Bacterial catalase (from Micrococcus lysodeikticus)

Sample A I. This had an activity corresponding to a *Kat.f.* of 99,000 (Herbert, private communication). It gave a single homogeneous component with some heavier and lighter impurities (Fig. 1). The S_{20} (corr.) was 11.0×10^{-13} , and integration of the boundary gave 85% of the initial refracting material.

Sample A II. This was another preparation of slightly lower activity, *Kat.f.* = 90,000 (Herbert, private communication). It also gave a single homogeneous component with heavier and lighter impurities (Fig. 2), but integration gave only 70% of the initial refracting material. The S_{20} (corr.) was again 11.0×10^{-13} .

Human erythrocyte catalase

A single sample was examined. The *Kat.f.* when first prepared was 63,000, but during keeping it dropped to 44,300 (Herbert, private communication), suggesting that some denaturation had taken place. The sedimentation diagram was similar to that of the two previous samples except for a

larger proportion of the heavier impurity, possibly denatured catalase. This impurity can be seen clearly in an early photograph (Fig. 3a), but it dispersed as the run proceeded (Fig. 3b) suggesting heterogeneity. Some of the pigment was seen to be associated with it. The S_{20} (corr.) of the main component was 11.2×10^{-13} .

CONCLUSION AND SUMMARY

1. The ultracentrifugal data suggest that, while none of the preparations is fully homogeneous, there is in each case a main component which is associated with most of the pigment.

2. The sedimentation constants of this component in preparations from *Micrococcus lysodeikticus* and from human blood are similar (11.0 and 11.2×10^{-13} respectively), though the absolute difference is probably outside the experimental error.

3. The value of 11.2×10^{-13} for the sedimentation constant of human-blood catalase agrees with Agner's (1938) value of 11.2×10^{-13} for horse-liver catalase and with Sumner & Gralén's (1938) value of 11.3×10^{-13} for cow-liver catalase.

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Studies in Detoxication

17. THE FATE OF CATECHOL IN THE RABBIT AND THE CHARACTERIZATION OF CATECHOL MONOGLUCURONIDE

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(Received 21 January 1948)

The main object of this investigation was to find out if conjugation of catechol occurs *in vivo* on one or both of its hydroxyl groups. The study of the fate of catechol was also necessary because it had a bearing on other problems being studied in this laboratory, namely, the metabolism of adrenaline (cf. Dodgson, Garton & Williams, 1947) and of benzene (cf. Porteous, Smith & Williams, 1947).

Despite the importance of catechol compounds in biochemistry, no significant study of the fate of catechol in the intact animal has been made other than that of Baumann (1876) who showed that

administration of catechol to dogs caused an increased output of ethereal sulphate. However, numerous studies have been carried out *in vitro* of the formation and oxidation of catechol by enzymes of the tyrosinase type (see review by Nelson & Dawson, 1944).

From a consideration of the structure of catechol and of the results of enzyme studies, it is clear that it may form in the body (1) an ethereal mono- or disulphate or both, (2) a mono- or di-glucuronide or both, (3) *o*-benzoquinone by oxidation, (4) hydroxy-quinol (1:2:4-trihydroxybenzene) and its conju-

gates, by oxidation, and (5) muconic acid by ring fission as suggested by Drummond & Finar (1938). These possibilities are formulated as follows:

where X = an ethereal sulphate or glucuronic acid group.

Evidence will be presented to show that the metabolites excreted are mainly the monoglucuronide and monosulphate of catechol together with small amounts of conjugated hydroxyquinol.

stomach tube. We found that in five rabbits 100 mg./kg. had no apparent effect; at 175 mg./kg. slight toxic effects were apparent; at 200 mg./kg. the rabbits showed immediate signs of distress such as rapid breathing and twitching, with occasional paralysis of the limbs and squeaking. After about 3 hr. the animals appeared to be normal. One experiment was carried out using a dose of 250 mg./kg. which was fatal within 24 hr.

EXPERIMENTAL

Animals. Giant chinchilla rabbits were used and fed on a diet of 50 g. Lever's cubes and water daily unless otherwise stated.

The effect of catechol on rabbits. Catechol is a relatively toxic substance. For cats and rats an oral dose of 50 mg./kg. is lethal (Dietering, 1938), whereas for rabbits the lethal

Quantitative results

Ethereal sulphate and glucuronic acid outputs. Ethereal sulphate excretion was determined by the method of Williams (1938) and glucuronic acid excretion according to Hanson, Mills & Williams (1944). The results are given in Table 1 and Fig. 1 illustrates a single experiment.

Fig. 1. The effect of feeding catechol (300 mg.) on the glucuronic acid and ethereal sulphate output of rabbit no. 113 (3 kg. wt.). Upper curve, glucuronic acid; lower curve, ethereal sulphate. Dotted lines show average normal values for (A) glucuronic acid, and (B) ethereal sulphate.

Table 1. The glucuronic acid and ethereal sulphate excretion of rabbits receiving catechol orally

Rabbit no.	Weight (kg.)	Dose (mg.)	Normal values of		Extra ethereal sulphate (mg.)	Extra glucuronic acid (mg.)	% of dose excreted	
			Ethereal sulphate (mg./day)	Glucuronic acid (mg./day)			As sulphate	As glucuronide
97* ♂	2.6	262	28.5	—	31.5	—	16.5	—
98* ♂	3.1	322	28.5	—	54.2	—	18.9	—
113† ♀	3.0	300	15.3	141	38.8	412	17.7	77.9
115† ♀	2.6	260	—	171	—	266	—	60.7
117† ♀	2.7	270	—	117	—	341	—	71.6
Average							17.7	70

* Diet, 50 g. Lever's cubes + 100 g. cabbage per diem.
† Diet, 50 g. Lever's cubes + water *ad lib.* per diem.

Table 1 shows that about 17–18% of the catechol fed is excreted as a sulphate and about 70% as a glucuronide, assuming that monoconjugates are excreted. Furthermore, about 87–88% of the catechol fed can be accounted for by these conjugates excreted within 24 hr. of dosing. In the case of rabbit 113, it had been possible to carry out both estimations simultaneously and here the sum of the ethereal sulphate and glucuronide output is 95%. These results indicate that the main bulk of the catechol fed is excreted conjugated. A small amount of free catechol is also excreted which we assess at no more than 5%, for we isolated about 2% of the dose as derivatives of catechol (see below).

The isolation of catechol metabolites

(a) *Catechol urine.* Rabbits receiving oral doses of catechol excrete a slightly alkaline urine, which is normal in colour but darkens on standing. It gives the green colour characteristic of free catechol with FeCl_3 , but does not reduce Fehling's or Benedict's reagents. It gives a strongly positive naphthoresorcinol reaction.

(b) *Detection of free catechol.* Urine (550 ml. in 24 hr.) from three animals after the feeding of a total of 0.68 g. of catechol was acidified with a few drops of conc. HCl, and extracted with ether for 1 hr. in a continuous extractor. The ether was removed at 20° under reduced pressure leaving 300 mg. of a brownish gum. This was dissolved in a few ml. of 40% (w/v) NaOH and benzoylated in the usual manner. Repeated recrystallization of the product from 95% ethanol yielded 10 mg. (0.7% of dose) of catechol dibenzoate, m.p. 83°, or 84–5° mixed with synthetic material (m.p. 85°).

(c) *Isolation and characterization of catechol monoglucuronide.* (i) *Preparation of the glucuronide gum.* A total of 7.59 g. of catechol was fed to 12 rabbits and a 24 hr. urine (1750 ml.) collected. The urine was clarified by centrifuging and made faintly acid (grey with congo red paper) by adding glacial acetic acid. Saturated lead acetate solution (350 ml.) was then added and the precipitate filtered off. The filtrate was made faintly alkaline with a little ammonia (sp.gr. 0.88) and treated with 200 ml. saturated basic lead acetate solution. The white precipitate was filtered off, washed well with cold water, made into a thin cream with water and the lead was removed completely with H_2S . The filtrate from the lead sulphide was taken to dryness *in vacuo* at 45–50°, and there remained a brown gum (12.5 g.) which showed a very slight reducing action towards Benedict's reagent and contained free catechol as indicated by its reaction with FeCl_3 . The gum was taken up in absolute ethanol and on standing overnight a sludge of inorganic matter separated. This was removed by filtration and then the ethanolic solution was reduced to 30 ml. *in vacuo*. This solution was now poured into 300 ml. ether. Most of the glucuronide remained in solution but about 2 g. of gummy material containing much inorganic matter were thrown out. After removal of this, the ethanol-ether solution was concentrated *in vacuo* to a clear brown gum (10 g.). At this stage the purified gum gave a strong green colour with FeCl_3 , a strong naphthoresorcinol reaction and reduced Benedict's reagent very slightly. Attempts to crystallize the glucuronide or to prepare crystalline salts with benzylamine, *o*- or *p*-toluidine or benzidine were unsuccessful.

(ii) *Removal of free catechol from the glucuronide gum.* The gum (6 g.) was dissolved in 75 ml. water and the solution

was extracted with 3 × 100 ml. portions of ether. The extract was dried with anhydrous Na_2SO_4 and taken to dryness *in vacuo* at 20°. The semi-crystalline residue (730 mg.) gave intense colour reactions for catechol but gave no tests for hydroxyquinol (see below). It was dissolved in 12 ml. 2N-NaOH and immediately mixed with acetone (10 ml.) containing *p*-toluenesulphonyl chloride (1 g.). The mixture, which turned purple, was shaken vigorously for 15 min. and then poured into 30 ml. cold water. The white crystalline precipitate (300 mg., m.p. 159°) was collected and recrystallized from absolute ethanol. The product formed colourless needles and was identified as *catechol di-p-toluenesulphonate*, m.p. 161°. (Found: C, 57.8; H, 4.6; S, 14.95. $\text{C}_{20}\text{H}_{18}\text{O}_8\text{S}_2$ requires C, 57.4; H, 4.3; S, 15.3%.) This appears to be a new compound; an authentic specimen, m.p. 162–163°, and 161–162° when mixed with the material described above, has been prepared in another investigation (Porteous & Williams, unpublished). The yield of catechol ester corresponded to 1.8% of the catechol fed.

(iii) *Conversion of the glucuronide gum into the methyl ester of guaiacol triacetylglucuronide.* The solution after removal of catechol was taken to dryness and the residual gum (5.2 g.) was dissolved in 30 ml. absolute ethanol and treated with 250 ml. of a saturated ethereal solution of diazomethane. The mixture was kept at room temperature overnight. A slight precipitate of inorganic material was removed by filtration. The solution on evaporation yielded a pale yellow gum. Since the gum was acidic to litmus and incompletely soluble in ether, the treatment with diazomethane was repeated and on removal of the solvents a pale yellow, neutral, ether-soluble gum was obtained. This gum, which is presumably guaiacol glucuronide methyl ester, could not be induced to crystallize. It was therefore dissolved in 10 ml. pyridine followed by 15 ml. acetic anhydride and the mixture kept at room temperature overnight. On pouring into 50 ml. ice water a semi-solid gum was precipitated. This gum was now exhaustively extracted with chloroform yielding an orange-yellow solution which was washed twice with 2N-HCl, then twice with 10% (w/v) Na_2CO_3 solution and finally with water. The washed solution was now treated with charcoal, filtered and dried over CaCl_2 . Removal of the solvent on the water bath left a clear yellow gum soluble in ethanol, acetone, ether and benzene but insoluble in water. Crystallization of the gum was achieved by dissolving in a minimum of acetone and then adding it to a large volume of warm water. Any cloudiness was dispersed by addition of a little acetone. After standing several days at room temperature, crystals contaminated with gum gradually appeared. The crystals were again recrystallized from a large volume of acetone-water (yield 2.83 g.). The compound was now easily recrystallized (charcoal) from aqueous ethanol and formed thick colourless needles, m.p. 107°. It gave the correct analysis for *o*-methoxyphenyltriacetylglucuronide methyl ester (*guaiacol triacetylglucuronide methyl ester*). (Found: C, 54.9; H, 5.5; OMe, 13.0. $\text{C}_{20}\text{H}_{24}\text{O}_{11}$ requires C, 54.5; H, 5.5; OMe, 14.1%.) $[\alpha]_D^{18} = -49.3^\circ$ ($c=1$ in ethanol). It gave a positive naphthoresorcinol reaction for glucuronic acid on prolonged boiling (5 min.) with the reagents.

(iv) *Acid hydrolysis of the acetylated methyl ester. Isolation of guaiacol.* The methyl ester (0.75 g.) was heated under reflux for 6 hr. with 4N-HCl (50 ml.) and ethanol (5 ml.). The mixture, which smelt of guaiacol, was cooled and then

extracted with 3 × 75 ml. portions of ether. The combined ether extracts were extracted with small portions of 10% (w/v) NaOH solution (15 ml. in all). This alkaline extract which now contained the guaiacol was shaken for 10 min. with 5 ml. acetone containing 0.75 g. *p*-toluenesulphonyl chloride. The resulting brown solution was poured into 200 ml. water, but no solid appeared. The mixture was therefore heated at 60° for 1 hr. when a white precipitate appeared. After cooling, the precipitate (200 mg.) was collected and repeatedly recrystallized from ethanol. The product finally had m.p. 81–82°, and 82° mixed with authentic guaiacol *p*-toluenesulphonate, m.p. 82°. (Found: C, 60.3; H, 5.0; S, 11.65. Calc. for $C_{14}H_{14}O_4S$: C, 60.4; H, 5.1; S, 11.5%.) The residual solution, after ether extraction of the guaiacol, gave a positive naphthoresorcinol reaction and after neutralization reduced Fehling's and Benedict's reagents, thus showing the presence of glucuronic acid.

(d) *Detection of hydroxyquinol (1:2:4-trihydroxybenzene) in catechol urine.* Other work in this laboratory (Porteous & Williams, unpublished) had shown that benzene is converted in the rabbit to hydroxyquinol and we believe that this phenol is formed from benzene via catechol. It seemed likely therefore that on feeding catechol itself some hydroxyquinol might be produced.

Hydroxyquinol can be readily detected in the presence of catechol by two colour reactions.

(i) *The sulphuric acid test.* If small amounts of hydroxyquinol are gently warmed with conc. H_2SO_4 , an intense red colour develops immediately. Phenol, catechol, quinol, pyrogallol and phloroglucinol do not give colours with H_2SO_4 under these conditions, whilst resorcinol gives a faint orange colour. By this test small amounts of hydroxy-

peroxide-free ether. Catechol and hydroxyquinol were detected in the extract up to 24 hr. after beginning the extraction. After 27 hr. extraction neither catechol nor hydroxyquinol was detected. The combined extracts were evaporated to dryness *in vacuo* and yielded 2.42 g. of semi-crystalline material. Catechol was removed by exhaustive extraction with dry benzene (4 × 40 ml.) in which hydroxyquinol is insoluble. Colour tests showed that the benzene extract contained no hydroxyquinol and on evaporation it yielded crystalline catechol. The residue (1.9 g.) after benzene extraction gave intense colorations in the H_2SO_4 and 2:6-dichloroquinone chloroimide tests for hydroxyquinol, but attempts to obtain crystalline derivatives were unsuccessful. The extracted urine was now warmed to expel ether and then made 3N with respect to HCl. The mixture was boiled under reflux for 1 hr. to hydrolyze glucuronides. After cooling, the urine was continuously extracted for 10 hr. with peroxide-free ether. The extract contained much catechol but no trace of hydroxyquinol. All extracts were also tested for pyrogallol without positive results.

It can, therefore, be concluded from these experiments that, in the rabbit, catechol is oxidized in small amounts to hydroxyquinol which appears in the ethereal sulphate, but not in the glucuronide, fraction of catechol urine.

DISCUSSION

It is clear from the above that the metabolism of catechol can be represented as follows:

quinol can be detected readily in the presence of large amounts of catechol.

(ii) *The 2:6-dichloroquinone chloroimide test.* Traces of hydroxyquinol when added to 2–3 ml. of a saturated aqueous solution of 2:6-dichloroquinone chloroimide give a red colour which is intensified rapidly when the pH of the solution is raised to 8–9 by the addition of small amounts of $NaHCO_3$. This reagent also gives colours with phenol (blue) and catechol (blue-purple), but the hydroxyquinol colour is always dominant in a mixture at pH 8–9 (Porteous & Williams, unpublished).

Eleven rabbits were fed a total of 6.36 g. catechol (dose 200 mg./kg.) and urine (2 l.) collected over 24 hr. The urine was clarified by centrifuging and then made acid to litmus with a little dilute HCl. It was then continuously extracted for 5 hr. with peroxide-free ether. The extract contained catechol but no hydroxyquinol. From this we concluded that no hydroxyquinol was excreted in the free state. Ether was now expelled from the urine by gentle warming on the water bath, and the urine then was made N with respect to HCl and heated on a water bath for 0.5 hr. to hydrolyze ethereal sulphates but not glucuronides. The product was now subjected to continuous extraction with

About 90% of catechol can be accounted for as catechol monoglucuronide, catechol monosulphuric acid, a small amount of free catechol and traces of conjugated hydroxyquinol, and these are excreted within 24 hr. after dosing (see Fig. 1).

The free catechol actually isolated as its crystalline di-*p*-toluenesulphonate was 1.8% of the dose and this must be considered as a minimum value owing to the losses inherent in the isolation of a small quantity of such a phenol. A figure of 5% of the dose would no doubt be nearer the true value for the free catechol excreted. The catechol glucuronide excreted has been proved to be a monoglucuronide. Methylation of the glucuronide (I) with diazomethane introduced two methyl groups thus indicating the presence of two acidic groups in the molecule, i.e. one free phenolic OH group and the carboxyl group of glucuronic acid. Methylation with diazomethane of the gummy catechol glucuronide did not yield a crystalline compound which could be characterized by melting point and optical rotation.

However, acetylation of the methylated glucuronide (II) yielded a crystalline product, viz. *o*-methoxyphenyl 2:3:4-triacetyl- β -D-glucuronide methyl ester (III). Hydrolysis of this ester produced guaiacol (IV) characterized as its *p*-toluenesulphonate. The isolation of guaiacol proves that the catechol glucuronide contains one free phenolic hydroxyl group and is therefore a monoglucuronide.

catechol; see also Ball & Chen (1933) and Florence & Schapira (1943) on dissociation constants). It is interesting to note that there are many instances known of compounds carrying two or more similar groups capable of conjugation *in vivo* but in which conjugation occurs only on one of these groups, e.g. orcinol (Sera, 1913, 1914), phloroglucinol (Sera, 1914), phenolphthalein (Di Somma, 1940), stilb-

We have not provided direct proof that the ethereal sulphate excreted is a monosulphate but there is indirect evidence that this is so. Sulphuric esters of phenols are often extremely difficult to isolate from a complex fluid such as urine. Both the mono- and di-sulphuric esters of catechol have been prepared synthetically by Baumann (1878) who obtained the mono-ester as a crystalline potassium salt and the di-ester as an amorphous potassium salt. Neither of these esters has been isolated from biological sources (cf. Baumann & Herter, 1877-8). The mono-ester gives a violet colour with ferric chloride and such a colour can be obtained with acetone extracts of catechol urine.

It can be argued on theoretical grounds that the formation of a disulphuric ester from the mono-ester is unlikely, since catechol monosulphate is a phenol carrying a strongly acidic group in the *ortho* position. The situation is similar to that found in salicylic acid which does not form an ethereal sulphate in the rabbit (Williams, 1938). The reactivity of the free hydroxyl is suppressed for various reasons (*ortho* effect) by the adjacent strongly acidic group. Furthermore, Williams (1938) has shown that if, in an *o*-substituted phenol, the substituent group is a neutral one, e.g. the $-\text{OCH}_3$ group in guaiacol and the $-\text{CH}_3$ in *o*-cresol, then the extent of sulphate conjugation of that phenol is the same as found in phenol itself, i.e. c. 19% of the dose is excreted as sulphate. Assuming that catechol forms a monosulphate, the extent of its sulphate conjugation is 18% (see Table 1), in complete accord with the assumption that the second OH group is acting as a neutral group. There is also physico-chemical evidence which suggests that the reactivity of one hydroxyl group in catechol may be suppressed (e.g. Pauling (1945) on hydrogen bond formation in

oestrol, hexoestrol and dionoestrol (Dodgson, Garton, Stubbs & Williams, 1948), etc.

Although the main fate of catechol in the rabbit involves conjugation only, it does undergo further oxidation to hydroxyquinol (1:2:4-trihydroxybenzene). Small amounts of conjugated hydroxyquinol were found in the urine, probably as an ethereal sulphate for it was only found in the ethereal fraction of the urine. From studies *in vitro* it has been shown that catechol is oxidized to hydroxyquinol by enzyme preparations containing catecholase (see review by Nelson & Dawson, 1944). Our results indicate that this oxidation also takes place in the intact animal. However, it is not clear from our experiments whether the hydroxyquinol arises by oxidation of free catechol or of a catechol conjugate. It is possible that it could arise by the oxidation of catechol monosulphate.

SUMMARY

1. The fate of catechol in the rabbit has been studied. At dose levels of 100 mg./kg., 70% of orally administered catechol is excreted in 24 hr. conjugated with glucuronic acid and 18% conjugated with sulphuric acid. A small amount, c. 2%, is excreted in the free state. Some catechol is also oxidized to hydroxyquinol which appears in the urine as an ethereal sulphate.

2. The catecholglucuronide has been proved to be a monoglucuronide, by conversion into crystalline *o*-methoxyphenyltriacetylglucuronide methyl ester which on hydrolysis yields guaiacol. Evidence is also presented to show that the ethereal sulphate of catechol formed in the rabbit is a monosulphate.

The expenses of this work were in part defrayed by a grant from the Medical Research Council.

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Metabolism of Derivatives of Toluene

1. THE METABOLISM OF ACETOTOLUIDIDES IN THE RABBIT

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(Received 2 February 1948)

In an investigation of the metabolism of the acetotoluidides, Jaffe & Hilbert (1888) found that all three isomers were oxidized in the dog and rabbit. *p*-Acetotoluidide was shown to be converted almost quantitatively to *p*-acetamidobenzoic acid in both animals; similarly, *m*-acetamidobenzoic acid was isolated as a metabolite of *m*-acetotoluidide and it was suggested that an aminocresol might also be formed. *o*-Acetotoluidide gave rise in the dog to a compound claimed to be a methylbenzoxazolone. The constitution of this compound was not elucidated, but on treatment with ammonia in a sealed tube at 130-140° it gave a compound melting at 148-150° which appeared to be an aminocresol. Seven aminocresols were known to Jaffe & Hilbert, but it is now apparent that the isomer they obtained was in fact 2-amino-*m*-cresol which was not again described until 37 years later (Hodgson & Beard, 1925). The nature of the aminocresols formed as metabolites of acetotoluidides was of interest to us in view of the general rule concerning the position of entry of the hydroxyl group in biological hydroxylations which had been suggested by other investigations (e.g. Bray, Ryman & Thorpe, 1947; Bray, Lake, Neale, Thorpe & Wood, 1948). Consequently, we have carried out a further investigation of the fate of these compounds in the rabbit and included certain quantitative studies, the results of which are also reported here.

EXPERIMENTAL

Materials and methods

Materials. The acetotoluidides used in this study were prepared by the action of acetic anhydride upon the corresponding toluidines (Light and Co. Ltd.), the products being recrystallized from water. The reference compounds, 2-amino-5-hydroxytoluene (6-amino-*m*-cresol) and 5-amino-2-hydroxytoluene (5-amino-*o*-cresol), were prepared by the method of Noelting & Cohn (1884), which involves the reductive fission of azo compounds prepared from benzenediazonium chloride and the appropriate cresol. The azo compounds were dissolved in 2*N*-NaOH and reduced by means of Na₂S₂O₄. The properties of the compounds obtained agreed with those described in the literature.

Diet and feeding. The rabbits used were does of 2-3 kg. weight. A constant diet of rabbit pellets, with water *ad lib.*, was given throughout the study, as previously described (Bray *et al.* 1947). The acetotoluidides were administered by stomach tube as suspensions in water. The *p*-isomer showed no toxic properties at a dose level of 0.5 g./kg., but, since at this dose level the *m*-isomer was noticeably toxic and *o*-acetotoluidide slightly so, these two compounds were usually administered at a dose level of 0.25 g./kg.

Estimation of diazotizable material. This was carried out in acetotoluidide urines as collected, and after hydrolysis under the conditions described by Bratton & Marshall (1939). Compounds which might theoretically be included in the term 'diazotizable material' are aminocresols, with their glucuronides and ethereal sulphates, toluidines, and aminobenzoic acids. We found, however, that the two aminocresols