CXVIII. STUDIES IN DETOXICATION

I. THE INFLUENCE OF (*a*) DOSE AND (*b*) *o*-, *m*- AND *p*-SUBSTITUTION ON THE SULPHATE DETOXICATION OF PHENOL IN THE RABBIT

By RICHARD TECWYN WILLIAMS

From the Physiology Department, University of Birmingham

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Several factors influence the mode and extent of conjugation of a foreign compound in the animal body. The following are of significance.

Species. The mode of detoxication depends upon the species of animal under observation (cf. the detoxication of phenylacetic acid in man by glutamine [Ambrose *et al.* 1933], in the dog by glucuronic acid and glycine [Quick, 1928] and in the bird by ornithine [Totani, 1910]).

Diet. The nature of the diet can play a considerable role in detoxication [Braunstein *et al.* 1931; for further references see Pryde & Williams, 1936].

Dose. The magnitude of the dose of the foreign compounds affects the extent and method of disposal [Quick, 1924; 1926; 1933; Sherwin, 1922].

Administration. The mode of administration influences the method of detoxication [Hele, 1923; Pelkan & Whipple, 1922].

Fatigue. Fatigue has been found to decrease the synthesis of conjugated phenol compounds in rabbits [Palladin & Palladina, 1935].

Temperature. The influence of this factor has been demonstrated by Ito [1916] who showed that abnormal temperatures lower the conjugating power of an animal.

The nature of the compound. This factor will obviously have a great effect not only on the method but also upon the extent of detoxication.

Amongst other possible factors which may influence detoxication is the condition of such organs as the liver and intestines [Quick, 1937]. Under normal circumstances, these may be left out of account. However, when the liver is affected by disease or poisoning the detoxicating power of an animal is lowered [for literature, see Quick, 1937].

The fate of phenol

When phenol is administered to an animal it is partly oxidized, partly conjugated with sulphuric and glucuronic acids and partly excreted free. What actually happens to phenol during oxidation in the body is somewhat obscure. However, small amounts of quinol and catechol are formed particularly when large doses are fed [Baumann & Preusse, 1879]. Other products of phenol oxidation are pigments of unknown nature, probably produced by the action of phenolases, which oxidize phenols to complex quinonoid pigments. The relative amounts of phenylsulphuric and phenylglucuronic acids formed from a given dose of phenol are not known with certainty, owing to the absence of a satisfactory method of estimating phenylglucuronic acid in urine.

In order to compare the influences of *o*-, *m*- and *p*-substitution on the extent of the sulphate conjugation of phenol, it was necessary to find the extent of

conjugation in the case of unsubstituted phenol. A dose equivalent to 0·25 g./kg. was ordinarily adopted for comparison, since the sulphate response from this dose was reasonably large and easily estimated. Some of the substituted phenols used had however to be fed in smaller doses owing to their toxicity, so that the sulphate response for the equivalent dosage of unsubstituted phenol had to be determined. Doses varying from 25 to 250 mg./kg. were used; the sulphate response to doses smaller than 25 mg./kg. could not be estimated with accuracy, since the normal value of the ethereal sulphate could vary to the extent of 10 mg. SO_3 or more from the mean normal value. Doses greater than 250 mg. caused distress.

The only data on the feeding of varying doses of phenol are those of Tauber [1878], who found in the dog that the percentage of phenol excreted in the urine on a constant diet increased with dose. From 60 mg. (to a 20 kg. dog) none was recovered, whilst the largest dose used, 480 mg., gave a recovery of 55%, estimated as tribromophenol. Tauber, however, made no estimation of conjugated phenol.

The results of the present experiments on rabbits are contained in Fig. 1, which shows that for doses of 100–250 mg./kg. the percentage of the dose conjugated with sulphate is constant, i.e. the ethereal sulphate produced is proportional to the dose. About 20% of the phenol is excreted conjugated with

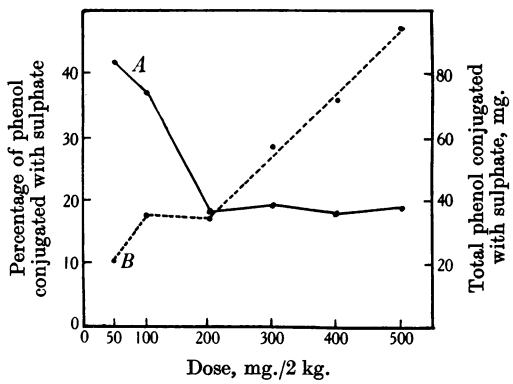


Fig. 1. Graph showing the variation in the percentage and total phenol conjugated with sulphate with different doses. *A*, % phenol conjugated with sulphate. *B*, total phenol conjugated with sulphate.

sulphate. Below 100 mg./kg. the percentage conjugated rises rapidly with falling dose and at 25 mg./kg. more than 40% of the dose is conjugated with sulphate, indicating that this conjugation plays an important role in the detoxication of small doses of phenol. It is probable that at these low levels glucuronic acid conjugation is negligible and that a considerable proportion of the phenol is completely oxidized. Christiani [1878] found that on giving 262 mg. of phenol with milk to a small rabbit (1 kg. ?) 26·7% was excreted in combination with sulphate. The conjugated sulphate of phenol is not metabolized, since it has been shown that potassium phenylsulphate administered to rabbits is almost quantitatively recovered in the urine [Rhode, 1923; Christiani, 1878].

The fate of *o*-, *m*- and *p*-substituted benzene derivatives has been previously studied by Jaffe & Hilbert [1888], Cohn [1893; 1894], Meyer [1905] and particularly by Quick [1932, 1]. The results of these workers indicate that *o*-compounds behave somewhat differently from their *m*- and *p*-isomerides. In such studies,

ceteris paribus, two factors must be considered, a structural factor, which in the present case is the *o*-, *m*- and *p*-position of the group under consideration and a chemical factor, involving the chemical nature or polarity of the substituent. Thus Quick found *o*-substitution to inhibit glycine conjugation with substituted benzoic acids, and the inhibition was independent of the nature of the group, but when the glucuronic acid conjugation was considered it was found that the nature of the group, in addition to its position, was of the utmost importance. Quick also found for the glucuronic acid conjugation, but not for glycine, a similarity between *o*- and *p*-substituted benzoic acids.

Experimental

All sulphate values in this investigation are expressed as SO_3.

Rabbits. Female rabbits weighing approximately 2 kg. were used throughout these experiments. Their weights were checked before and after each experiment.

Cages. The rabbits were placed singly in cages which were set in large supported tinned-copper funnels and the urine was collected in a beaker placed at the orifice of the funnels. The funnels were changed every 2 days for cleaning. The cages were kept in a room maintained at 50–60° F.

Diet. The rabbits were given daily a "mixed diet" of 100 g. cabbage and 50 g. bran, these amounts being found to be sufficient without being excessive. This quantity of foodstuff was maintained throughout the experiments. The phenol was administered in aqueous solution by stomach tube.

The normal value of the ethereal sulphate. Using the above diet the normal value of the ethereal sulphate excretion averaged about 39 mg./2 days, with limiting values varying from 20 to 70 mg., and most commonly varying from 30 to 50 mg. The total sulphate excreted varied from 0·3 to 0·9 g./2 days, the most common values being 0·5–0·7 g.

The course of an experiment. The experimental period extended over 6 days; the urine was collected and analysed every 2 days for total and inorganic sulphate by the gravimetric ($BaSO_4$) method of Folin [Peters & Van Slyke, 1932]. The phenol was administered on the morning of the 3rd day and the urine for the 3rd and 4th days counted as that which contained the metabolic products of the administered phenol. The normal value of the ethereal sulphate was taken as the mean of the values found for the 1st–2nd and 5th–6th days. The difference between the ethereal sulphate value for the 3rd–4th days and the normal value gives the increase due to the administered phenol. In calculating the percentage of phenol conjugated with sulphate, 1 mol. of phenol is equivalent to one of SO_3. It is realized that small amounts of dihydric phenols may be formed and may be singly or doubly conjugated with sulphate. It is probable, however, that the amounts would be very small and would make very little difference to the results. The following protocol shows how an experiment is run.

Exp. 33*a*. Phenol. Dose 0·5 g. Rabbit 29. Weight 2 kg. Phenol given in water by stomach tube on 11. x. 37.

Date	Urine vol. ml.	Inorganic SO_3 mg.	Ethereal SO_3 mg.
9. x. 37 10. x. 37	192	558	41
11. x. 37 12. x. 37	237	523	115
13. x. 37 14. x. 37	280	415	39·5

Average normal SO_3 = 40·3; increase in SO_3 = 74·7 mg. Phenol conjugated with SO_3 = 87·8 mg.; % detoxicated = 17·6.

The excretion of the administered phenol is usually complete within 24 hr.; a 2-day collection of urine was made in order to ensure this, particularly in the case of the substituted phenols and also to lessen the number of analyses. To make sure that no ethereal sulphate is hydrolysed during a 2-day collection of urine, one experiment was carried out on all substances used in which the urine was analysed every day. The results were the same as for the 2-day collections, so that no appreciable loss of ethereal sulphate occurred during the longer period of collection. The results for phenol are shown in Table I.

Table I. *The percentage of phenol conjugated with sulphate with varying doses*

Rabbit no.	Dose mg./kg.	% conjugated	Rabbit no.	Dose mg./kg.	% conjugated
37	25	40·3	23	150	17
41		45·3	27		20·7
44		41·2	28		19·2
46		35·0			Mean 19
47		43·9			
		Mean 41	14	200	16·2
			32		18·1
23	50	37·8	33		20·5
29		36·8			Mean 18
33		35·8			
		Mean 37	4	250	17·3
			1		18·5
28	100	19	23		22·7
29		15·1	26		19·9
30		19·4	29		17·6
		Mean 18			Mean 19

Experiments with substituted phenols. The substituted phenols were administered to the rabbits in aqueous solution or suspension. They were fed in doses equivalent to 0·25 g. phenol per kg. The doses used are given in Table II.

Table II. *Equivalent doses of monosubstituted phenols per kg*

Substituent	Dose mg.	Substituent	Dose mg.	Substituent	Dose mg.
H	250	CH_2OH	325	NO_2	370*
NH_2	290	$CONH_2$	325	$COOCH_3$	405
CH_3	290	†$NHCH_3$	325	CH_2COOH	405
CHO	325	Cl	340	Br	460
OCH_3	325	COOH	365		

* See text. † As metol 0·457 g.

All substituted phenols were purchased except *m*- and *p*-hydroxybenzamides, *m*-chlorophenol and *o*-hydroxyphenylacetic acid. The *m*- and *p*-hydroxybenzamides were prepared by heating the corresponding methyl hydroxybenzoates with aqueous ammonia in a sealed tube. *m*-Chlorophenol was prepared by diazotization, followed by hydrolysis, of *m*-chloroaniline. *o*-Hydroxyphenylacetic acid was obtained by the action of boiling HI on *o*-methoxymandelonitrile obtained from *o*-methoxybenzaldehyde [Czaplicki *et al.* 1909]. Three experiments were carried out simultaneously using three isomeric substituted phenols. The following protocol illustrates the course of an experiment.

Exp. 22*c*. *p*-Chlorophenol. Rabbit 19. Weight: initial 2 kg., end 1·95 kg. Diet: 50 g. bran and 100 g. cabbage *per diem*. Given 0·68 g. *p*-chlorophenol with water by stomach tube on 31. v. 37.

Date	Urine vol. ml.*	Inorganic SO_3 mg.	Ethereal SO_3 mg.
29. v. 37 30. v. 37	209	605	35
31. v. 37 1. vi. 37	259	776	146
2. vi. 37 3. vi. 37	234	531	43

Normal value of SO_3 = 39 mg. Increase after chlorophenol = 107 mg. *p*-Chlorophenol equivalent to the increase = 172 mg. Chlorophenol conjugated with SO_3 = 25 %.

* Urine vol. includes funnel washings which may be about 20–30 ml.

The results of the experiments are given in Table IV. Duplicate results only are quoted (about 120 experiments were carried out).

Table III. *The sulphate conjugation of* p-*nitrophenol*

Rabbit no.	Dose mg./kg.	Fed with Na_2CO_3	% conjugated
24	322	Yes	—*
13	296	No	—*
21	264	Yes	23·4
27	220	No	—*
23	220	No	39
45	200	Yes	15·5
29	182	No	32
44	182	Yes	24

(370 mg./kg. of *p*-nitrophenol ≡ 250 mg. phenol.)

* Dose fatal.

The results with *p*-nitrophenol are uncertain because it is highly toxic to rabbits. These are given in Table III. It will be noted that when *p*-nitrophenol was fed with Na_2CO_3 solution (i.e. as Na salt) the percentage conjugated with sulphate was about 20 %, but when given in aqueous suspension the figure obtained was over 30 %.

Discussion

The results, summarized diagrammatically in Fig. 2, show that the con jugation of phenol with sulphuric acid is profoundly influenced by substitution and by the nature of the substituent group. The distance of the substituent from the —OH is important, since effects progressively diminish from the *o*- to the *p*-positions. In the *p*-position all effects are at a minimum whilst the maximum effects are in the *o*-position, the *m*-position being intermediate. *o*-Acidic groups decrease and *o*-basic groups increase the sulphate conjugation, whilst *o*-neutral groups have little effect. In the *m*-position similar conditions to those of the *o*-position prevail but the effect is less pronounced, whilst in the *p*-position only the COOH and $CONH_2$ groups have any pronounced effect.

The most important factor in the *o*-position is the chemical nature of the group operating nearer to the OH than in either the *m*- or *p*-positions. Acidic groups will tend to prevent conjugation of the OH with the highly acidic —OSO_3H group, whilst basic groups would tend to attract such a group and

Table IV. *The conjugation of monosubstituted phenols with sulphate in the rabbit*

Results expressed as a percentage of the dose fed. Phenol = 19 %

Substituent group	*o*-	*m*-	*p*-
COOH	0	3	6·0
	0	4·5	6·5
	0	5	8
$COOCH_3$	3	4·5	13
	6	6	14
	—	—	15·5
CHO	6	5·5	10·5
	6·5	5·5	13·5
NO_2	7	13	23*
	7	14	24*
CH_2COOH	8·5	—	—
	10·5	—	—
CH_2OH	6	—	—
	10	—	—
Cl	9·5	18	22·5
	10·5	—	25
Br	12	—	19
	16	—	20
OCH_3	17·5	20	19·5
	18	22	21·5
CH_3	20·5	21·5	14·5
	23·5	22	16·5
NH_2	26	31	19·5
	29	36	20·5
$NHCH_3$	—	—	22·5
	—	—	25·5
$CONH_2$	29	32	30
	30	34	36
	40	—	—

* See also Table III.

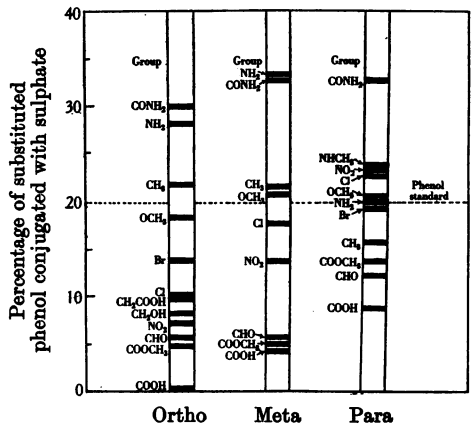
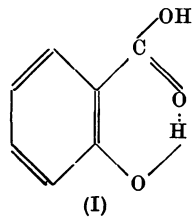


Fig. 2. Diagram showing the influence of substitution on the sulphate detoxication of phenol in the rabbit.

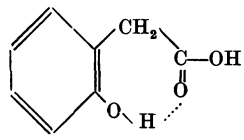
thereby increase the conjugation. The neutral CH_3 and OCH_3 will have little polar effect and therefore have little influence on conjugation; thus the conjugation of *o*-cresol is 22%, guaiacol 18%, compared with 19% for phenol. In the cases of *o*-acidic groups, COOH, NO_2, CHO, CH_2COOH, Cl, Br, CH_2OH, some lower the conjugation more than others, but it will be noted that there is no relation between the lowering of conjugation and the size of the group, for a large group such as Br only lowers the conjugation to 14% whilst much smaller groups such as CHO and COOH lower it to 6 and 0% respectively. In organic chemistry it is found that there is no definite relation between the influence of a group in the *o*-position and its weight or volume [Dippy *et al.* 1937]. One is therefore led to suspect that there may be other factors operating in the *o*-position and these may be related to the *ortho*-effect.

According to Dippy *et al.*, the *ortho*-effect may be due to a combination of several factors, which include Victor Meyer's conception of steric hindrance and the formation of hydrogen bonds [Sidgwick & Callow, 1924]. The formation of hydrogen bonds (chelation) was visualized by Sidgwick & Callow to explain abnormalities in the physical properties of certain *o*-substituted phenols. The *ortho*-effect appears to operate only when the reacting group contains a powerful electron donor [Dippy *et al.*], e.g. COR, CO_2R, NR_2; at the same time the OH group is predominantly an electron-accepting group. The COOH and CHO groups are those which cause the greatest depression of sulphate conjugation. For salicylic acid, it is zero and the formation of a H-bond might well explain this. In salicylic acid the H-bond is part of a six-membered stable ring (I) and therefore the OH group



(I)

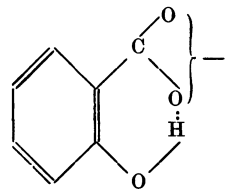
is not entirely free for conjugation; this is supported by the very small glycine and glucuronic acid conjugations of salicylic acid [Quick, 1932, 2]. The *ortho*-effect does not operate if the reacting group is separated from the nucleus by one or more carbon atoms, hence one would expect that conjugation with *o*-hydroxyphenylacetic acid would occur. When this acid is fed to rabbits, 9–10% of it is excreted conjugated with sulphate, a result which is comparable with most of the *o*-substituted phenols used. In *o*-hydroxyphenylacetic acid H-bond formation is improbable since it would necessitate the formation of an unstable seven-membered ring (II).



(II)

If one accepts Quick's theory of detoxication [Quick, 1932, 2] together with H-bond formation, then another interesting explanation of the low detoxication of salicylic acid arises. Quick suggests that the fundamental factor in a conjugation process may be the conversion of a weak acid, which the body cannot

readily excrete, into a strong acid which it can, since a sufficiently strong acid can be eliminated without conjugation. Salicylic acid is a relatively strong acid ($10^5K = 105$) and according to Dippy *et al.* [1937] this is due to H-bond formation, which can take place more readily with the salicylate ion (III) than in the undissociated molecule.



(III)

Formation of this complex prevents reassociation with the H^+ ion making salicylic acid a relatively strong acid (cf. *m*- and *p*-acids; $10^5K = 8{\cdot}7$ and $2{\cdot}8$). The fact that salicylic acid is a relatively strong acid and at the same time its OH is not free for conjugation (both due to H-bond formation) provides a good explanation for its negligible conjugation in the body.

The case of *o*-hydroxybenzaldehyde is complicated by the fact that it is oxidized in the body to salicylic acid, but it is probable, since 6% of it is conjugated, that this conjugation takes place before oxidation of the aldehyde group. Methyl salicylate also exhibits a low sulphate response, but this behaviour is no doubt due to its being considerably hydrolysed in the intestine giving free salicylic acid, which is not conjugated for reasons already mentioned. Since, however, ethereal sulphate corresponding to 5% of the ester fed is excreted, some must escape hydrolysis before conjugation. A considerable amount of hydrolysis of ester does occur during absorption from the intestine, since Hanzlik & Wetzel [1920] found that oral administration of the ester to dogs (0·2 g./kg.) resulted in the appearance of 0·2–0·5% of it in the urine, whilst intramuscular injection of the same amount results in some 14% of the ester appearing in the urine.

Other *o*-acidic groups depress the conjugation to about one-half of the unsubstituted phenol value, the average figure being 9–10% (phenol 19%), i.e. NO_2, 7; CH_2OH, 8; CH_2COOH, 10; Cl, 10; Br, 14%. In these cases it appears that the depression is a purely polar effect and that the *ortho*-effect is absent.

For *o*-basic groups one would expect, in the absence of any effect other than a purely polar one, the percentage conjugation to be greater than for *m*-basic groups. However, it has been found that the *o*-NH_2 and *o*-$CONH_2$ groups give values (29%) slightly lower than *m*-NH_2 and *m*-$CONH_2$ (33%). But the possibility of any other effect than a purely polar one must be ruled out, since in some experiments the *o*-$CONH_2$ group gave definitely higher values (e.g. 40%) than the *m*-$CONH_2$, although the average was lower. The lower values are probably due to experimental error.

In the *m*-position the effect seems to be purely polar but less than in the *o*-position.

In the *p*-position all influences are at a minimum; *p*-substitution has little effect on the sulphate conjugation, even though the groups are of different polarity. Groups like NO_2, Cl, Br, NH_2, $CHNH_2$ and OCH_3 are conjugated to the same extent as phenol. The CHO (12%), $COOCH_3$ (14%) and CH_3 (15·5%) groups give values somewhat lower than the general average for *p*-substitution. This is no doubt due to the fact that in the body they are partially

converted into *p*-COOH which has a low conjugation (7–8%) for a *p*-group. *p*-Cresol is partially oxidized to *p*-hydroxybenzoic acid [Preusse, 1881]. *p*-Hydroxybenzoic acid is not conjugated to a large extent in the dog or in man with either glycine or glucuronic acid [Quick, 1932, 2], although Quick is of the opinion that the free acid found in the urine has not passed through the body unchanged, but is probably derived from a strongly acidic glucuronic acid conjugate. It must be noted, however, that the hydroxybenzoic acids are relatively much stronger acids than any of the other phenols, which are extremely weak acids. The $CONH_2$ group increases the conjugation by more than 50% irrespective of its position, e.g. *o*-, 30%; *m*-, 33%; *p*-, 33%. No explanation is offered until the fate of these amides in the body has been further investigated.

Summary

The influence of dose on the sulphate conjugation of phenol in the rabbit has been studied. For doses from 100 to 250 mg./kg. the percentage of phenol conjugated with sulphate is constant at about 20%. For doses lower than 100 mg./kg. the percentage conjugated rises with falling dose.

The influence of *o*-, *m*- and *p*-substitution on the sulphate conjugation of phenol in the rabbit has also been studied. It is found that the conjugation is influenced by the nature and the relative position of the substituent.

1. In the *o*-position, acidic groups depress the conjugation by 50%, whilst basic groups increase it by 50%; neutral groups have no effect. The *o*-COOH group abolishes the conjugation entirely, this being probably due to the operation of the *ortho*-effect in addition to the usual influence of an *o*-acidic group.

2. In the *m*-position, the influence is similar to that in the *o*-position, but less pronounced.

3. In the *p*-position, groups of varying nature have little, if any, effect, with the exception of the COOH and $CONH_2$ groups.

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