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THE RELATIVE POTENCIES OF SOME SUBSTITUTED SALICYLIC ACIDS AS METABOLIC STIMULANTS IN THE INTACT RAT

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The effects of eighteen substituted benzoic acids on the rate of oxygen consumption have been studied in rats. 2:3-Dihydroxybenzoic acid, phthalic acid and 6-methylsalicylic acid were, at the doses used, inactive; *m*- and *p*-hydroxybenzoic acid, 2:4-, 2:5-, 2:6-, 3:4-, and 3:5-dihydroxybenzoic acid, *o*-aminobenzoic acid, salicyluric acid, salicylamide and 5-aminosalicylic acid decreased the rate of oxygen consumption. Only salicylic acid and *o*-, *m*- and *p*-cresotic acid (3-, 4- and 5-methylsalicylic acid respectively) increased the rate of oxygen consumption. Molar potency ratios of the cresotic acids as metabolic stimulants relative to salicylic acid were determined; *o*-cresotic acid was the most powerful with a ratio of 2.61, *m*- and *p*-cresotic acid had values of 1.78 and 1.89 respectively. Two possible explanations of the higher potencies of the cresotic acids were considered. No difference in the primary action of the drug was established by determining the effect on rate of oxygen consumption of a mixture of *o*-cresotic and salicylic acids. An alternative possibility was that the rates of detoxication and excretion of the cresotic acids differed among themselves and from salicylic acid. No such differences were found.

It is well established that sodium salicylate, in moderate dosage, increases the rate of oxygen consumption of experimental animals (Singer, 1901 ; Reid, 1957) and man (Barbour and Devenis, 1919 ; Cochran, 1952).

Meade (1954) found that only salicylic acid, among all the mono- and di-hydroxybenzoic acids, increased the rate of oxygen consumption of rats ; *m*-hydroxybenzoic acid was a depressant and the other acids were inactive. Hall, Tomich, and Woollett (1954) investigated a number of antirheumatic compounds and other substances chemically related to salicylic acid. They found that only salicylic acid and acetylsalicylic acid increased the rate of oxygen consumption of rats or mice ; 2:5- and 2:6-dihydroxybenzoic acid, *m*- and *p*-hydroxybenzoic acid and salicylamide were inactive.

The present work compares eighteen substituted benzoic acids with respect to their ability to stimulate oxygen consumption ; the relative potencies of the active compounds are compared and commented upon.

The rat was chosen as the most practical experimental animal, the smallest in which changes in oxygen consumption can be easily detected over 1 hr. periods.

METHODS

Oxygen consumption was measured with a closed-circuit manometric method described by Cameron (1958).

In the first experiments Wistar albino rats weighing 230 to 290 g. were used. The animals were paired for sex and weight, and in each run one animal received the test solution while the other received an identical volume of normal saline. The drugs were administered by intraperitoneal injection as solutions of the sodium salts at *p*H 7 to 9 ; the doses administered were the highest that could be tolerated.

Dose/Response Experiments. — The compounds used in these experiments were salicylic acid and *o*-, *m*-, and *p*-cresotic acid (3-, 4-, and 5-methylsalicylic acid respectively). Wistar albino rats of 170 to 250 g. weight were used and the animals were paired for sex and weight. As before, one animal was given the drug while the other received the same volume of normal saline. The difference in rate of oxygen consumption between the members of each pair ($\triangle O_2$, expressed in ml./hr. at s.t.p.) was determined. The animals were injected intraperitoneally with the sodium salts of the four acids in four doses ranging from an arbitrary low dose to the maximum dose generally tolerated by the rat. The order of observations was randomized, in respect of drug and dose, within a Graeco-Latin square and 12 estimates of $\triangle O_2$ (6 males, 6 females) were obtained for each drug at each dose.

The combined action of salicylic and *o*-cresotic acids was studied on Wistar albino rats of 200 to 250 g. weight. Six rats (three pairs of the same sex and weight) were used for each trial; one from each pair received normal saline while the other received an intraperitoneal injection of the sodium salt of salicylic acid, or the equipotent dose of *o*-cresotic acid, or a mixture of half of both, and $\triangle O_2$ was determined as before.

Time/Concentration Experiments. — Wistar albino rats of 150 to 350 g. weight were injected with the highest dose of salicylic acid or the cresotic acids used previously, and killed by decapitation at varying times after injection over a 16 hr. period. Eight determinations (4 males, 4 females) of the plasma drug concentration were made for each drug at each time. Plasma concentrations of salicylic acid and the three cresotates were determined by the method of Trinder (1954), which was found to be applicable to the methyl-substituted salicylic acids as well as to salicylic acid itself.

Results

The first experiments were made to determine which of eighteen compounds, related in their structure to salicylic acid, increased the rate of oxygen consumption of rats.

The composite hypothesis that the mean difference in the rates of oxygen consumption by the members of each pair of rats was zero (H_0) was tested against the single alternative that it differed from zero (H_1), using the sequential test proposed by Wald (1947) and the tables of Arnold (1951). Formally, in each case, where μ was the mean $\triangle O_2$ and σ^2 was its variance, it was decided whether $|\mu| < \delta\sigma$ or whether $|\mu| \geqslant \delta\sigma$. In these experiments δ, which determines the critical $\triangle O_2$, was assigned the value 1. The maximum probability of a decision in favour of either hypothesis when in fact the other was true was chosen as 0.05. Where H_1 was accepted, the sign of the mean $\triangle O_2$ was formally established from its 95% fiducial limits, since in no instance did these limits include zero. Table I summarizes these results.

It was previously decided that the results would be discarded if the animals were restless, convulsed, or died during the 60 min. experimental period. In fact, only one of over 400 estimations had to be discarded and this was due to death.

The rate of oxygen consumption of 164 control rats (weight range 230 to 290 g.) showed a nearly normal distribution. The mean rate of oxygen consumption was 420.9 ml./hr. ($\pm$60.3 : s.d.). A quality control chart confirmed that the experiments were in statistical control.

Only four compounds were found to increase the rate of oxygen consumption of rats. These compounds were salicylic acid and the three cresotic acids. Qualitatively these acids were very similar, the maximum doses tolerated were of the same order and the toxic effects observed with very high doses, such as hyperventilation and convulsions, were the same. The relative potencies of the cresotic acids as metabolic stimulants were therefore next compared with salicylic acid.

The dose/response curves were simultaneously determined and are shown in Fig. 1. There was for each compound an approximately linear relation between dose and response and there was no gross difference in intercept on the *y* axis. It seemed likely that these results would therefore permit a comparative slope ratio assay. Before proceeding to the analysis, homogeneity of variance was confirmed by Bartlett's test, $0.50 > P > 0.10$. The requirements for a valid slope/ratio assay are linearity and common intercept; the analysis of variance (Table II) shows that there was no significant deviation from linearity nor was there a significant component of variance due to difference in the intercepts on the *y* axis of the four regression lines.

TABLE I

EFFECT OF SALICYLIC ACID AND RELATED COMPOUNDS ON THE RATES OF OXYGEN CONSUMPTION OF WISTAR ALBINO RATS

$\triangle O_2$ is the difference in rates of oxygen consumption between paired treated and control rats (ml./hr.); H_0 is the hypothesis that the mean $\triangle O_2$ is zero, H_1 the alternative; n is the number of trials required for termination of the sequential test of H_0 against H_1.

Compound	Dose (mg.)	Mean O_2 Uptake of Control Rats (ml./hr.)	$\triangle O_2$: Mean and 95% Fiducial Limits (n)	Hypothesis Accepted
Salicylic acid ..	120	418·9	+53·5± 34·6 (10)	H_1
m-Hydroxybenzoic acid	500	457·7	−173·0±103·9 (6)	H_1
p-Hydroxybenzoic acid	500	409·8	−78·1± 42·1 (8)	H_1
2 : 3-Dihydroxybenzoic acid ..	100	430·0	−25·5± 55·9 (12)	H_0
2 : 4-Dihydroxybenzoic acid ..	300	456·6	−84·4± 44·5 (7)	H_1
2 : 5-Dihydroxybenzoic acid ..	500	474·3	−332·9± 71·6 (6)	H_1
2 : 6-Dihydroxybenzoic acid ..	200	438·4	−121·5± 71·0 (7)	H_1
3 : 4-Dihydroxybenzoic acid ..	500	454·8	−102·9± 67·4 (10)	H_1
3: 5-Dihydroxybenzoic acid ..	500	466·6	−104·5± 69·9 (7)	H_1
5-Aminosalicylic acid	100	399·5	−95·1± 55·4 (7)	H_1
Salicyluric acid ..	100	388·6	−46·9± 29·9 (8)	H_1
Salicylamide ..	50	409·4	−128·6± 46·8 (6)	H_1
o-Cresotic acid (3-methylsalicylic acid)	100	395·9	+176·1± 76·7 (7)	H_1
m-Cresotic acid (4-methylsalicylic acid)	100	385·1	+109·9± 33·6 (6)	H_1
p-Cresotic acid (5-methylsalicylic acid)	105	383·2	+186·6± 62·9 (8)	H_1
6-Methylsalicylic acid	24	442·6	+21·6± 61·7 (10)	H_0
o-Aminobenzoic acid	100	379·0	−68·7± 40·7 (7)	H_1
Phthalic acid ..	100	452·9	−13·6± 48·9 (9)	H_0

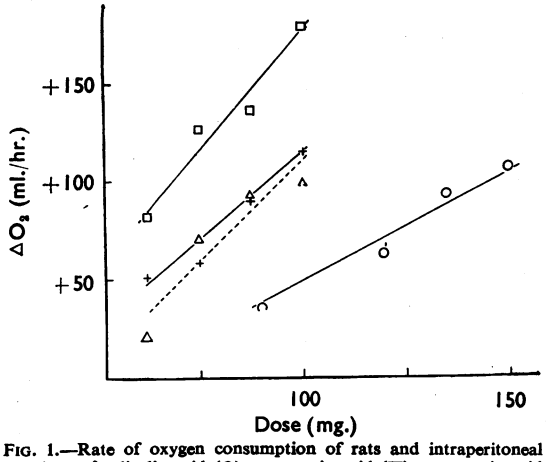

FIG. 1.—Rate of oxygen consumption of rats and intraperitoneal dose of salicylic acid (O); *o*-cresotic acid (□), *m*-cresotic acid (△ - - - - - -) and *p*-cresotic acid (+). For definition of ΔO_2 see text. Each point is mean of twelve determinations.

TABLE II

SLOPE/RATIO ASSAY OF SALICYLIC ACID AND THE CRESOTIC ACIDS: GENERAL ANALYSIS OF VARIANCE

Source of Variance	Sum of Squares	d.f.	Variance
Rectilinear regressions	148,493·81	4	37,123·45
Deviation from regressions ..	17,861·85	8	2,232·73
Between intercepts	3,672·35	3	1,224·12
Residual	704,831·22	176	4,004·72

The potency ratios of the cresotic acids with respect to salicylic acid were therefore calculated in molar units and are shown in Table III. The 95% confidence limits of these ratios were obtained by a maximum likelihood method (Silvey, personal communication).

TABLE III

MOLAR POTENCY RATIOS OF THE THREE CRESOTIC ACIDS RELATIVE TO SALICYLIC ACID AS METABOLIC STIMULANTS

Compound	Potency Ratio	95% Confidence Limits
o-Cresotic acid	2·61	2·50–2·72
m-Cresotic acid	1·78	1·69–1·87
p-Cresotic acid	1·89	1·79–1·99

In these experiments the rats weighed less than in the previous experiments. The rates of oxygen consumption of 252 control rats (weight range 170 to 250 g.) showed an apparently normal distribution, and a quality control chart confirmed that the experiment was in statistical control. The mean rate of oxygen consumption was 389.2 ml./hr. ($\pm$48.5: s.d.).

The distributions of the rates of oxygen consumption for the two series of experiments were similar, and although the rate of oxygen consumption depends on body weight there was no significant correlation between ΔO_2 and body weight for any drug or dose ($P>0.05$).

The preceding observations permit a quantitative study of the combined action of salicylic acid and the cresotic acids. Such experiments might point to a difference in the primary action of the drugs which in turn might explain the greater potencies of the cresotic acids.

TABLE IV

COMBINED ACTION OF SALICYLIC ACID AND *o*-CRESOTIC ACID

Results of the sequential tests of the hypothesis that the mean difference in ΔO_2 of rats treated with 150 mg. salicylic acid (S), 70 mg. *o*-cresotic acid (O), and a mixture of 75 mg. salicylic acid and 35 mg. *o*-cresotic acid (SO) were equal. Formally where each mean difference was μ and its variance σ^2, the hypothesis $H_0: |\mu| < 0{\cdot}7\sigma$ was tested against $H_1: |\mu| \geqslant 0{\cdot}7\sigma$, the probability of accepting the wrong hypothesis was 0·05.

Difference Tested	No. of Trials Required	Hypothesis Accepted	Mean Difference and 95% Fiducial Limits of the Mean
(S)–(O)	14	H_0	+1·6±38·5
(S)–(SO)	14	H_0	+6·0±41·6
(O)–(SO)	14	H_0	+4·4±43·5

The nature of the combined action of salicylic acid and *o*-cresotic acid was determined as described by Gaddum (1953). The effects of a selected dose of salicylic acid on the rate of oxygen consumption, an equipotent dose of *o*-cresotic acid, and a mixture of half the dose of each drug were compared. Table IV summarizes these results. In every case H_0 was accepted. Thus administration of a mixture of salicylic acid and *o*-cresotic acid gave a response which was indistinguishable from an additive effect, and therefore no difference in the primary actions of the two drugs was established by this experiment.

The possibility that differences in rates of degradation and excretion between salicylate and the

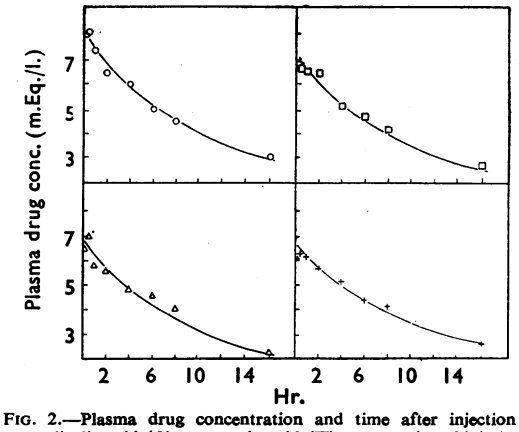

FIG. 2.—Plasma drug concentration and time after injection of salicylic acid (O), *o*-cresotic acid (□), *m*-cresotic acid (△) and *p*-cresotic acid (+). Each point is mean of eight determinations.

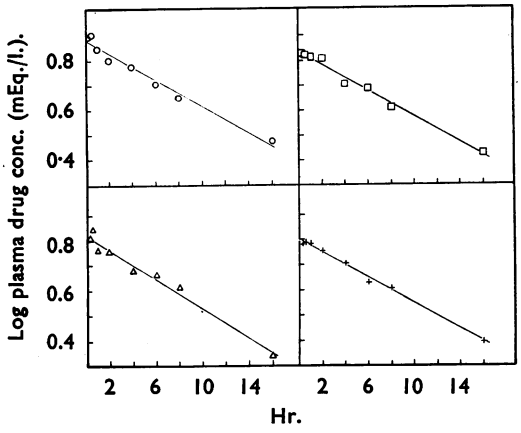

FIG. 3.—Log plasma drug concentration and time after injection of salicylic acid (O), *o*-cresotic acid (□), *m*-cresotic acid (△) and *p*-cresotic acid (+). Each point is mean of eight determinations.

cresotates might account for the difference in potencies was examined by comparing the rates of disappearance of the drugs from the blood after single intraperitoneal injections.

Rats which had received the previous maximum dose of salicylic acid (150 mg.) or a cresotic acid (100 mg.) were killed between 15 min. and 16 hr. later and the plasma drug concentration was determined. The mean plasma concentrations for each drug are shown in Fig. 2. In each case the plasma drug concentration showed an apparently exponential decline. Transformation of the concentrations to a logarithmic scale made the relationship rectilinear (Fig. 3).

The homogeneity of variance of the transformed observations was confirmed by Bartlett's test, which gave $0.50>P>0.10$; analyses of variance failed to show a significant deviation from rectilinear regression for any of the drugs (Table V). The regression lines were then formally compared.

TABLE V

ANALYSES OF VARIANCE OF LOG PLASMA DRUG CONCENTRATIONS AND REGRESSION EQUATIONS OF LOG PLASMA DRUG CONCENTRATION ON TIME AFTER INJECTION FOR SALICYLIC ACID AND THE THREE CRESOTIC ACIDS

x=time in hr.; y=log plasma drug concentration (m.Eq./l.).

Compound	Mean Squares			Regression Equation
	Due to Regression d.f.=1	Deviation from Regression d.f.=6	Residual d.f.=56	
Salicylic acid ..	1·1433	0·0047	0·0055	$y=0·89-0·027x$
o-Cresotic acid ..	1·0823	0·0027	0·0045	$y=0·84-0·026x$
m-Cresotic acid ..	1·3777	0·0070	0·0051	$y=0·82-0·029x$
p-Cresotic acid ..	1·0210	0·0058	0·0067	$y=0·80-0·025x$

TABLE VI

DISAPPEARANCE FROM BLOOD OF SALICYLATE AND CRESOTATES

Regressions of log plasma drug concentration on time after injection: analysis of covariance.

Source of Variance	Regression Coefficient	Deviation from Regression		
		Sum of Squares	d.f.	Variance
Salicylic acid	−0·0268	0·3342	62	0·00539
o-Cresotic acid	−0·0261	0·2674	62	0·00431
m-Cresotic acid	−0·0294	0·3299	62	0·00532
p-Cresotic acid	−0·0253	0·3787	62	0·00611
Within drugs		1·3102	248	0·00528
Regression coefficient ..		0·0151	3	0·00503
Common line	−0·0269	1·3253	251	0·00528
Elevation		0·2764	3	0·092
Total ..		1·6017	254	0·00631

Any rectilinear regression is fully defined in terms of two parameters, elevation and gradient. In the present experiments the elevations were mainly dependent on the initial dose of the drug injected; any difference in rates of disappearance of the drugs from the blood would, after logarithmic transformation of concentration, appear as a difference in gradient. The analysis of covariance which is summarized in Table VI confirmed that there was no significant difference between the four regression coefficients of log concentrations of the various drugs on time after injection.

There was thus no evidence that the rates of disappearance of the cresotates from blood differed among themselves or from that of salicylate.

DISCUSSION

In accordance with the practice in this laboratory the variability of biological material was accepted. No attempt was made to impose "basal" conditions; variations in exercise, feeding, etc., were assumed to be randomly distributed. The animals were, however, paired for sex and weight, and all the animals were chosen within an arbitrary weight range. Quality control charts showed that the experiments were in satisfactory statistical control.

The drugs which at the dose administered did not alter the rate of oxygen consumption of rats were 2:3-dihydroxybenzoic acid, phthalic acid, and 6-methylsalicylic acid.

Many of the compounds were tolerated at high doses. At such doses *m*- and *p*-hydroxybenzoic acid, 2:4-, 2:5-, 2:6-, 3:4-, and 3:5-dihydroxybenzoic acid depressed the rate of oxygen consumption of rats. 5-Aminosalicylic acid, salicylamide, salicyluric acid, and *o*-aminobenzoic acid were also metabolic depressants in doses similar to salicylic acid.

Gentisic acid (2:5-dihydroxybenzoic acid) and salicyluric acid have been isolated in the urine as metabolites of salicylic acid (Kapp and Coburn, 1942). Thus in the body some salicylic acid is converted from a metabolic stimulant to a depressant, which suggests that these two acids, salicyluric and gentisic, are true detoxication products in respect of their metabolic stimulant action.

It was already known that the antituberculous drug, *p*-aminosalicylic acid, depressed the rate of oxygen consumption in man (MacGregor and Somner, 1954) and it is interesting to note that the 5-aminosalicylic acid possessed a similar depressant property.

The only compounds which increased the rate of oxygen consumption of rats were salicylic acid and *o*-, *m*-, and *p*-cresotic acid.

These acids have many chemical, physical, and pharmacological properties in common. Chemically they are all phenolic acids with the hydroxyl group in the *ortho* position relative to the carboxyl group and they all give a purple colour with ferric chloride. May (1909) found that the cresotic acids resembled salicylic acid in their action as antifermentatives, as bactericides, as antipyretics, and as specifics in acute rheumatism. Stockman (1912) used the sodium salts of the three cresotic acids in the treatment of rheumatic fever and concluded that for practical purposes they were inferior to sodium salicylate.

Sodium gentisate (Meyer and Ragan, 1948) and sodium γ-resorcylate (sodium 2:6-dihydroxybenzoate) (Reid, Watson, Cochran, and Sproull, 1951) have been reported as effective in the treatment of acute rheumatism, but neither increases the rate of oxygen consumption of rats. These results indicate that metabolic stimulant action in rats is not essential for therapeutic activity, although, within this series, the ability to increase the rate of oxygen consumption may be sufficient for anti-rheumatic activity.

The dose/response curves of the three cresotic acids and salicylic acid were rectilinear. There was therefore no need to transform dose to a logarithmic scale. The present dose/response relationship is consistent with the results of Alexander and Johnson (1956) on man and of Reid (1957) on rabbits. The comparative assay of the four metabolic stimulants described in the present investigation was novel both because the common intercept on the *y*-axis was negative and because it was a comparative assay, and therefore better not treated in terms of multiple regression. A procedure devised by Silvey (personal communication) was therefore used.

The cresotic acids were found to be more powerful metabolic stimulants than salicylic acid when administered in single doses intraperitoneally to rats; *o*-cresotic acid was the most powerful with a potency ratio relative to salicylate of 2.61, *m*-cresotic acid had a ratio of 1.78, and *p*-cresotic acid a ratio of 1.89. The fiducial limits of the ratios showed that these differences were significant at the 95% level. They were calculated on a molar basis, and would have been numerically lower, but equally significant, if computed weight for weight. Such a basis would have taken no account of the chemical equivalence of the drugs.

The introduction of the methyl group into the benzene ring therefore increased the potency of salicylic acid as a metabolic stimulant; the *ortho* position was the most effective. This raises the question of the effect of other alkyl or aryl substituents in the *ortho* position. It is of interest to note that preliminary observations with 3-phenylsalicylic acid suggest that it is even more potent than *o*-cresotic acid.

The present results establish that these differences in the potencies of the cresotic acids relative to salicylate are not dependent on variations in the rates of degradation or excretion of the drugs nor on differences in the primary actions. It is concluded that the tissues themselves might therefore be more sensitive to the cresotates than salicylate.

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