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The Identification of the Major Diazotizable Metabolite of 4:4'-Diaminodiphenyl Sulphone in Rabbit Urine

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Titus & Bernstein (1949), while studying the pharmacology of water-soluble disubstituted derivatives of 4:4'-diaminodiphenyl sulphone (DADPS) in mice and dogs, found that DADPS itself was excreted mainly as a water-soluble substance which was readily hydrolysed by dilute acid to free DADPS. Lowe (1952) also reported a similar change in the drug given to man for the treatment of leprosy and showed that as much as 80% of the diazotizable material excreted in the urine was present in this form.

DADPS is regarded as the most effective drug available for the treatment of leprosy and in view of the low proportion of the drug excreted in the free form it seemed probable, especially if the water-soluble derivative were also the major constituent in the blood, that the antileprotic activity of the drug was due to this acid-labile metabolite. We therefore examined by paper chromatography and paper ionophoresis the excretion products of DADPS, and in a preliminary communication (Bushby & Woiwod, 1955) we reported the isolation of the major diazotizable excretion product from rabbit urine and its identification as a monoconjugate with glucuronic acid. We also reported the synthesis of the conjugate. In this paper the synthesis of this conjugate is described, and the evidence for its presence in urine and blood is presented.

EXPERIMENTAL

Paper chromatography. The apparatus and procedures used were those of Woiwod (1949). The solvents used were *n*-butanol saturated with water, *n*-butanol-acetic acid-water (12.5:3:12.5, by vol.), phenol saturated with water and *n*-propanol-*n*-butanol-water (2:3:5, by vol.). No. 4 Whatman papers were washed with several changes of distilled water in an enamelled tray and dried at 37° before use.

Ionophoresis. Samples were run on strips of no. 4 Whatman paper with 0.05 M- Na_2HPO_4 as electrolyte and a potential gradient of 7.0 v/cm.

Detection of primary aromatic amino groups. Two reagent sprays were used. (1) A 1% (w/v) solution of *p*-dimethylaminobenzaldehyde in 20% (v/v) conc. HCl-ethanol, diluted with 19 vol. of ethanol just before use. Colour was developed at room temp. To avoid background coloration the ethanol was redistilled from KOH and KMnO_4 . (2) A solution of NaNO_2 [0.5% (w/v) in 20% (v/v) *N*-HCl-ethanol], followed, after the paper had nearly dried, by 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride in the same solvent.

Detection of glucuronic acid. Aniline hydrogen phthalate, benzidine and naphthoresorcinol sprays were used. The sensitivity of the last two reagents was markedly improved by using phosphoric acid in the spray as suggested by Bryson & Mitchell (1951).

Estimation of glucuronic acid. Glucuronic acid in material eluted from paper was determined either by the copper-reduction method of Shaffer & Somogyi (1933) or the colorimetric procedure of Jones (1954). The latter gave much less colour with glucuronic acid than with glucose and other reducing sugars owing to the SnCl_2 in the reagent. When this was replaced by Na_2SO_3 the colour obtained with glucuronic acid equalled that given by glucose. The modified reagent consisted of 0.2% (w/v) benzidine in acetic acid containing 0.05% (w/v) Na_2SO_3 . The fine precipitate which formed was filtered off after 30 min. and the reagent was used immediately.

Synthesis of the sodium salt of the acid-labile conjugate of DADPS with glucuronic acid

Sodium glucuronate (4.3 g.) in 15 ml. of water adjusted to pH 5.5 was added to diaminodiphenyl sulphone (5 g.) in 15 ml. of acetone. The mixture was refluxed for 1 hr., 100 ml. of water added, the precipitated DADPS removed by filtration and the filtrate concentrated *in vacuo* to about 50 ml. After adjustment to pH 7.0, the solution was extracted three times with equal volumes of phenol equilibrated with 0.067 M phosphate buffer (pH 7.4). This extracted a large

part of the sodium salt of the monoconjugate of DADPS and glucuronic acid, and left a considerable amount of the unchanged sodium glucuronate and the sodium salt of the diconjugate. The phenol in the extract was removed with ether and the resulting solution freeze-dried. Chromatograms of a solution of this solid run in *n*-propanol-*n*-butanol-water showed, besides the diconjugate (R_F 0.05) and monoconjugate (R_F 0.33), whose identities were established by determining in eluates the ratio of DADPS to glucuronic acid, two minor substances (R_F 0.38 and 0.45) reacting with *p*-dimethylaminobenzaldehyde. Purification of the sodium salt of the monoconjugate was carried out by preparative paper chromatography with the same solvent; the monoconjugate was eluted with water and freeze-dried immediately to avoid decomposition. The amorphous white solid obtained (about 1.25 g.) contained a little impurity from the paper, but was 98% pure as judged by its content of DADPS and glucuronic acid. (Found: C, 47.8; H, 4.3; N, 6.0. $C_{18}H_{19}O_8N_2Na$ requires C, 48.5; H, 4.3; N, 6.3%.) The sodium salt was readily soluble in water, sparingly soluble in methanol and hot ethanol but insoluble in chloroform, carbon tetrachloride and benzene. All attempts to crystallize the product were unsuccessful. The free acid could not be prepared from the sodium salt, as at pH values below 4.5 rapid decomposition to DADPS and glucuronic acid occurred.

Isolation and analysis of the glucuronic acid conjugate of DADPS from rabbit urine

The urine of rabbits given DADPS (100 mg./kg. by stomach tube) did not apparently contain DADPS in acetylated form, since the concentration of the drug as determined by the method of Bratton & Marshall (1939) was the same before and after acid hydrolysis. However, paper chromatography in *n*-butanol-water, *n*-propanol-*n*-butanol-water and phenol showed that the drug was excreted both free and as a conjugate running considerably slower than the free drug. This conjugate was acid-labile and was decomposed on chromatograms run in *n*-butanol-acetic acid to yield glucuronic acid and a substance with an R_F the same as that of DADPS. *N*-HCl at room temperature also decomposed the conjugate to glucuronic acid and a substance chromatographically and ionophoretically identical with DADPS.

The amount of free DADPS in urines varied with the pH; no free DADPS was detected, either by chromatography or by solvent extraction in neutral or alkaline urines. In acid urine, however, the free DADPS content was high, and increased with increasing acidity and on storage; this suggests that breakdown could occur in the bladder. The best yields of the metabolite for preparative work were obtained by injecting sodium citrate intraperitoneally (600 mg./kg.) 0.5 hr. before the DADPS (100 mg./kg.) was administered. The urine was collected for 4-6 hr., the pH was checked and the DADPS content estimated; the samples were then freeze-dried.

The pooled freeze-dried urine of several rabbits was dissolved in distilled water, insoluble matter was removed by centrifuging and the supernatant chromatographed with *n*-propanol-*n*-butanol-water on paper (about 1 g. of solids/16 sheets of paper). The conjugate was eluted from the dried sheets with distilled water and the eluate was checked for DADPS equivalent by the Bratton & Marshall (1939) procedure. A second chromatographic separation was

carried out on the pooled freeze-dried eluates. The final eluate, which was between 60 and 70% pure on a DADPS basis, assuming a monoconjugate with glucuronic acid, was freeze-dried. A solution of the solid decomposed on chromatograms run in *n*-butanol-acetic acid to glucuronic acid and DADPS. The identity of DADPS was confirmed by isolation of crystals with m.p. 176°, unchanged by admixture with authentic DADPS (m.p. 177°). The diacetyl derivative had m.p. 284-286°, unchanged by admixture with the diacetyl derivative of DADPS (m.p. 284-286°). The glucuronic acid:DADPS ratio of the conjugate was obtained by determining the glucuronic acid content of the acidified solution after the DADPS had been extracted. The colorimetric method gave a ratio of glucuronic acid to DADPS of 1:1, and the copper-reduction method 1.15:1. The latter suggested that small quantities of reducing substances which did not react in the colorimetric method were extracted with the conjugate. On ionophoresis in 0.05M- Na_2HPO_4 the conjugate migrated towards the anode; DADPS moves towards the cathode. From this it was concluded that the carboxyl group of the glucuronic acid was free. The synthetic monoconjugate of glucuronic acid and DADPS matched the isolated conjugate exactly, both chromatographically and ionophoretically.

Detection and estimation of conjugate in rabbit plasma

A rabbit given DADPS (100 mg./kg.) orally was killed and bled out after 2 hr. The blood had activity, approximately equal to 1 mg. of DADPS/100 ml. as measured by the method of Bratton & Marshall (1939); 70% of this was not extracted by organic solvents. The plasma (53 ml.) was freeze-dried and 1 g. of the solid was extracted with methanol. The extract was dried down and the residue re-extracted with a small volume of methanol; much solid material remained undissolved. When chromatographed in *n*-propanol-*n*-butanol-water the extract showed, besides free DADPS, a substance with the same R_F as a sample of synthetic monoconjugate which had been added to serum and extracted in a similar manner. In both cases the spot was displaced from the usual position, due to the high content of methanol-soluble material in the extract. Both samples decomposed on butanol-acetic acid chromatograms and had identical ionophoretic mobilities in 0.05M- Na_2HPO_4 . The very small amounts available precluded further characterization, but these results suggested that the monoconjugate of DADPS and glucuronic acid isolated from urine was also circulating in the blood of the rabbit.

DISCUSSION

These investigations show that after oral administration to the rabbit detectable DADPS is present in the plasma and the urine almost entirely as a monoconjugate with glucuronic acid. The proportion present in the urine in this form depends upon the pH of the urine; in acid urine breakdown occurs in the bladder so that the proportion of free DADPS is relatively high. Although the exact nature of the bond uniting DADPS and glucuronic acid is unknown, the ionophoretic behaviour of the conjugate indicates that the carboxyl group of the glucuronic acid is free and the isolation of intact DADPS after

mild acid hydrolysis precludes any form of phenolic glucuronide formation. Bushby & Woiwod (1955) assumed from analogy with 4:4'-diaminodiphenyl sulphone *NN'*-didextrose sulphonate (Promin) that the linkage was through an anil bond. Such a linkage might explain the sugar-containing metabolite of 3:5-dibromosulphanilamide isolated from human urine by McChesney (1947), and also the so-called labile glucuronides found by Smith & Williams (1949*a, b*) in the urine of rabbits fed on aniline and phenetidine. However, Boyland & Manson (1955) have found in the urine of rabbits given naphthylamine a conjugate of naphthylamine with glucuronic acid which resembles our conjugate in its lability to acid and to which they ascribe an *N*-glucosiduronic acid structure. This they suggested could also be the structure of the labile glucosiduronates reported by Smith & Williams (1949*a, b*).

Although this report is concerned with the metabolism of DADPS in the rabbit and the monoconjugate of DADPS and glucuronic acid has so far been identified only in this animal, preliminary experiments (Bushby & Woiwod, 1955) show that the drug is excreted by man as a monoconjugate with glucuronic acid, though the linkage may possibly be that of a phenolic glucuronide rather than that of an anil. Monosubstituted derivatives of DADPS are known to have antileprotic and antitubercular activity (Cochrane, 1952; Smith, Jackson & Bauer, 1949). Therefore, in view of the high proportion of DADPS present in the body in a monoconjugated form, these observations may be of chemotherapeutic importance.

SUMMARY

1. The major diazotizable metabolite of 4:4'-diaminodiphenyl sulphone (DADPS) has been isolated from rabbit urine and identified as a glucuronic acid conjugate.

2. One molecule of DADPS is combined with one of the acid, and ionophoresis shows that the carboxyl group of the acid is free. The metabolite is acid-labile and breaks down to glucuronic acid and DADPS. The conjugation is therefore probably between the aldehydic carbon and the nitrogen of one of the amino groups, and the linkage may be either that of an anil or an *N*-glucosiduronic structure.

3. The metabolite has been synthesized.

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Studies in Carotenogenesis

21. THE NATURE OF THE CHANGES IN CAROTENOID SYNTHESIS IN *RHODOSPIRILLUM RUBRUM* DURING GROWTH*

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The studies of Engelmann (1883, 1888), later confirmed by Molisch (1907) and Buder (1919), had established that purple bacteria can respond by phototaxis, reversing the direction of their motion when experiencing a sudden drop in light intensity. Hence the bacteria tend to accumulate in areas where the light intensity is perceived as highest. In a spectrum they aggregate in bands whose position

indicates which wavelengths are most effectively absorbed. Pertinent experiments had indicated that the accumulation bands coincide with regions of maximum absorption by both the green (chlorophyllous) and the red (carotenoid) pigments of the organisms. Thus a simple and satisfactory picture had emerged. However, as our knowledge of the carotenoids of the purple bacteria developed, some curious inconsistencies became apparent.

* Part 20: Goodwin & Land (1956*a*).