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SALICYLATE AND LIVER GLUTATHIONE

BY

D. H. SPROULL*

From the Clinical Chemotherapeutic Research Unit of the Medical Research Council, Western Infirmary, Glasgow

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The effects of sodium salicylate, benzoate, gentisate, m- and p-hydroxybenzoates, antipyrine, and 2:4-dinitrophenol on the reduced glutathione concentration of rat liver were determined. An increase in concentration in the male, but not in the female, rats was found after salicylate, benzoate and gentisate; after antipyrine, a comparable increase occurred in the females only. There is a relation, in these compounds, between capacity to increase the concentration of reduced glutathione in the rat liver in one sex and therapeutic activity in rheumatic fever.

In continuation of a search for pharmacological actions of salicylate which are correlated with its therapeutic properties, the effect of salicylate on the reduced glutathione concentration of liver was examined. A two-fold increase in the reduced glutathione concentration of rat liver after administration of sodium salicylate has been reported by Lutwak-Mann (1942). The present results do not confirm this finding: a moderate increase in the reduced glutathione concentration of rat liver was induced by salicylate, but in the male sex only.

An attempt has been made to evaluate this finding, by comparing the activity of salicylate on rat liver glutathione with that of a variety of other compounds. In addition to salicylate, the following were studied: other antirheumatic drugs of the same series (benzoate and gentisate), and of a different series (antipyrine); m- and p-hydroxybenzoates, which are therapeutically inert isomers of salicylate, and 2:4-dinitrophenol, which, like salicylate, is a peripheral metabolic stimulant.

In the first experiments, an interval between administration of the drug and termination of the experiment was arbitrarily selected as adequate and convenient for salicylate. This interval was unlikely to be suitable for the rapidly excreted compounds such as benzoate. Rather than find the optimal interval for each compound, a second series of observations was made, in which two doses were given of each of the compounds which in single doses of 200 mg/100 g body weight were tolerated without noticeable effect. Salicylate was included for comparative purposes.

A relation was then found, within the present group of compounds, between therapeutic activity in rheumatic fever and capacity to increase rat liver glutathione concentration in one sex.

* Present address: National Institute for Medical Research, Mill Hill, London, N.W.7.

Since the quantity of oxidized glutathione extractable from rat liver is negligible (Bhattacharya, Robson & Stewart, 1955), the present investigation was confined to the reduced (GSH) form, which will be referred to as "glutathione."

METHODS

The drugs were given by intraperitoneal injection to young mature Wistar albino rats of 150–250 g body weight; with the exception of antipyrine, all the drugs were given as neutral solutions of the sodium salt.

Dosage. In the first series of experiments, each rat received a single dose of the drug, 17 hr before being killed; the doses were, in mg/100 g body weight: sodium gentisate, benzoate, m- and p-hydroxybenzoates, 100; sodium salicylate and antipyrine, 50; sodium 2:4-dinitrophenate, 2. In the second series, from which antipyrine and 2:4-DNP were omitted, two equal doses of each drug were given, 24 and 17 hr before the rats were killed; the doses were, in mg/100 g body weight: sodium gentisate, m- and p-hydroxybenzoates, 100; sodium benzoate, 75; sodium salicylate, 35.

General procedure. In both series of experiments, each drug was given to 14 rats, 7 of each sex. Each series included a control group of 28 rats, 14 of each sex; these were given 1 ml. 0.9% saline intraperitoneally when the corresponding treated animals were injected. A further control group of 14 was given no saline. The rats were put up daily in batches of 9 (first series) or 7 (second series) of the same sex; two rats of each batch were assigned to the control group, and one to each of the drugs; access to food (M.R.C. diet 41) and water was permitted throughout. Next day, the animals were killed between 9.00 and 9.10 a.m. by decapitation, the carcasses bled, and liver samples taken for the estimation of reduced glutathione.

Estimation of reduced glutathione. Reduced glutathione estimation was by a modification of the specific glyoxalase method (Woodward, 1935), where the course of the reaction was followed in terms of the residual methyl glyoxal, determined colorimetrically as its bis-2:4-dinitrophenylhydrazone.

The tissue extracts were prepared in 3% (w/v) sulphosalicylic acid (Bhattacharya, Robson & Stewart, 1955), using an all-glass Potter & Elvehjem (1936) type tissue grinder, with a pestle clearance of 0.002 to 0.004 in., operated at 8,000 rev/min. The glutathione standards were prepared from Reduced Glutathione (Sigma), m.p. 194.5° C. The glyoxalase reaction was conducted according to Schroeder & Woodward (1939), except that the initial concentration of methyl glyoxal in the reaction mixture was reduced from 1.0 to 0.75 mg/ml.

Estimation of the methyl glyoxal as its bis-2:4-dinitrophenylhydrazone depended on isolation of the precipitate by filtration. A filter stick was made for this purpose by connecting to a water pump a 7 in. length of 8.5 mm diameter × 6 mm bore glass tubing, the free end of which was cut squarely and lightly fire-polished. A glass bead of 6.0 to 6.5 mm diameter was sucked on to the end of this tube, which was then dipped into an aqueous suspension of short fibrous asbestos. The asbestos packed at the junction of the bead and tube, forming an efficient filter, which was washed by the passage of 20 to 30 ml. water.

The method finally adopted was as follows: The incubation of each glyoxalase reaction mixture was terminated by the addition of 10 ml. 0.025 N sulphuric acid; after spinning, 1 ml. of the supernatant was added to 2 ml. of 10% trichloroacetic acid, and 1 ml. of this mixture was added to 1 ml. of 2:4-dinitrophenylhydrazine reagent (made freshly every day by dissolving 120 mg 2:4-dinitrophenylhydrazine in 100 ml. of 2 N hydrochloric acid), and left standing for 18 hr. Using the filter stick, the supernatant was then removed, and the precipitate washed with 4 to 5 ml. water. Chloroform (10 ml.) was pipetted into the precipitation test-tube, the filter stick was disconnected from the water-pump, and the glass bead and asbestos discharged into the chloroform by gently blowing down the filter stick, the end of which was finally washed with a little water on to the chloroform. The precipitate was

dissolved in the chloroform, using a high-speed stirrer, and the bulk of the asbestos in the small aqueous layer was removed by suction tube and wash bottle. The solution of the hydrazone in chloroform was centrifuged briefly to ensure adequate separation of the aqueous layer, and a 1 ml. aliquot was added to a further 3 ml. chloroform.

The optical densities of the resulting solutions were measured at 435 mμ ; it was advantageous to set the spectrophotometer to an optical density of 0.350 for the zero glutathione standard. A curve, relating optical density directly to glutathione concentration, was drawn for each batch of analyses, and the unknown glutathione values were finally obtained by interpolation. The liver glutathione concentrations are expressed as mg/g wet weight.

Statistical. The two series of observations are treated separately. The variances and means found are compared, using the two-tailed F-test and the t-test (Snedecor, 1956). Comparable data, homogeneous in variance according to Bartlett's (1937) test, are pooled for purposes of estimation.

RESULTS

The precision of the estimation of liver glutathione was established by quadruplicate determinations in four rats: the estimated standard deviation of a single replicate was ±0.08 mg/g.

TABLE 1

THE CONCENTRATIONS OF REDUCED GLUTATHIONE IN THE LIVERS OF WISTAR ALBINO RATS

Intraperitoneal injections of the compounds were made 17 hr before.
M=male. F=female.

Drug	Dose mg/100 g body weight	Sex	No. of rats	Glutathione concentration mean±s.d., mg/g
None	—	M	7	2·58±0·44
		F	7	2·64±0·24
0·9% NaCl, 1 ml.	—	M	14	2·48±0·40
		F	14	2·55±0·40
Sodium m-hydroxybenzoate	100	M	7	2·77±0·56
		F	7	2·56±0·32
Sodium p-hydroxybenzoate	100	M	7	2·79±0·67
		F	7	2·64±0·28
Sodium benzoate	100	M	7	2·75±0·28
		F	7	2·71±0·36
Sodium gentisate	100	M	7	2·47±0·32
		F	7	2·64±0·32
Sodium salicylate	50	M	7	3·63±0·73
		F	7	2·51±0·44
Antipyrine	50	M	7	2·31±0·65
		F	7	3·32±0·73
Sodium 2 : 4-dinitrophenate	2	M	7	2·49±0·56
		F	7	2·58±0·36

All drugs were tolerated in apparent comfort by the rats ; no distinctive signs were seen, except after antipyrine, which induced for some hours a mild stupor, from which the animals could be easily roused.

Single-dose experiments

The liver glutathione concentrations of the controls shown in Table 1 are homogeneous, and on pooling the data of the 14 untreated rats with those of the 28 rats given an injection of saline, the grand mean liver glutathione concentration of the controls of the first series is 2.55 mg/g, and the standard deviation (s.d.) ± 0.36.

A single dose of benzoate or gentisate did not affect rat liver glutathione concentration 17 hr later. The glutathione concentrations after the injection of these drugs are homogeneous, and these data, summarized in Table 1, when pooled, give a mean of 2.64 mg/g and s.d. ± 0.33, which are similar to the control values.

No distinction was found between the liver glutathione concentrations of the rats given m- or p-hydroxybenzoates. The female data resemble the controls, while the males show an elevated variance (see Table 1). On pooling the glutathione concentrations of each sex, the following estimates of mean and s.d. are obtained: male, 2.78 ± 0.60 mg/g; female, 2.60 ± 0.29 mg/g. The variance of the glutathione concentrations of the males is significantly greater than that of the controls, $0.05 > P > 0.01$.

After the single dose of salicylate or antipyrine, an increase in the mean liver glutathione concentration was found, but in one sex only (see Table 1). The mean glutathione concentration of the livers of the salicylate-treated males is 1.12 mg/g higher than the corresponding female figure, and this difference is significant, $0.01 > P > 0.001$. The mean concentration of glutathione in the livers of the antipyrine-treated females is 1.01 mg/g higher than that of the males, and this sex difference is also significant, $0.02 > P > 0.01$. With the exception of the salicylate-treated females, the variances of these groups are greater than that of the controls.

TABLE 2

THE CONCENTRATIONS OF REDUCED GLUTATHIONE IN THE LIVERS OF WISTAR ALBINO RATS

Two equal doses, shown in the second column, were given by intraperitoneal injection 24 and 17 hr previously. M=male. F=female.

Drug	Dose, mg/100 g body weight	Sex	No. of rats	Glutathione concentration mean±s.d., mg/g
0·9% NaCl, 1 ml.	—	M	14	2·59±0·17
		F	14	2·61±0·22
Sodium m-hydroxybenzoate	100	M	7	2·69±0·39
		F	7	3·08±0·75
Sodium p-hydroxybenzoate	100	M	7	2·79±0·48
		F	7	2·97±0·60
Sodium benzoate	75	M	7	3·33±0·30
		F	7	2·27±0·75
Sodium gentisate	100	M	7	3·07±0·33
		F	7	2·59±0·41
Sodium salicylate	35	M	7	3·08±0·39
		F	7	2·42±0·36

The single dose of sodium 2:4-dinitrophenate had no effect on the liver glutathione of the rat.

Two-dose experiments

The mean glutathione concentration in the livers of the 28 rats given two injections of saline, shown in Table 2, scarcely differs from the mean of those given one injection of saline. However, the variance of the control group of the second series of experiments is significantly less than that of the first, $P<0.01$.

The glutathione concentrations after 2 doses of m- or of p-hydroxybenzoate, shown in Table 2, are homogeneous in variance and on pooling yield a mean and s.d. of 2.88 ± 0.56 mg/g. The variance of the pooled data is significantly greater than that of the corresponding controls, $P<0.01$.

The glutathione concentrations, shown in Table 2, of livers from the rats given 2 doses of benzoate, gentisate, or salicylate are alike. In each case, the female mean is similar to that of the controls, while the male mean is substantially greater. Each of these sex differences in liver glutathione concentration is significant: $0.01>P>0.001$ (benzoate); $0.05>P>0.02$ (gentisate); $0.02>P>0.01$ (salicylate). The data for each sex are homogeneous, and, on pooling, the following estimates of mean and s.d. are obtained: male, 3.14 ± 0.35 mg/g; female, 2.43 ± 0.53 mg/g. The estimates of variance are significantly greater than that of the controls: male, $0.01>P>0.001$, female $P<0.001$.

DISCUSSION

The reduction in variance of the control glutathione concentrations after two injections of saline, as compared with one or none, suggests that the emotional disturbance of unaccustomed handling may affect liver glutathione; it is consistent with the increased turnover rate of glutathione in the liver of rats given adrenaline, observed by Henriques, Henriques & Mandelbaum (1957). The utility of the two-dose experiments is enhanced by this feature.

The increase in rat liver glutathione concentration produced by salicylate has been found, in the present experiments, not only to be smaller than that reported by Lutwak-Mann (1942), but also to be confined to the male sex. The apparent discrepancy between the present results and those of Lutwak-Mann (1942) may be attributable to differences in technique, since the method of estimating glutathione used by Lutwak-Mann was not specific (Patterson & Lazorow, 1955). The interpretation of the sex difference is a matter for further investigation: in particular, it would be desirable to determine whether the turnover rates of glutathione in the liver after salicylate are the same in both sexes.

The failure of a single dose of benzoate or gentisate to affect liver glutathione could be attributed to prompt excretion. In contrast, salicylate is excreted rather slowly in the rat, with a half-time of plasma concentration of 10 hr (Andrews, 1958).

When two doses were given, salicylate, benzoate and gentisate were indistinguishable in their effect on rat liver glutathione concentration; this effect was

not seen after the injection of the therapeutically inert m- and p-hydroxybenzoates, nor after 2:4-dinitrophenol. The effect of antipyrine on liver glutathione was comparable to that of salicylate, but the sex difference was reversed: elevation of liver glutathione concentration occurred only in the females. Although it is not possible, at present, to explain these results, it is worth considering whether the effect, particularly of salicylate, benzoate and gentisate, on liver glutathione concentration is related to some other property of these compounds.

The identity of the effects of two doses of salicylate, benzoate or gentisate on glutathione concentration in the rat liver cannot be accounted for in terms of conjugation, since glutathione is not the source of glycine in hippurate formation by the rat (Waelsch & Rittenberg, 1941). Exhaustion of the "first glycine pool" of Arnstein & Neuberger (1951) by massive doses of hippurate precursors might nevertheless affect liver glutathione concentration. The increased variance of liver glutathione concentration after m- and p-hydroxybenzoates could be accounted for in this way. However, the increases in mean glutathione concentration are not dependent on glycine conjugation, since this is improbable with antipyrine and gentisate, and since the rat forms only a negligible proportion of salicylurate (Quilley & Smith, 1952).

The calorigenic action of salicylate is not relevant to the present findings, because no sex difference in this effect was found in the rat by Andrews (1958), and because neither benzoate nor gentisate possesses this property (Meade, 1954; Andrews, 1958). Furthermore, 2:4-dinitrophenol, which is a powerful metabolic stimulant, had no effect on rat liver glutathione concentration.

The classical instance of increase in liver glutathione occurs in thyroid deficiency (Lazorow, 1954), and both salicylate (Austen, Rubini, Meroney & Wolff, 1958) and gentisate (Wolff & Austen, 1958) have definite antithyroid activity. However, 2:4-dinitrophenol is also a thyroid depressant (Wolff, Rubin & Chaikoff, 1950; Goldberg, Wolff & Greep, 1955), but did not affect liver glutathione, which eliminates this factor as an explanation of the present results.

Within the hydroxybenzoate series of compounds, therapeutic activity in rheumatic fever is not peculiar to salicylate. m- and p-Hydroxybenzoates are therapeutically inert, but benzoate has an unmistakable, if weak, anti-rheumatic action (Stockman, 1920). Gentisate, although active, is also inferior to salicyate (Horder & Bywaters, 1955). It is apparent, therefore, that in these compounds there is an association between anti-rheumatic activity and ability to increase glutathione concentration in the liver of male rats. This contention is reinforced by the unquestionable therapeutic superiority of salicylate, since this compound was outstanding in its effect, in single dose, on liver glutathione.

The significance of the apparent relation between anti-rheumatic activity and effect on rat liver glutathione is obscure. The metabolic reactivity and rapid turnover rate of rat liver glutathione (Waelsch & Rittenberg, 1941) suggest that changes in its concentration simply reflect variation in the volume and quality of metabolic activity for which glutathione is necessary. The present findings therefore imply the existence of a common metabolic effect after the administration of salicylate, gentisate or benzoate, and it is suggested that this primary metabolic

effect, although hypothetical and undefined, may be relevant to the anti-rheumatic action of these compounds.

The association between anti-rheumatic activity and effect on rat liver glutathione is not confined to the benzoate series, but extends to antipyrine. Antipyrine is pharmacologically an inert compound (Greenberg, 1950), which, like benzoate and gentisate, lacks the dramatic side actions of salicylate, yet is as effective as salicylate in rheumatic fever (Bouchut & Levrat, 1937). The effect of antipyrine on rat liver glutathione concentration differs, however, from that of the benzoate series of anti-rheumatic compounds, in the reversal of the sex difference. The underlying metabolic change induced by antipyrine is apparently not the same as that due to salicylate, benzoate or gentisate. Further investigation is necessary to clarify this matter. Nevertheless, the relevant actions of antipyrine, salicylate, benzoate and gentisate must all occur in the same metabolic field.

That two compounds as chemically diverse yet as therapeutically equivalent as antipyrine and salicylate should both have an effect on liver glutathione concentration is surely more than a coincidence. The question arises, therefore, of whether the inference drawn from the present results may be extended: does increased glutathione concentration in rat liver occur after the administration of other anti-rheumatic compounds, such as cinchophen ? May effect on glutathione concentration of rat liver be used as a screening test for therapeutic activity ?

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