TABLE 3. ZOXAZOLAMINE METABOLISM IN RAT LIVER (9000 g FRACTION)

No. of rats	Group	Time after tumor transplantation days	Body weight (g ± S.E.)	Tumor weight (g ± S.E.)	μg Zoxazolamine metabolized/100 mg/60 min ± S.E.	μg Zoxazolamine metabolized/mg protein/60 min ± S.E.
5	Normal	—	198 ± 6	—	11·7 ± 2·8	0·59 ± 0·13
5	Walker	4	152 ± 8	—	8·4 ± 2·0	0·45 ± 0·10
5	Normal	—	191 ± 6	—	10·8 ± 1·6	0·53 ± 0·08
5	Walker	11	176 ± 6	3·3 ± 0·5	4·4 ± 0·9*	0·23 ± 0·06*
5	Normal	—	236 ± 13	—	13·5 ± 1·9	0·66 ± 0·07
5	Walker	16	182 ± 15	15·5 ± 3·2	2·7 ± 0·5†	0·14 ± 0·03†
5	Normal	—	284 ± 18	—	12·0 ± 2·2	0·66 ± 0·13
5	Walker	22	182 ± 29	34·7 ± 5·0	3·0 ± 0·7†	0·18 ± 0·04†

* 0·01 < P < 0·05.
† P < 0·01.

These results agree with previous results obtained with Pentobarbital and *d*-Amphetamine.[1,2] Further studies to elucidate the possible mechanisms involved and their specificity are in progress.

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The separation and detection of metabolites of guanethidine

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THE ANTIHYPERTENSIVE drug guanethidine has been widely used in investigations of the adrenergic neurone. Although several studies[1–3] suggest that the drug is metabolised in animals, until recently little was known about its metabolites.

Furst[4] showed that in rats, radioactivity labelled guanethidine was extensively converted into what appeared to be a single polar metabolite and that oxygen-dependent metabolism of the drug took place in rat and rabbit liver microsomal preparations.[5]

Further information about the metabolites of guanethidine was required for an investigation of the therapeutic implications of the distribution and fate of this drug in man. The experiments described below were undertaken to define some of the properties of the metabolites of guanethidine so that methods for isolating and identifying them could be considered.

Samples containing radioactively labelled metabolites were obtained by incubating [^{14}C]-guanethidine,[4] with liver homogenates or by collecting urine from rats which had been injected with the drug.

In a typical liver homogenate preparation 100 g of fresh rabbit or pig liver was homogenised in 200 ml of 0·1 M pH 7·4 phosphate buffer using the Ultra-Turrax for 1 min at 60 per cent of maximum speed. The homogenate was centrifuged at 14,000 *g* for 20 min and 2 ml aliquots of the supernatant incubated with 3 ml of 0·1 M pH 7·4 phosphate buffer containing 3 mg of [^{14}C]-guanethidine sulphate (sa 15 μc/mg) for 1 hr at 37°. Incubation was terminated by the addition of 3 ml of a 50:50 mixture of 10% TCA and 0·1 N HCl and the mixture blended with the Ultra-Turrax and centrifuged at 4000 rpm for 20 min to give a protein-free supernatant containing metabolites and some unchanged drug.

Urine was collected in the period 0–8 hr after i.p. injection of male Wistar rats with 10 mg/kg [^{14}C]-guanethidine sulphate (sa 15 μc/mg).

A Tri-Carb Liquid scintillation spectrometer series 314E was used to assay samples for radioactivity. Up to 0·5 ml of aqueous samples were added to vials containing 15 ml of scintillator made by mixing 2·5 l. toluene, 1·7 l. methylcellosolve, 30 g BBOT and 340 g naphthalene.

The first step was to establish methods for separating the metabolites from major contaminating compounds in the sample. Since the metabolites could not be readily extracted into chloroform, alternative procedures were explored. When ion exchange resins were tested, it was found that at least twenty bed-volumes of liver homogenate supernatant or urine adjusted to pH 2 could be passed through a column of AG50 × 4 sulphonic acid ion exchange resin (H^+ form) with complete retention of radioactive drug and metabolites on the column.

Radioactive material could be eluted with 4N ammonia from a column which had been loaded with metabolites. A typical result shown in Fig. 1. shows a small radioactive peak (A) followed by a larger symmetrical peak (B).

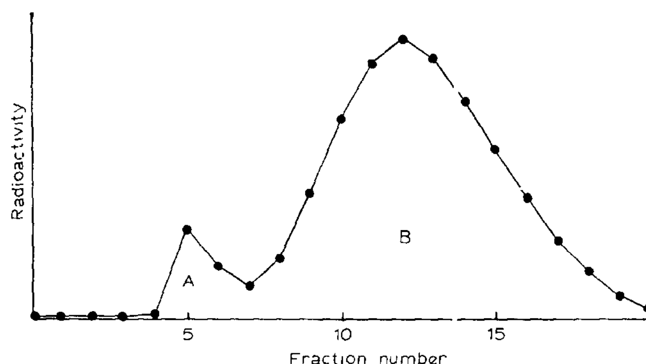


Fig. 1. Radioactivity in 1 ml fractions eluted from a 7 × 1 cm column of AG 50 × 4 with 4N NH_4OH at 1 ml/min. The column had been loaded with metabolites formed by rabbit liver homogenate (2 ml of protein-free supernatant) A. Transformation product of metabolite 1; B. Metabolite 1.

The compounds appearing in the eluate were examined by low voltage paper electrophoresis. After the run, the paper was dried and cut into sections which were assayed by liquid scintillation spectrophotometry. When fractions in the main peak (B) of the ammoniacal eluate from a column were cooled soon after collection and freeze dried, the product obtained gave a single peak (C) on electrophoresis at pH 6·1 (Fig. 2(a)). If the eluate was heated during evaporation a second peak (D) appeared (Fig. 2(b)).

Electrophoresis at different pH values and paper chromatography suggested that the product obtained by freeze drying the column eluate contained a single radioactive compound. Electrophoresis applied directly to fresh urine samples (Fig. 2(c)) showed the presence of a radioactive product (E)

of similar mobility. Heating of the urine sample (pH 7, 1 hr, 100°) prior to electrophoresis (Fig. 2(d)) caused a decrement of the peak due to this product and the appearance of a peak which corresponded to that (D) found in heated column eluate.

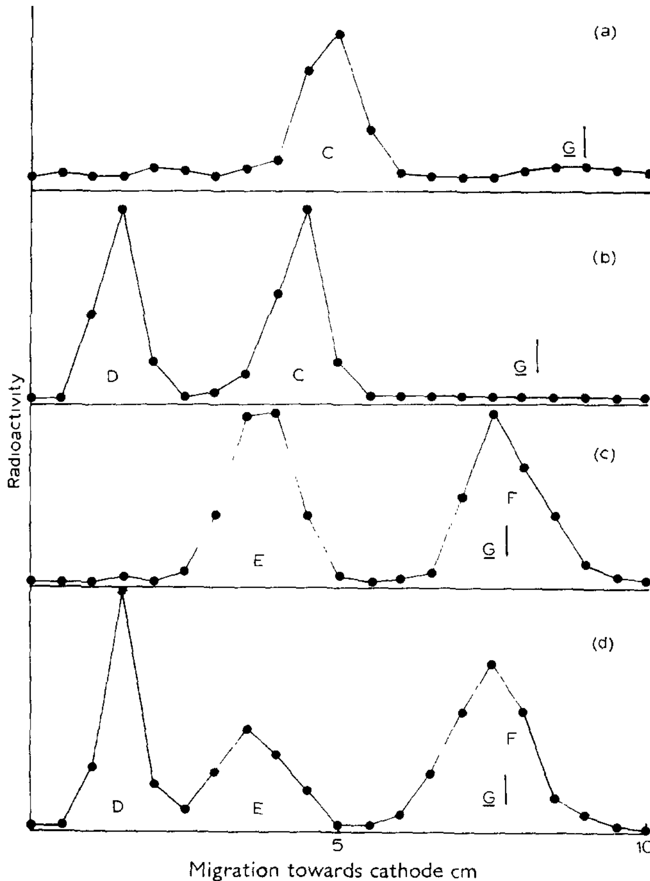


Fig. 2. Electrophoresis on Whatman 3 mm paper. Conditions: Pyridine/Acetate buffer (pH 6·1), initial voltage 250V, constant current, time of run, 15 hr.
(a) Sample obtained by freeze drying the main radioactive fractions (B) eluted from an ion exchange column loaded with metabolites formed by pig liver homogenate; (b) Sample obtained by rotary-evaporation (60–80°) of the main radioactive fractions (B) eluted from an ion exchange column loaded with metabolites formed by pig liver homogenate; (c) 10μl of rat urine collected after i.p. injection of [^{14}C]-guanethidine; (d) 10 μl of a portion of the rat urine used for (c) which had been heated at 100° for 1 hr at pH 7.
G shows the position of marker guanethidine added so that variation between runs could be taken into account.
C—Metabolite 1.
D—Transformation product of metabolite 1.
E—Peak containing metabolite 1 and metabolite 2.
F—Unchanged guanethidine.

For these reasons, the main radioactive fractions (B) eluted from the column were assumed to contain a single authentic metabolite (metabolite 1) of guanethidine which underwent transformation when heated in aqueous solutions. This metabolite was present in rat urine, in rabbit liver homogenates and to a lesser extent in pig liver homogenates.

When reapplied to a column of ion exchange resin 96% of metabolite 1 was eluted with 4N ammonia in the position of peak (B) (Fig. 1). The transformation product when reapplied to the column was eluted in the position of the small peak (A) shown in Fig. 1. No radioactive products were eluted with ammonia when pure [^{14}C]-guanethidine was applied to the column.

Quantitative aspects of the elution of metabolites were investigated further to see whether all the metabolites were being eluted from the column with ammonia. The total amount of metabolites in samples was estimated by radioactive assay of samples from which excess guanethidine had been quantitatively removed by chloroform extraction. Paper chromatography and electrophoresis of extracts of rat urine suggests that only guanethidine is extracted with chloroform (10 vol.) from strongly alkaline aqueous solutions (1 vol. sample + 0·2 vol. 60% KOH).

It was found that the radioactive material recovered from the column with ammonia did not account for the total quantity of metabolites present in urine and liver homogenate samples. Typical results are shown in Table 1.

TABLE 1. TOTAL METABOLITES ESTIMATED BY ASSAY OF RADIOACTIVITY LEFT IN SAMPLES FROM WHICH GUANETHIDINE HAS BEEN REMOVED BY CHLOROFORM EXTRACTION, COMPARED WITH METABOLITE 1 AND ITS TRANSFORMATION PRODUCT ESTIMATED AS RADIOACTIVITY ELUTED FROM AG50 × 4 ION EXCHANGE RESIN WITH 4N AMMONIA

Sample	% total metabolites	% metabolite 1 and its transformation product
Rat urine	75	55
Rabbit liver homogenate	55	25

Solvents which might elute further metabolites from columns were tested. When a column which had been loaded with sample and eluted with 4N ammonia was subsequently eluted with 6 N HCl a single broad radioactive peak was obtained.

The products in the 6N HCl eluate were examined by high voltage paper electrophoresis. A typical result (Fig. 3) showed that in addition to unchanged guanethidine (J) a metabolite (H) with the same mobility relative to guanethidine (0·58 as metabolite 1 was present. A small amount of metabolite (I)

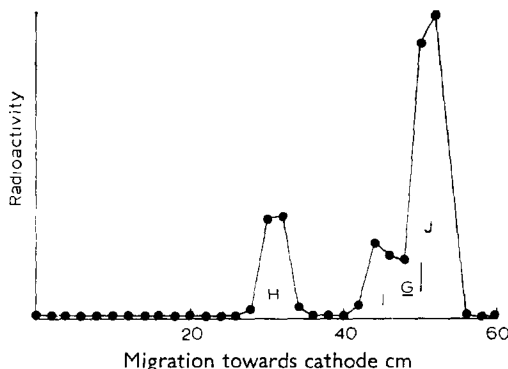


FIG. 3. High voltage electrophoresis on Whatman 3 mm paper of a 60 μl aliquot of the radioactive material eluted with 20 ml of 6N HCl from an ion exchange column which had previously been loaded with metabolites formed by rabbit liver homogenate (2 ml of protein-free supernatant) and washed with $4NNH_4OH$. Conditions: Pyridine/Acetate buffer (pH 6·1), 6 kV constant, time of run: 1 hr. G shows the position of marker guanethidine.

H—Metabolite 2.

I—Metabolite 3.

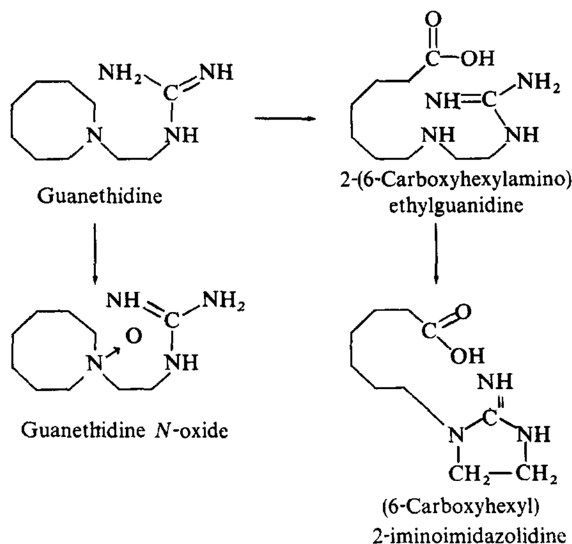
J—Unchanged guanethidine.

which was poorly resolved from guanethidine was also present. This metabolite (metabolite 3) is probably partially extracted into chloroform from alkaline solution.

Metabolite 1 was not, however, present in these acid fractions because when samples of these were reapplied to a column an insignificant amount of radioactive material was eluted with 4N ammonia. Thus, the radioactive peak (H) appeared to be due to a different metabolite (metabolite 2). Since metabolite 1 and metabolite 2 have the same mobility they are probably both present in peak (E) in Fig. 2(c). High voltage electrophoresis at a number of pH values failed to give conditions under which metabolite 1, metabolite 2 and unchanged guanethidine could be completely resolved into three components.

Further metabolites might be left on the ion exhange column since the recovery of radioactivity from columns which have been eluted with 4N ammonia followed by 6N HCl is still not complete. However it is more likely that this is due to the kinetics of desorption of material from resin in highly acidic media. When pure guanethidine was applied to columns, only about 80% of the material could be readily recovered by elution with 6N HCl.

In further investigations,[6] to be published the isolation and identification of metabolites 1 and 2 has been achieved. The structures of these compounds (Scheme 1) have been established by elemental



analysis, potentiometric titration, the study of model transformation reactions and by synthesis of compounds with proposed structures which have mpts and IR spectra in agreement with those of the metabolites. Pharmacological tests carried out by Dr. Brunner (CIBA Basle) have shown that these metabolites have less than 1/10th of the antihypertensive activity of guanethidine in the renal hypertensive rat.

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Inhibition of bacterial macromolecular syntheses by the polyamine POX-3

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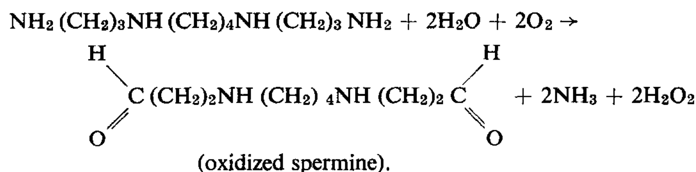
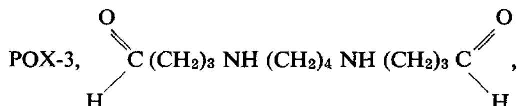
The naturally occurring polyamine, spermine, is oxidized by purified serum amine oxidase (amine: O_2 oxidoreductase; deaminating) as follows[1]:



The product, oxidized spermine, was shown to inhibit bacterial growth,[2] to inactivate bacterial, plant and mammalian viruses[3–6] and to be toxic for Ehrlich ascites cells.[7] Very recently, the chemical synthesis of oxidized spermine and related iminodialdehydes has been described by Fukami *et al.*[8] These investigators also confirmed our finding that oxidized spermine inactivates coliphages of the T-odd series.

The antimicrobial action of enzymatically prepared oxidized spermine has been explained on the basis of the interaction of the drug with microbial DNA.[9–12] This interaction was shown to lead to an immediate inhibition of RNA synthesis;[9,13] on the other hand, protein synthesis was arrested only after a definite lag period.[14]

The antiviral and antitumor activities of oxidized spermine make it a potential therapeutic agent. However, further pharmacological studies were hampered by the instability of the biologically prepared iminodialdehyde and by the tediousness of the enzymatic oxidation. The chemical synthesis of stable diacetals eliminates these difficulties and also permits the examination of related oxidized polyamines. The present work deals with the effect of the commercially available oxidized polyamine,



on the synthesis of macromolecules in *Escherichia coli*. It will be shown that POX-3 behaves like oxidized spermine and inhibits the synthesis of bacterial nucleic acids within 3 min. On the other hand, protein synthesis is inhibited by POX-3 only after a lag period.

POX-3, in the form of the diacetal of the oxalic acid salt, was synthesized by Fine Organics Inc., Lodi, New Jersey. The compound, in the form of the oxalate salt, was recrystallized from aqueous ethanol; it decomposed at 230–240°. The phosphate salt, however, had a melting point of 148–150°. The diacetal was converted into the free aldehyde by incubating for 3 hr at 37° with 0·05 N H_2SO_4