other substances	
				Ether	Ester		
Dog	50	10	4 to 5	25	absent		Alpen, Mandel, Rodwell and Smith[3]
Rat	present	present	present			2 unidentified compounds	Schayer[5]
	41 to 63	<1	18 to 34	1 to 3	absent	Conjugated gentisic acid probably gentisyl glucuronide	Present work
Rabbit	85 Ether soluble acid fraction	5	4 to 5	5 to 14	3 to 4	(*a*) 2:3 dihydroxybenzoic acid (*b*) Conjugated gentisic acid	Bray, Ryman and Thorpe[15] Bray, Thorpe and White[7]
Man	20	55	4 to 8	25		Uraminosalicylic acid	Kapp and Coburn[2]
	10 to 85	0 to 50	1	15 to 40		No uraminosalicylic acid detected	Alpen *et al.*[3]

alloxan-diabetic rats causes a reduction of an average of 5 g. per day in the glycosuria.[1] Conjugation of the salicylate as glucuronide cannot provide an explanation of this effect.

Lutwak-Mann[4] reported that the administration of salicylates to normal rats caused an almost complete disappearance of glycogen from the liver 4 to 7 hours after the injections and this was accompanied by the excretion of glucose in the urine. The effect of salicylate on the liver glycogen content has been confirmed[1] but in the present work the normal rat when injected with salicylate only excretes traces of glucose (up to 2·5 mg.) in the urine in 24 hours.

SUMMARY

1. The metabolism of salicylic acid in the rat has been studied by a paper-partition chromatographic method.

2. After the administration of a single dose of sodium salicylate only salicylate could be detected in the plasma. The urine contained salicylic, salicyluric and gentisic acids together with an ether-linked salicylglucuronide and an acid labile conjugate of gentisic acid which was probably a glucuronide.

3. Quantitative estimation of the metabolites showed that 41 to 63 per cent. of the ingested salicylate was excreted in the free form, 18 to 34 per cent. as gentisic acid, 1 to 3 per cent. as salicyl glucuronide and traces as salicyluric acid and conjugated gentisic acid.

4. Small amounts of glucose (less than 2·5 mg. per 24-hour collection) were found in the urine of rats receiving salicylates.

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