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REFERENCES

Alekseeva, M. V. (1940). *Chem. Z.* **1**, 1712.
Baernstein, H. D. (1943). *Industr. Engng Chem.* (Anal. ed.), **15**, 251.
Bykhovskaya, M. S. (1945). *Zavodskaya Lab.* **11**, 537.
Drummond, J. C. & Finar, I. V. (1938). *Biochem. J.* **32**, 79.
Lazarev, N. V., Brusilovskaya, A. J. & Lavrov, J. N. (1931). *Arch. Hyg., Berl.*, **106**, 112.
Lehmann, K. B., Stohr, O., Kleiner, R. & Gundermann, S. O. (1910). *Arch. Hyg., Berl.*, **72**, 307.
Munk, I. (1876). *Pflüg. Arch. ges. Physiol.* **12**, 142.
Nencki, M. & Sieber, N. (1883). *Pflüg. Arch. ges. Physiol.* **31**, 319.
Pearce, S. J., Schrenk, H. H. & Yant, W. P. (1936). *Rep. U.S. Bur. Min.* no. 3302.
Peltzer, J. (1933). *Chem. Z.* **57**, 162.
Peronnet, M. (1935). *J. Pharm. Chim., Paris*, **21**, 503.
Porteous, J. W. & Williams, R. T. (1949). *Biochem. J.* **44**, 46.
Schrenk, H. H., Pearce, S. J. & Yant, W. P. (1935). *Rep. U.S. Bur. Min.*, no. 3287.
Schultzen, O. & Naunyn, B. (1867). *Arch. Anat. Physiol., Lpz.*, p. 349.
Wyler, O. (1931). *Helv. chim. Acta*, **15**, 23.

Studies in Detoxication

31. THE ISOLATION OF *m*- AND *p*-CYANOPHENOLS AS METABOLITES OF CYANOBENZENE (BENZONITRILE) AND THE PROBLEM OF THE ORIENTATION OF HYDROXYL GROUPS FORMED *IN VIVO*

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When monosubstituted benzenes carrying the classical *ortho-para*-directing groups (e.g. NH_2, NHAc, Cl, OH) are fed to animals, it is found that the urine contains conjugates of the corresponding *ortho*- or *para*-substituted phenols, or both, e.g. acetanilide (Smith & Williams, 1948), aniline (Smith & Williams, 1949), phenol (Garton & Williams, 1949) and chlorobenzene (Spencer & Williams, unpublished observations). Little definite information, however, is available about the position of biological hydroxylation of monosubstituted benzenes carrying the classical *meta*-directing groups (e.g. NO_2, CN, COOH). According to Meyer (1905) small amounts of *p*-nitrophenol occur in the urine of rabbits receiving nitrobenzene, but this was not unequivocally proved. Baumann (1883) isolated small amounts of *o*- and *p*-hydroxybenzoic acids from the acid-hydrolysed urines of dogs dosed with cyanobenzene. Evans, Parr & Evans (1949) have suggested that *p*-hydroxybenzoic acid may be the first product of the oxidation of benzoic acid by certain bacteria (see also Parr, Evans & Evans, 1949). The information available suggests that monosubstituted benzenes, if oxidized to phenols *in vivo*, give rise to *ortho*- or *para*-substituted phenols, or both, irrespective of the nature of the substituent.

Now Smith & Williams (1948) pointed out that the orientation of hydroxylation of acetanilide and aniline in the rabbit was similar to that found during nitration, i.e. *para* in acetanilide and mainly *ortho* and *para* in aniline. This suggested to us that one possible interpretation of the orientation of biological hydroxylation could be along the lines used to explain aromatic substitution in pure organic chemistry, i.e. that substitution is either ionic or free-radical in nature (for discussion of free-radical substitution see Hey & Waters, 1937, 1948; Waters, 1948; Dewar, 1949). Whilst other interpretations of the orientation of biological oxidation are also possible, we studied the fate of cyanobenzene and benzoic acid with the purely chemical theory of aromatic substitution in mind. Working on these assumptions we thought that if the orientation of hydroxylation *in vivo* were known for both *ortho*-, *para*- and *meta*-directing groups, then we could decide whether biological hydroxylation had the characteristics of ionic substitution or of free-radical substitution, assuming, of course, that the hydroxy-

lation was a substitution and not an addition reaction. The idea that free radicals may be involved in enzymic processes has been discussed by Michaelis (1946), Kalckar (1946), Delbrück (1944) and Waters (1946) among others. Regarding the question of whether it is possible to explain enzyme catalysis in terms of mesomeric free radicals, Kalckar (1946) makes the following statement: 'There are observations which may provide confirmation for such an explanation. Some years ago, Haas (1937) found that riboflavin phosphate, when linked to a protein, forms a semiquinone when undergoing reduction. This semiquinone is not observed during the reduction of free riboflavin phosphate in neutral solution, but accumulates when the reaction is acid enough to insure complete ionization. In other words, the enzyme is able to stabilize a product at neutral reaction which otherwise would exist only at strongly acid reaction. This observation may be taken as a clear indication that oxidation reduction enzymes in some way or other are concerned with the formation of mesomeric free radicals.'

EXPERIMENTAL

Preparation of reference compounds

Benzylamine salts of the cyanophenols

Equal weights of the cyanophenol and benzylamine were warmed together until a homogeneous liquid was obtained. On cooling, the mixtures containing the *o*- and *p*-isomers crystallized, whereas that containing the *m*-isomer remained liquid. The crystalline products were recrystallized from ethyl acetate, thus affording the *benzylamine salt* of o-*cyanophenol* which formed needles, m.p. 110–111°, sparingly soluble in light petroleum but soluble in water, ether, ethanol and ethyl acetate (Found: C, 73·9; H, 6·2; N, 12·3. $C_7H_5ON.C_7H_9N$ requires C, 74·3; H, 6·2; N, 12·4%); and the *benzylamine salt* of p-*cyanophenol* which formed prisms, m.p. 80°, which were very soluble in water, ether, ethanol and ethyl acetate and less soluble in light petroleum (Found: C, 73·9; H, 6·2; N, 11·9%). The *p*- was much more soluble than the *o*-derivative.

We failed to isolate a salt from *m*-cyanophenol, and the reason for this became apparent when we measured the dissociation constants of the three isomers. *m*-Cyanophenol was more feebly acidic than its isomers. The approximate pK_a's of the three cyanophenols found from their titration curves, determined with the glass electrode, were *o*-, 7·3, *m*-, 8·8 and *p*-, 8·2.

The azobenzene-4-carboxylic esters of the cyanophenols

Since it was possible that the cyanophenols of cyanobenzene urine could be separated by chromatography, it was decided to synthesize coloured derivatives of the phenols. We therefore synthesized the azobenzene-4-carboxylic esters. Equimolecular amounts of the cyanophenol and azobenzene-4-carbonyl chloride were refluxed for a few minutes with about 15 equiv. of pyridine. The solution was diluted with water and the precipitate collected and dried *in vacuo*. The solid was now extracted with $CHCl_3$ and the extract purified by passing through a short column of alumina (Peter Spence, Widnes; Grade H). The solvent was then evaporated and the residue recrystallized from a mixture of $CHCl_3$ and CCl_4.

o-Cyanophenyl azobenzene-4-carboxylate was obtained as clumps of orange needles, m.p. 153–155°, sparingly soluble in ethanol, light petroleum and CCl_4, but soluble in $CHCl_3$. (Found: N, 12·5. $C_{20}H_{12}O_2N_2$ requires N, 12·8%.) *m-Cyanophenyl azobenzene-4-carboxylate* formed sheaves of orange needles, m.p. 143–147°, of similar solubility to the 2-isomer. (Found: N, 13·1%.) *p-Cyanophenyl azobenzene-4-carboxylate* formed orange rectangular plates, m.p. 175°, with similar solubility to the other isomers. (Found: N, 13·0%.)

2:4-*Dinitrophenyl ethers of the cyanophenols*

These were prepared from cyanophenols and 1:2:4-chlorodinitrobenzene following directions given by Wild (1947).

2:4-*Dinitro-2'-cyanodiphenyl ether* formed small crossed rods, m.p. 115°. (Found: C, 55·0; H, 2·4; N, 14·9. $C_{13}H_7O_5N_3$ requires C, 54·8; H, 2·5; N, 14·7%.) 2:4-*Dinitro-3'-cyanodiphenyl ether* formed branching clusters of needles, m.p. 130°. (Found: C, 55·1; H, 2·4; N, 14·9%.) 2:4-*Dinitro-4'-cyanodiphenyl ether* formed clusters of plates, m.p. 134°. (Found: C, 55·0; H, 2·4; N, 15·2%.)

Metabolites of cyanobenzene

Preliminary experiments of a quantitative nature showed that when cyanobenzene (b.p. 188–190°/760 mm.) was fed to rabbits there was an increased output of ethereal sulphate and conjugated glucuronic acid. In two experiments it was found that 23 and 27% of the dose (145 mg./kg.) was excreted as ethereal sulphates, and 38 and 28% as conjugated glucuronic acids in 2 days. Determinations of mercapturic acid output also suggested that about 5% of the cyanobenzene was excreted as a mercapturic acid. A further metabolite appeared to be benzoic acid. It was also noted that the excretion of these metabolites persisted over 2–4 days after dosing, despite the fact that the dose was a relatively small one. It was clear, therefore, that about 60% of the cyanobenzene was hydroxylated and excreted as oxygen-conjugates.

Mercapturic acid formation

According to Baumann (1883) cyanobenzene gives rise to a mercapturic acid. Attempts were made to prove this by isolation and by quantitative estimation by the iodometric method of Stekol (1936) for dog and rat urines (see also Binkley, 1949), with slight modifications for rabbit urines. In adapting this method for various urines, attention had to be paid to the amounts of 0·5N-NaOH and 10% (w/v) $ZnSO_4$ used for the removal of interfering substances. The correct amounts of the $ZnSO_4$-NaOH precipitants were arrived at by finding out how much of the precipitants were necessary to give the same blank titration on the filtrate from normal urines before and after alkaline hydrolysis. Daily urines (average 140 ml.) were centrifuged and made up to 200 ml. with water. 50 ml. samples were then analysed for mercapturic acid by Stekol's method using 15 ml. of 0·5N-NaOH and 15 ml. of 10% (w/v) $ZnSO_4$ to precipitate interfering substances. Three female chinchilla rabbits, on a constant diet, were given cyanobenzene orally. The urine was analysed daily for mercapturic acid (Table 1) until the I titrations reverted to normal values. On the reasonable assumption that the I used up is due to a mercapturic acid, then rabbit

Table 1. *Mercapturic acid in the urine of rabbits receiving cyanobenzene*

(Cyanobenzene administered on the fourth day; MA = mercapturic acid.)

Rabbit no.	127			165			168		
Rabbit wt. (kg.) ...	2·8			2·5			2·9		
Cyanobenzene dose (mg.)	560			500			580		
	Titre (0·01 N-I_2)		MA	Titre (0·01 N-I_2)		MA	Titre (0·01 N-I_2)		MA
Day	Before hydrolysis (ml.)	After hydrolysis (ml.)	as PhCN (mg.)	Before hydrolysis (ml.)	After hydrolysis (ml.)	as PhCN (mg.)	Before hydrolysis (ml.)	After hydrolysis (ml.)	as PhCN (mg.)
1	0·21	0·24	—	0·22	0·23	—	0·21	0·23	—
2	0·21	0·24	—	0·22	0·25	—	0·20	0·22	—
3	0·23	0·20	—	0·20	0·22	—	0·23	0·26	—
4	0·21	0·70	16·2	0·21	1·10	29·3	0·22	0·87	21·4
5	0·23	0·62	12·9	0·21	0·32	3·6	0·22	0·36	4·6
6	0·23	0·23	—	0·23	0·24	—	0·20	0·22	—

no. 127 excreted 5·3%, no. 165, 5·7% and no. 168, 5·2% of the dose of cyanobenzene as a mercapturic acid, which may be *p*-cyanophenylmercapturic acid. Attempts at isolation of the compound from rabbit urine have so far been unsuccessful and until this has been done, there is no unequivocal proof that cyanobenzene forms a mercapturic acid.

Cyanobenzene urine

In most experiments cyanobenzene, dissolved in olive oil, was fed by stomach tube. At a dose level of 150 mg./kg. no toxic effects were observed but higher doses (250 mg./kg.) were liable to be fatal. The bulk of the metabolites appeared in the urine on the second and third day after feeding. The urine reduced Benedict reagent very slightly but gave no colour with $FeCl_3$. The naphthoresorcinol test was positive. Tests for thiocyanate by $FeCl_3$ and by the $CoSO_4$ procedure of Feigl (1947) were negative. On mild hydrolysis with acid followed by ether extraction, an extract was obtained which gave a weak green colour with $FeCl_3$ chloride and reduced ammoniacal $AgNO_3$ in the cold.

This latter test suggested that a cyanocatechol may be excreted in small amounts but we were unable to isolate it and compare it with synthetic 4-cyanocatechol.

Isolation of benzoic acid

The urine (1 l.) from five rabbits which had received collectively 4 g. of cyanobenzene in aqueous suspension was collected over 2 days. After adding 100 ml. of conc. HCl, the urine was heated for 20 min. at 80°. After cooling, it was extracted with 2 × 200 ml. of ether in a separating funnel. Evaporation of the ether left an oil smelling faintly of cyanobenzene. The oil partly crystallized and by pressing on a porous tile 500 mg. of benzoic acid, m.p. and mixed m.p. 121°, were isolated.

The ethereal sulphate fraction. Isolation of m- *and* p-*cyanophenols*

(i) *Isolation of the mixed phenols.* A total of 2·8 g. of cyanobenzene in olive oil was fed to six rabbits. The urine (1·9 l.) collected over 4 days was brought to pH 4 with HCl and continuously extracted with ether for 6 hr. Evaporation of the ether left a brown tar from which nothing crystalline was isolated.

The urine was now brought to pH 1, heated at 85° for 40 min. to hydrolyse ethereal sulphates, and continuously extracted with ether for 3 hr. Evaporation of the ether left a brown tar which gave a weak green colour with $FeCl_3$ and a blue colour at pH 8 with 2:6-dichloroquinone chloroimide. Purification, but no separation, of the phenols in the tar was achieved by passing the tar dissolved in ether through a column of alumina (Peter Spence, Widnes; grade H). From the eluate 680 mg. of mixed cyanophenols (19% of dose) were obtained. Further elution of the column with methanolic HCl gave a brown tar which gave a green colour with $FeCl_3$, but we were unable to isolate cyanocatechol or protocatechuic acid from it.

The mixture of cyanophenols gave a purple colour with $FeCl_3$ and a blue colour at pH 8 with 2:6-dichloroquinone chloroimide. These tests indicated that the mixture contained an *o*- or *m*-substituted phenol, for we found that pure *o*- and *m*-cyanophenols gave a blue colour with 2:6-dichloroquinone chloroimide at pH 8, whereas the pure *p*-isomer gave no colour. 4-Cyanocatechol also gives a blue colour with this reagent. All three cyanophenols give a weak purple colour with $FeCl_3$. 4-Cyanocatechol gives the usual catechol colours with $FeCl_3$. The mixture also had a sweet taste, and it has been reported by Griess (1876) that of the three cyanophenols only the *m*-isomer has a sweet taste, a fact which we have confirmed.

(ii) *Isolation of* p-*cyanophenol.* The mixed phenols (680 mg.) were dissolved in 1 ml. of benzene and 1 ml. of light petroleum added. A crystalline precipitate of *p*-cyanophenol (125 mg. or 3·5% of dose) separated. After recrystallization from benzene it had m.p. and mixed m.p. 110–112°. (Found: N, 11·7. Calc. for C_7H_5ON: N, 11·8%.)

In another experiment 140 mg. of the mixed phenols were treated with 0·15 ml. of benzylamine and warmed until fluid. On cooling, the mixture set to a paste of crystals which, when filtered with suction and washed with light petroleum, gave 140 mg. (5% of dose and 53% of the mixed phenols) of the benzylamine salt of *p*-cyanophenol. After recrystallization from a small amount of ethyl acetate, the salt had m.p. and mixed m.p. 80° with an authentic specimen (cf. p. 244). (Found: C, 74·8; H, 6·3; N, 11·8%.)

(iii) *Isolation of* m-*cyanophenol.* The mixed phenols (600 mg.) were warmed with benzylamine (500 mg.) until liquid and the product was cooled to −15° for 0·5 hr. Crystals of the benzylamine salt of *p*-cyanophenol (420 mg. or 37% of the mixture) were washed with a mixture of equal volumes of light petroleum and benzene and dried with suction. The filtrate was evaporated to dryness and the residue dissolved in 10 ml. of ether and extracted with 2 × 5 ml. of 2 N-HCl to

remove benzylamine. The ether was extracted with 8 × 2 ml. of 2N-Na_2CO_3, and the alkaline extracts were acidified with HCl and extracted with ether. After drying, removal of the solvent left a crystalline residue (140 mg., m.p. 50–55°) which gave a weak purple colour with $FeCl_3$ and a strong blue colour with 2:6-dichloroquinone chloroimide in alkaline solution. The product (120 mg.) was then refluxed for 1 hr. with 0·5 ml. of 2N-NaOH and 200 mg. of 1:2:4-chlorodinitrobenzene, and the pasty precipitate (65 mg.) was washed with ether and filtered. On recrystallization twice from ethanol the characteristic branched needles of 2:4-dinitro-3′-cyanodiphenyl ether were obtained. The m.p. and mixed m.p. was 130–131°. The m.p. was considerably depressed by the corresponding *o*- and *p*-cyanophenol derivatives. (Found: C, 54·95; H, 2·1; N, 14·4%.) The amount of *m*-cyanophenol isolated here corresponded to 5% of the mixed phenols.

The presence of *m*-cyanophenol was confirmed by the isolation of its azobenzene-4-carbonyl ester. The ethereal solution left after extraction with 2N-Na_2CO_3 (see above) was evaporated to dryness. The residue (100 mg.) was boiled for a few minutes with 200 mg. of azobenzene-4-carbonyl chloride in 2 ml. of pyridine. Dilution to 50 ml. with water yielded a precipitate which was collected and dried *in vacuo*. The dried material was now purified by dissolving in $CHCl_3$, filtering and passing through a short column of alumina (Peter Spence, Widnes, grade H). Evaporation of the eluate after washing the column with $CHCl_3$ left an orange residue (50 mg. or 3% of the mixed phenols) which crystallized from $CHCl_3$ as orange plates (26 mg.), m.p. 145–146° not depressed by authentic *m*-cyanophenyl azobenzene-4-carboxylate but considerably depressed by the corresponding esters of *o*- and *p*-cyanophenol. (Found: C, 73·0; H, 4·1; N, 12·7%.)

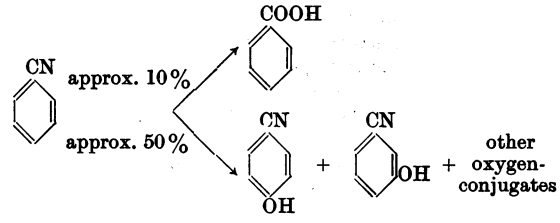
Search for oxidation products of benzoic acid

The main metabolites of benzoic acid are known to be hippuric acid and benzoylglucuronide, and since it does not cause increased excretion of ethereal sulphates in the rabbit (Bray, Neale & Thorpe, 1946) it seems unlikely that it is oxidized to phenolic acids. However, the possible oxidation products are *o*-, *m*- and *p*-hydroxybenzoic acids and protocatechuic acid. Now salicylic acid is known not to form an ethereal sulphate in the rabbit (Williams, 1938; Bray, Ryman & Thorpe, 1948), but it is easily detectable if present. The *m*- and *p*-hydroxybenzoic acids have low sulphate conjugations in the rabbit (Williams, 1938; Hartles & Williams, 1948; Bray *et al.* 1948). Should any of these acids be formed metabolically from benzoic acid, their formation could not be detected by ethereal sulphate determinations. Protocatechuic acid, however, does conjugate appreciably with sulphate in the rabbit (Dodgson, Garton & Williams, 1947; Dodgson & Williams, 1949). Benzoic acid was therefore fed to rabbits and the urine fractionated. The fractions were examined in detail for salicylic acid and protocatechuic acid by colour reactions and for *m*- and *p*-hydroxybenzoic acids spectroscopically.

Although the urine was examined in considerable detail, the only compounds found were hippuric acid, benzoylglucuronide and free benzoic acid. We found no hydroxybenzoic acids.

DISCUSSION

In the present paper we have not dealt with the glucuronic acid fraction of cyanobenzene urine. Although we have examined this fraction several times we have been unable to elucidate its nature except to show that a small part of it is benzoylglucuronide. However, as far as we have gone, the metabolites of cyanobenzene in the rabbit are *m*- and *p*-cyanophenols, which are excreted as ethereal sulphates, and benzoic acid, which is partly combined with glycine and glucuronic acid. Some of our evidence also suggests the presence of traces of a catechol derivative and small amounts of a mercapturic acid. We were unable to detect thiocyanates which are metabolites of aliphatic cyanides and derivatives of benzyl cyanide (Williams, 1947). Of the earlier work on cyanobenzene, Giacosa (1883) showed that it formed small amounts of benzoic acid in the dog; Baumann (1883) hydrolysed cyanobenzene urine from dogs and isolated small amounts of *o*- and *p*-hydroxybenzoic acids; and Adeline, Cerecedo & Sherwin (1926) failed to detect any metabolites in rabbits. Accordingly, the fate of cyanobenzene in the body may follow three paths: (*a*) oxidation to phenols, (*b*) hydrolysis to benzoic acid, and (*c*) possibly mercapturic acid formation. The third path is suggested tentatively and requires further confirmation. On the present evidence the following scheme may be suggested:



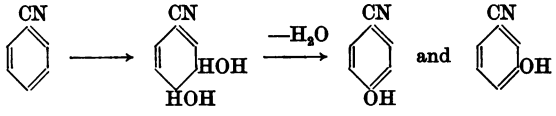
Although the work of Baumann (1883) suggests that cyanobenzene may undergo oxidation in the *ortho* position, we have not been able to detect *o*-cyanophenol or salicylic acid in our experiments.

There is a sharp distinction between benzoic acid and cyanobenzene in that it appears that the latter alone suffers oxidation in the body, a difference which obviously requires some explanation. Lederer (1949) (Lederer & Polonsky, 1948) has recently reported that *o*-, *m*- and *p*-hydroxybenzoic acids are constituents of normal urine. All three acids have been isolated from the urine of pregnant mares and the scent gland of the beaver. Furthermore, Lederer refers to the observation of Bielig & Hayasida (1940), who isolated *m*-hydroxybenzoic acid from rabbit urine. These acids could have arisen by the hydroxylation *in vivo* of benzoic acid, although other explanations are not excluded, and our negative results may have been due to not working on a large enough scale. However, it is clear that if benzoic acid is oxidized *in vivo*, then the extent of oxidation must be a very small one. Now it appears that benzoic acid is rapidly excreted, whereas cyanobenzene is

very slowly eliminated. There may therefore be a time factor involved, and consequently cyanobenzene has more opportunity of being oxidized than benzoic acid. Furthermore, the elimination of benzoic acid is assisted by its rapid conjugation with glycine and glucuronic acid to form conjugates which are quickly eliminated from the body. It is also possible that benzoic acid does not reach the site of hydroxylation in the body if this is different from that of conjugation, for relatively strongly ionized compounds do not penetrate cell membranes (Höber, 1946). Evidence in support of the suggestion that a time factor is involved can be gathered from the facts that salicylic acid, a substance which is slowly eliminated from the body (Quick, 1933; Kapp & Coburn, 1942) is in fact hydroxylated to gentisic acid (Baldoni, 1908; Angelico, 1921; Kapp & Coburn, 1942; Bray *et al.* 1948), and that benzoic acid is hydroxylated by certain soil bacteria when it is added to the medium in which the organisms are growing and is thus in contact with the organisms for a prolonged period (Evans, 1947).

The important observations made in this work are, first, that *meta*-hydroxylation appears to occur in the body, and secondly, that although cyanobenzene carries a *meta*-directing group it appears to undergo *para*-hydroxylation as do those monosubstituted benzenes carrying *ortho-para*-directing groups. Further work (unpublished) in this laboratory indicates that nitrobenzene is also *meta*-hydroxylated for we have isolated *m*-nitrophenol from the urine of rabbits receiving nitrobenzene and found strong indications that the *para*-isomer is also formed. The rest of this discussion will deal with how these observations could be interpreted in the light of current theories of diol formation *in vivo* (cf. Boyland, 1949; Boyland & Weigert, 1947) and of aromatic substitution.

In the first place it is possible that the *m*- and *p*-cyanophenols could have arisen during the isolation procedures as the result of dehydration by acid of a cyanodihydrobenzene-3:4-diol thus:



Dihydrodiols of polycyclic hydrocarbons are known to undergo this reaction (Young, 1947; Boyland & Wolf, 1948) although only one phenol, and not two as above, is produced in the cases of the diols of naphthalene and anthracene (for the action of dilute acid on the diols of other polycyclic hydrocarbons, see Cook & Schoental, 1948). However, we looked for a diol in cyanobenzene urine but did not detect it. It seems likely that the *m*- and *p*-cyanophenols were present in the urine mainly as ethereal sulphates because about 25% of the cyanobenzene fed was excreted as ethereal sulphate. It is possible that they were produced metabolically from a diol (cf. Berenblum & Schoental, 1949).

If our results are considered from the standpoint of the rules of aromatic substitution, the predicted orientation for anionic substitution of cyanobenzene would be *ortho* and *para*, whereas in the case of aniline, for example, the orientation would be *meta*. In the case of cationic substitution the predicted orientation would be *meta* for cyanobenzene and *ortho* and *para* for aniline. Now it has been found that, in the rabbit, cyanobenzene is *meta*- and *para*-hydroxylated, whereas aniline is *ortho*- and *para*-hydroxylated (Smith & Williams, 1949). These orientations cannot be explained in terms of either anionic or cationic substitution alone, although they can, if both mechanisms were assumed to act simultaneously, which, however, is very unlikely. However, it is known, in purely chemical reactions, that abnormal orientations occur when the reagent used gives rise to free radicals and in these cases all three positions may be substituted, the *para* being the more favoured, irrespective of the normal directing properties of the existing substituent for ionic substitutions. It appears, therefore, that the biological orientations found with cyanobenzene and aniline are analogous with those found during free-radical substitution. The question now arises whether the occurrence of abnormal substitution can be considered as evidence for the participation of neutral free radicals. It appears to us that there is a tendency to accept this for certain purely chemical reactions (cf. Hey, 1941; Dewar, 1949; Loebl, Stein & Weiss, 1949). Whether this is also true for biochemical reactions may be disputed. In this connexion it is interesting to note that Stein & Weiss (1948) have suggested that hydroxyl radicals may play an important part in certain biological oxidations. They found that when solutions of benzene and benzoic acid were submitted to the action of hydroxyl radicals produced by radiations, these compounds were hydroxylated and the products were similar to those obtained in the biological oxidation of these substances.

SUMMARY

1. A study has been made of the hydroxylation of cyanobenzene and benzoic acid in the rabbit.

2. No evidence was obtained of the oxidation of benzoic acid; the only metabolites obtained here were the unchanged acid, its glycine derivative and glucuronide.

3. Cyanobenzene is very slowly eliminated and undergoes considerable change *in vivo*. About 60% is excreted as oxygen conjugates, 10% is transformed to benzoic acid and it is suggested that another 5% forms a mercapturic acid.

4. The ethereal sulphate fraction of cyanobenzene urine contains *m*- and *p*-cyanophenols, which were isolated. The formation of *m*-cyanophenol is believed to be the first reported case of *meta*-hydroxylation *in vivo*. The nature of the glucuronide fraction was not elucidated.

5. The azobenzene-4-carbonyl esters, 2:4-dinitrophenyl ethers and benzylamine salts of the cyanophenols have been synthesized and used as reference compounds. The pK_a's of *o*-, *m*- and *p*-cyanophenols have been measured.

6. The results have been discussed in the light of current theories concerning diol formation *in vivo* and aromatic substitution *in vitro*.

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REFERENCES

Adeline, M., Cerecedo, L. R. & Sherwin, C. P. (1926). *J. biol. Chem.* **70**, 461.
Angelico, F. (1921). *Arch. Farmacol. sper.* **31**, 8.
Baldoni, A. (1908). *Arch. Farmacol. sper.* **8**, 174, 193.
Baumann, E. (1883). *Hoppe-Seyl. Z.* **8**, 195.
Berenblum, I. & Schoental, R. (1949). *Biochem. J.* **44**, 604.
Bielig, H. J. & Hayasida, A. (1940). *Hoppe-Seyl. Z.* **266**, 99.
Binkley, F. (1949). *J. biol. Chem.* **178**, 811.
Boyland, E. (1949). *Ann. Rev. Biochem.* **18**, 225.
Boyland, E. & Weigert, F. (1947). *Brit. med. Bull.* **4**, 359.
Boyland, E. & Wolf, G. (1948). *Biochem. J.* **42**, xxxii.
Bray, H. G., Neale, F. C. & Thorpe, W. V. (1946). *Biochem. J.* **40**, 134.
Bray, H. G., Ryman, B. E. & Thorpe, W. V. (1948). *Biochem. J.* **43**, 561.
Cook, J. W. & Schoental, R. (1948). *J. chem. Soc.* p. 170.
Delbrück, M. (1944). (Cited by Kalckar (1946).)
Dewar, M. J. S. (1949). *Electronic Theory of Organic Chemistry*, p. 278. Oxford: Clarendon Press.
Dodgson, K. S., Garton, G. A. & Williams, R. T. (1947). *Biochem. J.* **41**, 1.
Dodgson, K. S. & Williams, R. T. (1949). *Biochem. J.* **45**, 381.
Evans, W. C. (1947). *Biochem. J.* **41**, 373.
Evans, R. A., Parr, W. H. & Evans, W. C. (1949). *Biochem. J.* **44**, viii.
Feigl, F. (1947). *Qualitative Analysis by Spot Tests*, 3rd ed., p. 214. New York and Amsterdam: Elsevier.
Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **45**, 170.
Giacosa, P. (1883). *Hoppe-Seyl. Z.* **8**, 95.
Griess, P. (1876). *Ber. dtsch. chem. Ges.* **8**, 859.
Haas, E. (1937). *Biochem. Z.* **290**, 291. (Cited by Kalckar, 1946.)
Hartles, R. L. & Williams, R. T. (1948). *Biochem. J.* **43**, 296.
Hey, D. H. (1941). *Rep. Progr. Chem.* **37**, 282.
Hey, D. H. & Waters, W. A. (1937). *Chem. Rev.* **21**, 169.
Hey, D. H. & Waters, W. A. (1948). *J. chem. Soc.* p. 882.
Höber, R. (1946). *Physical Chemistry of Cells and Tissues*, p. 571. London: Churchill.
Kalckar, H. M. (1946). *Currents in Biochemical Research*, p. 229. New York: Interscience.
Kapp, E. M. & Coburn, A. F. (1942). *J. biol. Chem.* **149**, 549.
Lederer, E. (1949). *J. chem. Soc.* p. 2115.
Lederer, E. & Polonsky, J. (1948). *Biochim. Biophys. Acta*, **2**, 431.
Loebl, H., Stein, G. & Weiss, J. (1949). *J. chem. Soc.* p. 2074.
Michaelis, L. (1946). *Currents in Biochemical Research*, p. 216. New York: Interscience.
Meyer, E. (1905). *Hoppe-Seyl. Z.* **46**, 497.
Parr, W. H., Evans, R. A. & Evans, W. C. (1949). *Biochem. J.* **45**, xxix.
Quick, A. J. (1933). *J. biol. Chem.* **101**, 475.
Smith, J. N. & Williams, R. T. (1948). *Biochem. J.* **42**, 538.
Smith, J. N. & Williams, R. T. (1949). *Biochem. J.* **44**, 242.
Stein, G. & Weiss, J. (1948). *Nature, Lond.*, **161**, 650.
Stekol, J. A. (1936). *J. biol. Chem.* **113**, 279.
Waters, W. A. (1946). *Chemistry of Free Radicals*, p. 259. Oxford: Clarendon Press.
Waters, W. A. (1948). *J. chem. Soc.* p. 727.
Wild, F. (1947). *Characterization of Organic Compounds*, p. 70. Cambridge University Press.
Williams, R. T. (1938). *Biochem. J.* **32**, 878.
Williams, R. T. (1947). *Detoxication Mechanisms*, p. 125. London: Chapman and Hall.
Young, L. (1947). *Biochem. J.* **41**, 417.

The Oxidation of Pyruvate and Fatty Acids by *Mycobacterium ranae*

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A study of the intermediary metabolism of *Mycobacterium phlei* led Edson & Hunter (1947) to suggest that the aerobic breakdown of lactate may proceed through pyruvate to acetate by two routes, one of which involves a coupled non-enzymic oxidation of pyruvate. The alternative pathway via enzymic oxidation of pyruvate requires investigation, because it offers the possibility of partial assimilation at an early stage. Since added pyruvate is metabolized slowly by washed suspensions of *Mycobact. phlei*, we decided to study the oxidation of this substrate by *Mycobact. ranae* which utilizes both pyruvate and

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