The Isolation, Identification and Synthesis of Two Metabolites of Guanethidine Formed in Pig and Rabbit Liver Homogenates

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1. Two metabolites of radioactively labelled guanethidine were isolated from rabbit and pig liver homogenates by ion-exchange chromatography on a sulphonic acid resin. 2. One of the metabolites was eluted from the column with ammonia and identified as 2-(6-carboxyhexylamino)ethylguanidine on the basis of the elemental analysis, i.r. spectrum and pH titration curve of the pure compound, and the observed partial loss of tritium for ring-labelled guanethidine during the formation of this metabolite. 3. This identification was confirmed by synthesis. 4. 2-(6-Carboxyhexylamino)ethylguanidine underwent ring-closure in hot alkaline solution to 1-(6-carboxyhexyl)-2-iminoimidazolidine. 5. The other metabolite of guanethidine was eluted from the ion-exchange column with 6M-hydrochloric acid along with the unchanged drug. It was purified by countercurrent distribution and shown to be identical with synthetic *guanethidine N-oxide*. 6. The two metabolites and the product of ring-closure had less than one-tenth of the antihypertensive activity of guanethidine in the renal-hypertensive rat and are unlikely to contribute to the pharmacological properties of the drug.

Guanethidine, [2-(octahydro-1-azocinyl)ethyl]-guanidine, is a drug used in the treatment of hypertension. The prolonged action, the differences in the effective dose for individuals and certain side effects of the drug make information about its absorption, distribution, metabolism and rate of elimination in man desirable. For a study of these aspects of it, preliminary knowledge of the structures and chemical and pharmacological properties of metabolites of the drug was necessary.

Although metabolites of guanethidine have been mentioned in several publications (Dollery, Emslie-Smith & Milne, 1960; Bisson & Muscholl, 1962; Shanker & Morrison, 1965; Furst, 1967, 1968) their isolation and identification have not been reported.

In an investigation of methods for separating metabolites of guanethidine, McMartin (1969) showed that metabolites could be fractionated by using ion-exchange columns. Metabolites from large volumes of an acidified radioactive sample could be concentrated on a column of a sulphonic acid ion-exchange resin. Two partially resolved radioactive peaks could be eluted from the column with ammonia. The second peak, which was much larger than the first, consisted of a single radioactive metabolite. This metabolite (metabolite 1) was

unstable and underwent transformation in hot aqueous alkaline solution into a product with altered electrophoretic characteristics. When the column was subsequently eluted with 6M-hydrochloric acid a mixture of guanethidine and one other major metabolite (metabolite 2) was obtained.

The present paper describes evidence for the identity of these metabolites obtained by study of the metabolites produced from guanethidine labelled in different positions, by characterization of pure metabolites isolated from liver homogenates and by comparison of these metabolites with synthetic products.

MATERIALS AND METHODS

NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and hexokinase were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6), naphthalene (scintillator grade) from Nuclear Enterprises (G.B.) Ltd. (Edinburgh), methylCellosolve from Union Carbide Chemicals Co. Ltd. (London W.1), tritium gas from The Radiochemical Centre (Amersham, Bucks.), 2,5-bis-(5-*tert*.-butylbenzoxazol-2-yl)thiophen from CIBA (Duxford, Cambs.), AG 50 W (X4; 200–400 mesh; H⁺ form) from BioRad Laboratories, Richmond (Calif., U.S.A.), 2-(octahydro-1-azocinyl)ethylamine, octahydro-1-azocinylacetonitrile and 2-oxoheptamethyleneimine from CIBA (Basle, Switzerland) and guanethidine from CIBA (Horsham, Sussex). Where possible all other reagents were A.R. grade.

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Scheme 1.

Elemental analyses were carried out by Dr Weiler and Dr Strauss, the Microanalytical Laboratory, Oxford.

Synthesis of radioactively labelled compounds

The synthetic route used for labelling guanethidine is outlined in Scheme 1. Guanethidine labelled with tritium on the ring carbon atom next to the nitrogen atom and on the side-chain carbon atom next to the guanidine group was prepared by using LiAl^3H_4 at steps 1 and 3 respectively. Guanethidine (V) labelled with ^{14}C on the guanidine group was prepared by using *S*-methyl- ^{14}C isothiuronium sulphate at step 4.

Side-chain-labelled guanethidine sulphate. (a) LiAl^3H_4 . LiH was ground in a ball-mill and passed through a 200-mesh sieve. The sieved material (1g.) was placed in a 250ml. flask. Then $^3\text{H}_2$ gas (2c, 2ml. at s.t.p.) was introduced into the evacuated flask, which was sealed and heated at 200° for 48 hr. The powder was then transferred to a three-necked round-bottom 100ml. flask and 10ml. of dry ether was added. The suspension was stirred, 3g. of AlBr_3 in 20ml. of dry ether was carefully added and the mixture was heated under reflux for 6 hr. The ethereal solution of LiAl^3H_4 thus obtained was used as soon as possible.

(b) 2-(Octahydro-1-azocinyl)- ^3H ethylamine (IV). Octahydro-1-azocinylacetone (III) (1.35g.) was added to the ethereal solution of LiAl^3H_4 prepared as described above and the mixture was heated under reflux with stirring for 6 hr. The complex was decomposed by adding successively 1 ml. of water, 0.75 ml. of 20% (w/v) NaOH and 3.5 ml. of water. Excess of ether was then added and the mixture was shaken. The ether layer was separated and evaporated to dryness. Exchangeable tritium was removed from the residue by adding and removing 6*M*- HCl in ethanol several times under reduced pressure, and the residue was dissolved in dilute aqueous alkali and extracted with ether. The ether layer was dried over Na_2SO_4 and evaporated to give an oil. The product was dissolved in 60ml. of chloroform and the solution was extracted twice with 15ml. and once with 10ml. of 10% (v/v) acetic acid. The combined acid extracts were washed with 25 ml. of chloroform, made alkaline with

10ml. of 60% KOH and extracted three times with 60 ml of ether. The ether extracts were dried over Na_2SO_4 to give 336 mg. of a pale-yellow oil.

(c) 2-(Octahydro-1-azocinyl)- ^3H ethylguanidine (V). The tritiated amine prepared above was diluted with 433 mg. of unlabelled material and heated on a boiling-water bath under reflux with 2.2 ml. of water and 650 mg. of *S*-methylisothiuronium sulphate for 6 hr. Acetone was added to the cold solution to give a white precipitate of guanethidine hemisulphate, which was washed with acetone and ether and dried. A slight excess (3 ml.) of 0.5*M*- H_2SO_4 was added to convert this product into the sulphate, which was crystallized three times from ethanol-water and dried *in vacuo* to give 500 mg. of side-chain-labelled guanethidine sulphate [$132\mu\text{C}/\text{mg.}$; m.p. $243\text{--}248^\circ$ (decomp.)].

Chromatography on Whatman no. 4 paper in propan-2-ol-17*M*- NH_3 -water (20:2:3, by vol.) and butan-1-ol-acetic acid-water (12:3:5, by vol.) showed the presence in each system of a single radioactive component with R_F values 0.85 and 0.69 respectively, which correspond to those of authentic guanethidine. When the sample was diluted with unlabelled guanethidine sulphate the specific radioactivity changed by less than 3% after seven recrystallizations from ethanol-water.

Ring-labelled guanethidine sulphate. (a) [2- ^3H]Octahydroazocine (II). 2-Oxoheptamethyleneimine (I) (2g.) was added to an ethereal solution of LiAl^3H_4 prepared from 1g. of LiH as described above, and the mixture was stirred for 6 hr. The complex was decomposed as described for 2-(octahydro-1-azocinyl)-[1- ^3H]ethylamine (IV), but 2g. of carrier octahydroazocine was added before separation of the ether phase. The ethereal solution was dried over Na_2SO_4 and then evaporated to give 2g. of a colourless oil.

(b) [2- ^3H]Octahydro-1-azocinylacetone (III). The [2- ^3H]octahydroazocine (II) prepared as described above was dissolved in 12 ml. of toluene; 0.81g. of Na_2CO_3 and 1 ml. of chloroacetonitrile were added and the mixture was heated on a boiling-water bath under reflux for 6 hr. The reaction mixture was filtered and the filtrate evaporated to give a yellow oil, which yielded 1.8g. of a mobile oil on distillation.

(c) 2-([2-³H]Octahydro-1-azocinyl)ethylguanidine (V). The 2-([2-³H]octahydro-1-azocinyl)acetonitrile was reduced with unlabelled LiAlH₄ and amidinated under the conditions described for the preparation of side-chain-labelled guanethidine sulphate to give 1.5 g. of ring-labelled guanethidine sulphate (3.6 μC/mg.), which satisfied the criteria of purity described for side-chain-labelled guanethidine sulphate.

[guanidino-¹⁴C]Guanethidine sulphate (V). A mixture of [¹⁴C]isothiurea (307 mg., 20 mC), 0.13 ml. of water and 0.2 ml. of dimethyl sulphate was refluxed at 140° for 30 min. The resulting *S*-methyl[¹⁴C]isothiuronium sulphate solidified on cooling and was then used to amidinate 800 mg. of freshly distilled 2-(octahydro-1-azocinyl)ethylamine (IV) under the conditions described for the preparation of side-chain-labelled guanethidine sulphate, to give 690 mg. of a product (sp. radioactivity 15 μC/mg.) that satisfied the criteria of identity and purity described for side-chain-labelled guanethidine sulphate.

Incubation procedures

After removal of the liver from the animal and until incubation of the homogenates, all operations were carried out at about 4°. Tissues were homogenized in an Ultra-Turrax homogenizer (Janke und Kunkel, Staufen i. Br., West Germany). At 240 v the motor speed is 24 000 rev./min. For the present work 100 v was supplied through a variable transformer. All incubations were terminated by addition of 2 vol. of 5% (w/v) trichloroacetic acid in 0.05 M-HCl to 1 vol. of incubation mixture and blending in the Ultra-Turrax homogenizer. The mixture was centrifuged at 800 g for 20 min. and the acidified protein-free supernatant containing drug and metabolites was decanted and stored at 4°.

Experiments with pig liver. (a) Preparation of homogenate. Portions (100 g.) of minced liver were homogenized in 200 ml. of 0.1 M-sodium phosphate buffer, pH 7.4, in the Ultra-Turrax homogenizer for two periods of 30 sec., between which connective tissue was removed from the blade. The homogenate was centrifuged in 1 l. polythene bottles for 30 min. at 800 g and the supernatant was decanted through cheesecloth into a large polythene vessel.

(b) Tests for cofactor requirements. Portions (3 ml.) of supernatant were mixed with cofactors and enzymes as described in the Results section and with 6 mg. of side-chain-labelled guanethidine sulphate diluted to a specific radioactivity of 12 μC/g., and the mixture was incubated at 37° for 1 hr. in 25 ml. Erlenmeyer flasks.

(c) Large-scale preparation of metabolites. Homogenate from pig livers (3 kg.) was mixed with 18 g. (12 μC/mg.) of side-chain-labelled guanethidine sulphate, 60 g. of glucose 6-phosphate, 3 g. of NADP⁺, 600 units of glucose 6-phosphate dehydrogenase and 30 m-moles of MgCl₂. The mixture was heated by circulation through a large QVF condenser coil jacketed with water at about 40°.

Oxygenation was achieved by pumping the homogenate at a rate of 2–3 l./min. on to the top inside surface of a 20 l. round-bottomed flask inverted above the incubation tank so that a thin film of homogenate flowed down the walls of the flask and back into the tank. O₂ was passed into the flask at about 6 l./min. Incubation was continued for 1 hr. after the temperature had reached 30°.

Experiments with rabbit liver. (a) Small-scale experiments.

Rabbit liver was cut up into small pieces and homogenized with 2 ml. of 0.1 M-sodium phosphate buffer (pH 7.4)/g. of liver in the Ultra-Turrax homogenizer for 1 min. The homogenate was centrifuged at 14 000 g for 20 min. and the supernatant decanted into polythene tubes that were stored in liquid N₂. When required for use batches of supernatant were thawed rapidly and stored in ice until incubated. For the preparation of double-labelled metabolites and for experiments showing the effect of guanethidine concentration on the yield of metabolites, 4 ml. of supernatant was diluted with 3 ml. of 0.1 M-sodium phosphate buffer, pH 7.4, containing 1.8 mg. of NADP⁺, 20 mg. of glucose 6-phosphate and either 6 mg. of ring-labelled [³H]guanethidine sulphate (0.75 μC/mg.) or 0.6, 6 or 18 mg. of [¹⁴C]guanethidine sulphate (15 μC/mg.). The mixtures were incubated in 25 ml. Erlenmeyer flasks in air at 37° for 1 hr. For the preparation of samples to be tested for volatile radioactive products derived from guanethidine, 3 mg. of [¹⁴C]guanethidine sulphate (3.5 μC/mg.), side-chain-labelled [³H]guanethidine sulphate (30 μC/mg.) or ring-labelled [³H]guanethidine sulphate (3.6 μC/mg.) was incubated at 37° for 1 hr. with 2 ml. of stored 14 000 g supernatant from rabbit liver and 3 ml. of 0.1 M-sodium phosphate buffer, pH 7.4, containing 0.9 mg. of NADP⁺ and 1 mg. of glucose 6-phosphate.

(b) Large-scale preparations of metabolites. Six rabbits were killed by a sharp blow on the head and the livers were removed, freed of the gall bladder and placed in 0.1 M-sodium phosphate buffer, pH 7.4. After a rinsing with chilled buffer the pooled livers were damp-dried on Kleenex tissues and weighed. The pooled livers (350–450 g.) were minced, homogenized in 100 g. portions with 200 ml. of 0.1 M-sodium phosphate buffer, pH 7.4, and centrifuged as described for pig liver. The supernatants were decanted into a large beaker and 100 mg. of glucose, 10 units of hexokinase, 1 mg. of NADP⁺ and 9 mg. of guanethidine sulphate (12 μC/g.) were added/g. of liver used. After thorough mixing the solution was divided into 500 ml. Erlenmeyer flasks (approx. 100 ml. in each) and incubated in a water bath at 37° with rapid shaking for 1 hr.

Assay procedures

Radioactivity assay. Samples were assayed by using the Packard Tri-Carb model 314 EX liquid-scintillation counting system. Up to 0.5 ml. of aqueous samples were added to vials with 15 or 20 ml. of scintillator (containing 30 g. of 2,5-bis-(5-*tert*.-butylbenzoxazol-2-yl)thiophen and 340 g. of naphthalene in a mixture of 2.5 l. of toluene and 1.7 l. of methylCellosolve). The quantity of radioactive material in samples was calculated by reference to ³H and ¹⁴C standards added to appropriate blank samples. Counting efficiencies were about 50% for ¹⁴C and 10% for ³H with typical background counts of 20 and 30 c.p.m. respectively. For the simultaneous determination of ³H and ¹⁴C, samples were counted with the gain and discriminator settings adjusted so that ³H was counted with efficiencies of 10% and 0.03% and ¹⁴C was counted with efficiencies of 12% and 25% in the red and the green channel respectively. Typical background counts were 30 c.p.m. and 10 c.p.m. in the red and the green channel respectively. The ratios of the radioactivities (c.p.m.) above background in the two channels were determined for [¹⁴C]guanethidine (200 nc/vial) and for the double-labelled guanethidine used for

incubation (22.5nc of ^{14}C and 100nc of ^3H /vial) for each type of sample assayed. The $^3\text{H}/^{14}\text{C}$ ratio in the test sample compared with that in starting guanethidine (relative $^3\text{H}/^{14}\text{C}$ ratio) was calculated as follows: R_x, R_c, R_s are the radioactivities (c.p.m.) above background in the red channel for unknown, ^{14}C standard and double-labelled standard respectively. G_x, G_c, G_s are the corresponding radioactivities (c.p.m.) above background in the green channel.

$$\frac{^3\text{H}/^{14}\text{C unknown}}{^3\text{H}/^{14}\text{C guanethidine}} = \frac{(R_x - R_c G_x)/G_x}{(R_s - R_c G_s)/G_s} = (R_x/G_x - R_c/G_c)/(R_s/G_s - R_c/G_c)$$

Measurement of total metabolism. The procedure was based on methods for the separation of guanethidine from its metabolites, as described by Shanker & Morrison (1965) and Chang, Costa & Brodie (1965). Acidified protein-free supernatant (2ml.) was made strongly alkaline with 0.2ml. of aq. 60% KOH and 0.2ml. was removed for radioactivity assay. The mixture was shaken with 10ml. of chloroform, the plasma was allowed to separate and 0.2ml. of the aqueous phase was removed for radioactive assay. Calculation of the percentage of radioactivity left after chloroform extraction indicates the extent of metabolism, since 95–96% of unchanged guanethidine is removed from the aqueous phase under these conditions, and experiments by Chang *et al.* (1965) suggest that metabolites are not extracted into chloroform. The metabolites in the 6M-HCl eluate from ion-exchange columns were measured by making 1ml. of the eluate alkaline with 1ml. of 60% KOH and then proceeding as above.

Fractionation of metabolites. (a) Ion-exchange procedure. Up to 2ml. of acidified protein-free supernatant was applied to a column (7cm. $\times$ 1cm.) containing AG50W sulphonic acid ion-exchange resin (X4; 100–200 mesh; H^+ form). The column was eluted with 4M- NH_3 at 1ml./min. and 1ml. fractions were collected. All fractions after no. 4 were alkaline. Samples (0.1ml.) of each fraction were assayed for radioactivity. The radioactivity in fractions 8–20 was summed to give an estimate of the amount of metabolite 1 in the sample. The column was then eluted with 6M-HCl at 1ml./min. and 1ml. fractions were collected. Fractions 6–23 were pooled and evaporated at 40–50° in a rotary evaporator to give a viscous residue, which was dissolved in 2ml. of water. This solution was then examined by high-voltage electrophoresis.

(b) High-voltage electrophoresis. Small volumes (10–60 $\mu\text{l.}$) of each sample were applied to a strip (15cm. $\times$ 80cm.) of Whatman 3MM paper at points 3cm. apart and 10cm. from one end of the strip. The paper was then carefully wetted with pyridine-acetate buffer, pH 6.1 (pyridine-acetic acid-water, 25:2:625, by vol.), so that buffer did not run across the line of sample application, and the paper was damp-dried with Kleenex tissues. The paper was placed in a Locarte Mk. 4 high-voltage electrophoresis machine, sample end nearest the anode, and electrophoresis was carried out at 6000v for 1hr. The paper was dried in an oven at 60° and then cut lengthwise into 2cm. strips so that each sample lay in the centre of a strip. The strips were then divided lengthwise into two halves, one of which was tested by using the Sakaguchi colour reaction described by Smith & Horsewell (1960) and the other of

which was cut into 2cm. lengths, which were placed in counting vials containing 0.5ml. of 0.05M- H_2SO_4 . After agitation for 1min., 15ml. of scintillator was added and the vials were capped and mixed by using a Whirlimixer. After preliminary counting, the $^3\text{H}/^{14}\text{C}$ ratio was measured in samples that had been determined to have sufficient radioactivity, by removing the paper from the vials so that single-phase counting of material eluted from the paper could be carried out. Results obtained from ^{14}C -labelled and double-labelled standards added to 15ml. of scintillator and 0.5ml. of 0.05M- H_2SO_4 were used in calculation of the results. When this method was applied to double-labelled guanethidine standards that had been subjected to electrophoresis, relative $^3\text{H}/^{14}\text{C}$ ratios 0.95 and 0.97 were obtained for the radioactive peak.

Determination of tritiated water. A portion (1ml.) of protein-free supernatant obtained after incubating guanethidine with rabbit liver homogenate was added to 3ml. of water in a 25ml. round-bottomed flask and 2–3ml. of water was carefully distilled off by using a Claisen head and a single-walled water-cooled condenser. A 0.5ml. portion of this sample was assayed for radioactivity.

Isolation of metabolites

Metabolite 1. (a) Bulk fractionation. The pooled acidified protein-free supernatants from four batches of incubated rabbit liver homogenate were pumped on to 200ml. of AG50W sulphonic acid cation-exchange resin (X4; 100–200 mesh; H^+ form) in a 500ml. cylindrical separating funnel and the resin was washed with water until the eluate was neutral. The resin was then eluted with a 9% (v/v) solution of triethylamine in water and fractions were collected as soon as the alkaline front appeared. The eluate was tested for radioactivity and elution was stopped when the radioactivity (c.p.m.) had fallen to the background value. The total volume of these fractions was about 1l. and they contained about 5% of the radioactivity added to the column. These fractions were pooled, acidified with conc. HCl to pH 2 and pumped on to a 40ml. bed of AG50W resin (X4). The resin was washed with water and eluted as before. This time the radioactivity was concentrated in about 100ml. of eluate, which was deep yellow. The eluate was evaporated on a water bath at room temperature under high vacuum in a rotary evaporator fitted with a 'cold finger', cooled with acetone-solid CO_2 , to give a sticky yellow solid (approx. 1g.).

(b) Purification. The residue containing metabolite 1 was redissolved in 2–3ml. of 0.1M-HCl and run on to a column (30cm. $\times$ 0.7cm.) of AG50W resin (X4). The column was washed with water and then eluted with 4M- NH_3 . When the eluate became alkaline, fractions (5ml.) were collected. The first fraction was yellow-brown, the second was pale yellow and subsequent fractions were colourless. Samples (20 $\mu\text{l.}$) of these fractions were assayed for radioactivity and fractions 3–13 were pooled and evaporated as before, leaving a pale-yellow oil. A small amount of this oil was treated with saturated aqueous picric acid and the picrate formed was recrystallized from water to give 35mg. of a product, m.p. 177–178°. Most of the residual oil, however, was dissolved in 0.5–1ml. of 0.5M- H_2SO_4 and carefully transferred to a 10ml. centrifuge tube. On addition of ethanol a dense white precipitate was produced. This was centrifuged, the supernatant decanted and the

residue recrystallized in the same tube from ethanol-water. The product was washed three times by centrifugation with ether and decanting, and was then dried overnight *in vacuo* to give 240 mg. of white crystalline powder, m.p. 189–190°.

Metabolite 2. (a) Bulk fractionation. About 17 l. of protein-free supernatant from pig liver homogenates was pumped into a 1 l. cylindrical separating funnel containing 500 ml. of AG 50 W sulphonic acid ion-exchange resin (X4; 100–200 mesh; H⁺ form). The resin was washed with water until the eluate became almost colourless (5 l.). The eluate (0.5 ml. portions from every 5 l.) was tested for radioactivity during both washing and sample application. The column was eluted with 2 M-NH₃ until coloured material appeared in the eluate (after about 500 ml.) and then eluted with 4 M-NH₃. Fractions of the eluate were collected, 0.5 ml. of each of which was assayed for radioactivity. When no further radioactive material came off the column it was washed with 2 M-HCl until the eluate was acid and then fractions were collected during elution with 6 M-HCl. Samples (0.1 ml.) of these fractions were assayed for radioactivity and 1 ml. samples were tested for total metabolites. All fractions collected after 900 ml. had been eluted with 6 M-HCl and until 2900 ml. had been eluted were pooled. A small amount (10 μ l.) of this sample was examined by high-voltage electrophoresis.

(b) Countercurrent distribution. A 200 ml. sample of the pooled fractions eluted with 6 M-HCl was concentrated with a rotary evaporator on a water bath (50–60°) to give a viscous residue, which was neutralized with 4 M-NH₃ and applied to the first tube of a Quickfit Mk. 1a automatic steady-state distribution machine. Each tube contained 10 ml. of each phase of an aq. 0.1% NH₃-butan-2-ol (1:1, v/v) system. After 100 transfers of the upper phase the contents of tubes 17–23 were pooled and evaporated, and the residue was reapplied to tube 1 of the machine, which was loaded with fresh solvent. After 300 transfers 0.1 ml. of the top and bottom phases of each tube were combined for radioactive assay. The contents of tubes 62–78 were combined and evaporated under reduced pressure at 40° to give a white residue. This residue was dissolved in the minimum amount of water and treated with a saturated solution of picric acid in water to give a picrate, which was recrystallized from water to give approx. 100 mg. of a yellow crystalline product, m.p. 178–180°.

Synthesis of proposed metabolites and related compounds

2-(6-Carboxyhexylamino)ethylguanidine (metabolite 1).

(a) 2-(Benzamido)ethylguanidine. *N*-(2-Aminoethyl)benzamide (27 g.) prepared as described by Hill & Aspinall (1939) and *S*-methylisothiuronium sulphate (15.3 g.) were dissolved in water (70 ml.) and the mixture was heated for 2 hr. on a steam bath. After cooling, acetone (300 ml.) was added and the precipitate filtered off. Recrystallization from a small volume of water gave the required product as prisms, m.p. 107–109°, yield 22.3 g. Electrophoresis at pH 6.1 (pyridine-acetate buffer, 20 v/cm. on paper) gave a single Sakaguchi-positive ninhydrin-negative spot of lower mobility than that due to *N*-(2-aminoethyl)benzamide (ninhydrin-positive Sakaguchi-negative). 2-(Benzamido)-ethylguanidine sulphate was analysed and shown to be the hydrate [Found: C, 43.6; H, 6.13; N, 20.6; S, 6.1; (C₁₀H₁₄N₄O)₂.H₂SO₄.2H₂O requires C, 43.9; H, 6.23; N, 20.5; S, 5.9%], and 2-(benzamido)ethylguanidine also

formed a picrate, m.p. 198–200° (Found: C, 44.1; H, 4.2; N, 22.1; C₁₀H₁₄N₄O₂.C₆H₃N₃O₇ requires C, 44.1; H, 3.9; N, 22.5%).

(b) 2-Aminoethylguanidine. A solution of 2-(benzamido)-ethylguanidine sulphate dihydrate (20 g.) in 6 M-HCl (200 ml.) was boiled under reflux for 6 hr., cooled and extracted with ethyl acetate (3 $\times$ 100 ml.), and the acid layer was evaporated to dryness. The solid residue was recrystallized from aqueous ethanol to give prisms, m.p. 245° (decomp. with softening at 226°) (literature m.p. 298°; Adcock, Lawson & Miles, 1961), which were analysed and shown to be 2-aminoethylguanidine sulphate monohydrate (Found: C, 16.7; H, 6.0; N, 25.3. Calc. for C₃H₁₀N₄.H₂SO₄.H₂O: C, 16.5; H, 6.4; N, 25.7%).

(c) Reaction of 2-aminoethylguanidine with 7-bromoheptanoic acid. 2-Aminoethylguanidine (2 g.), 7-bromoheptanoic acid (2.1 g.) and Ba(OH)₂.8H₂O (3.9 g.) were stirred in 25 ml. of water for 3 hr. at 60°. The pH was maintained at 10–11 by addition of 0.3 g. of Ba(OH)₂.8H₂O during the reaction. After the mixture had cooled, the pH was adjusted to 4–4.5 with H₂SO₄, the precipitate was filtered off and the solution was put on to a column of AG 50 W resin (X4; 100–200 mesh; H⁺ form) and washed with water until the eluate was at pH 4–5. Elution with 2 M-NH₃ gave ninhydrin-positive Sakaguchi-negative material at the alkaline front, followed by, and overlapping to some extent with, Sakaguchi-positive ninhydrin-negative material of electrophoretic mobility (in pyridine-acetate buffer, pH 6.1) identical with that of natural metabolite 1. Fractions containing only Sakaguchi-positive ninhydrin-negative material were combined and evaporated (bath temp. less than 25°) to give a white solid. A portion was treated with saturated aqueous picric acid to form 2-(6-carboxyhexylamino)ethylguanidine picrate, m.p. 175–177° [Found: C, 38.4; H, 4.0; N, 20.5; C₁₀H₂₂N₄O₂.(C₆H₃N₃O₇)₂ requires C, 38.4; H, 4.1; N, 20.3%] and the remainder was treated with a slight excess of 2 M-H₂SO₄ in which it first dissolved and then crystallized out as 2-(6-carboxyhexylamino)ethylguanidine sulphate dihydrate, m.p. 188–190° (Found: C, 32.8; H, 7.3; N, 15.4; S, 9.4; C₁₀H₂₂N₄O₂.H₂SO₄.2H₂O, requires C, 33.0; H, 7.4; N, 15.4; S, 8.8%).

Guanethidine N-oxide. To a solution of 2.96 g. of guanethidine sulphate in 6 ml. of acetic acid, 1.5 ml. of 30% (w/v) H₂O₂ was added and the mixture was heated for 6 hr. at 70°. A piece of platinum wire was left in the cooled solution overnight to destroy excess of H₂O₂. The platinum wire was removed, 5 ml. of water added and the mixture evaporated to an oil, whereupon a further 5 ml. of water was added and evaporated. A white residue was obtained, which was washed with ethanol and ether and finally dried over CaO to give 3 g. of white powder, m.p. (in a sealed tube) 162° (Found: C, 38.0; H, 7.4; N, 17.7; S, 10.1; C₁₀H₂₂N₄O₂.H₂SO₄ requires C, 38.4; H, 7.7; N, 17.9; S, 10.3%). Low-voltage electrophoresis at pH 6.1 showed a single Sakaguchi-positive component with a mobility of 0.69 relative to guanethidine. A small amount of material dissolved in water was treated with a saturated solution of picric acid, and the precipitate was crystallized from water to give *guanethidine N-oxide dipicrate*, m.p. 178–180° [Found: C, 39.0; H, 4.3; N, 20.5; C₁₀H₂₂N₄O₂.(C₆H₃N₃O₇)₂ requires C, 39.3; H, 4.2; N, 20.8%].

2-(Methylamino)ethylguanidine. This compound and its transformation product, 1-methyl-2-iminoimidazolidine, were prepared by Mr P. Baldry. *S*-Methylisothiuronium

sulphate (27.8g.) was dissolved in 540ml. of water and 15.3g. of *N*-methylethylenediamine was added. After the mixture had stood at room temperature for 3 days, acetone was added, and, after vigorous stirring of the mixture, the solvent was decanted leaving an oily residue, to which was added 0.5M-H₂SO₄ until the solution was acid. Ethanol was added and, after the precipitate had been washed, filtered and dried, 13.5g. of 2-(methylamino)ethylguanidine sulphate was obtained, m.p. 235° (Found: C, 22.6; H, 6.7; N, 25.9; C₄H₁₂N₄.H₂SO₄ requires C, 22.4; H, 6.6; N, 26.2%). A portion (1g.) of this material was dissolved in water and saturated picric acid in water was added until precipitation was complete. The solution was boiled and cooled to give crystalline 2-(methylamino)ethylguanidine dipicrate, m.p. 223–226°, yield 2g. [Found: C, 33.3; H, 3.2; N, 24.2; C₄H₁₂N₄.(C₆H₃N₃O₇)₂ requires C, 33.5; H, 3.2; N, 24.4%]

Characterization of metabolites

Potentiometric titration. The sample was dissolved in 5ml. of water and the pH was adjusted to about 2.3 by adding M-HCl with a 10μl. syringe. Titration was then carried out under N₂ by using 0.1M-NaOH and a Radiometer titrimeter. For the comparison of metabolite 1 and its transformation product the curve for the titration of 3mg. of metabolite 1 up to pH11 was recorded. The solution was then heated at 100° for 30min. in a narrow tube with N₂ slowly bubbling through it. Moist pH paper held in the mouth of the tube became alkaline. The solution was cooled, the pH adjusted to 2.3 with M-HCl and then again titrated against 0.1M-NaOH.

Electrophoresis. Where mobilities relative to guanethidine are reported, the following conditions were used. Samples were spotted on to Whatman 3MM paper, which was then wetted carefully with pyridine-acetate buffer, pH6.1, and placed in a Shandon low-voltage electrophoresis apparatus so that the samples were 7cm. from the centre of the tank on the anode side. Electrophoresis was carried out for 1hr. at an initial voltage of 400v and at constant current; the paper was then dried and monosubstituted guanidines were located by using the Sakaguchi reaction as described by Smith & Horsewell (1960).

Transformation reaction. Synthetic 2-(6-carboxyhexylamino)ethylguanidine as the free base was converted into its transformation product by heating in 2M-NH₃ on a steam bath for 2hr. and evaporating the solution to dryness. The residue was recrystallized from water to give a product with m.p. 265° (decomp.). A portion of this product was converted into a crystalline picrate, m.p. 163–165°. A small amount of natural metabolite 1 similarly treated with 2M-NH₃ gave a crystalline picrate, m.p. 165–166°.

To prepare the transformation product of methylaminoethylguanidine sulphate, this compound was dissolved in 4M-NH₃ and the solution was heated on a steam bath for 1hr. and then evaporated to dryness. The residue was dissolved in a small amount of water and then treated with excess of saturated aqueous picric acid. The precipitated material was recrystallized from water to give a crystalline picrate, m.p. 186–189°.

The rate of transformation was measured by using the fact that the transformation product is not Sakaguchi-positive. Solutions containing 8mg. of synthetic 2-(6-carboxyhexylamino)ethylguanidine sulphate in 4ml. of 0.05M-H₂SO₄, 0.1M-sodium phosphate buffer, pH7.4 at 25°, and M-NH₃ or 0.1M-NaOH were prepared. Solutions

containing 8mg. of methylaminoethylguanidine sulphate, aminoethylguanidine sulphate or guanethidine sulphate in 4ml. of 0.1M-NH₃ were also prepared. Each solution was divided into two 2ml. portions, one of which was heated at 100° for 30min. and rapidly cooled and the other of which was kept at 0°. A 10μl. sample of each solution was then added to 2ml. of 0.05M-H₂SO₄ and this diluted solution was assayed by using the modified Sakaguchi reaction described by Van Pilsum, Martin, Kito & Hess (1956). The readings for each compound were in the linear range of the assay and the percentage of product transformed was calculated as:

$$\frac{\text{Reading for unheated solution} - \text{reading for heated solution}}{\text{Reading for unheated solution}} \times 100.$$

Nitrosation of metabolite 1. Synthetic metabolite 1 (0.5g.) was dissolved by warming in 0.5M-H₂SO₄ (3ml.), and 6ml. of 6% (w/v) NaNO₂ solution was added. The solution was heated for 1.5hr. on the steam bath, then a similar quantity of NaNO₂ was added and heating was continued for a further 1hr. Electrophoresis at pH6.1 (in pyridine-acetate buffer) gave a single Sakaguchi-positive (orange) spot of mobility identical with that of guanidoacetic acid and significantly less than that of metabolite 1. After the pH of the solution had been adjusted to 5 it was introduced on to a column (40ml.) of AG50W resin (X4) and the column washed well with water. Elution with M-NH₃ and collection of 10ml. fractions gave a Sakaguchi-positive peak emerging immediately after the alkaline front with a maximum at fraction 6. The fractions containing Sakaguchi-positive material were combined and evaporated to a crystalline solid of m.p. 230° (decomp.). Recrystallization from aqueous acetone gave a purer sample of 2-[N-(6-carboxyhexyl)-N-nitrosoamino]ethylguanidine, m.p. 238–240° (decomp.) (Found: C, 46.5; H, 8.2; N, 27.1; C₁₀H₂₁N₅O₃ requires C, 46.3; H, 8.1; N, 27.0%). A portion of this was converted with saturated aqueous picric acid into a picrate, m.p. 156–157° (Found: C, 39.2; H, 4.8; N, 22.7; C₁₀H₂₁N₅O₃.C₆H₃N₃O₇ requires C, 39.4; H, 4.9; N, 22.9%).

Action of concentrated hydrochloric acid on the nitroso derivative of metabolite 1. The nitroso compound (25mg.) was dissolved in 0.5ml. of conc. HCl and the solution boiled under reflux. Samples (5μl.) were removed at times 0, 5, 10, 20, 30, 75, 120, 180 and 280min., and subjected to paper electrophoresis at pH6.1 in pyridine-acetate buffer. With time the orange Sakaguchi-positive spot of mobility identical with that of guanidoacetic acid was replaced by an intense spot of colour and mobility identical with that of metabolite 1. In addition a much weaker Sakaguchi-positive spot of higher mobility appeared after 10min. and grew in intensity as the hydrolysis proceeded. After 1hr. the nitroso compound had been virtually all destroyed. That the minor Sakaguchi-positive spot arose from the nitroso compound was shown by a control experiment in which metabolite 1 was treated with boiling conc. HCl under similar conditions. No second Sakaguchi-positive spot was obtained after 3hr. of boiling.

Attempted condensation of benzoylaminoethylguanidine and 7-bromoheptanoic acid. To confirm that, in the synthesis of 2-(6-carboxyhexylamino)ethylguanidine, 7-bromoheptanoic acid did not condense with the guanidine group of aminoethylguanidine the following control reaction was carried out.

2-(Benzamido)ethylguanidine sulphate monohydrate

(0.5g.) was dissolved in 5ml. of water at 60° in a 25ml. Erlenmeyer flask and 2 μ l. taken for electrophoresis. Then 0.5g. of Ba(OH)₂·8H₂O and 0.42g. of 7-bromoheptanoic acid were added with stirring and 2 μ l. of this mixture was taken for electrophoresis. A small sample (10 μ l.) of this mixture was added to 2ml. of 0.5M-H₂SO₄ and, after centrifugation, 20 μ l. of the supernatant was added to 2ml. of 0.05M-H₂SO₄, and this sample was assayed by using the modified Sakaguchi reaction described for arginine by Van Pilsun *et al.* (1956).

The bulk of the reaction mixture was then heated at 60° for 3-5hr. with stirring and samples were then taken as above for electrophoresis and colorimetric assay. After the contents of the flask had cooled, the volume of the aqueous phase was measured so that allowance could be made for concentration due to water loss.

RESULTS

Incubation conditions. The rate of metabolism of guanethidine in whole pig liver homogenate was greatly increased by the addition of NADP⁺. Typical values for the total amounts of metabolites produced from 0.3mg. of guanethidine by 3ml. of pig liver homogenate assayed by the chloroform-extraction procedure are 8% without additional cofactors and 20% with the addition of 0.9mg. of NADP⁺ and 6mg. of nicotinamide. The importance of adding an adequate amount of NADP⁺ and an active enzyme system to reduce this cofactor is shown by the results in Table 1. It is shown that glucose 6-phosphate can be replaced by glucose and hexokinase. Since these preliminary experiments were carried out to establish a practical procedure for the large-scale production of metabolites the use of more than 1g. of NADP⁺/kg. of liver was not considered.

When the quantity of metabolites formed after incubation at 37° for different times was investigated it was found that metabolism of guanethidine had virtually ceased after 45min., and an incubation

period of 1hr. was therefore chosen for the preparation of metabolites.

In small-scale experiments carried out in Erlenmeyer flasks there was little difference in the yields of metabolites obtained when incubation was carried out in air or oxygen. However, in the large-scale pig liver preparations the surface-area/volume ratio appeared to limit the uptake of oxygen into the homogenate and the procedure described in the Materials and Methods section was developed to permit adequate oxygenation without the formation of large amounts of froth that occurred if oxygen was simply bubbled through a large volume of homogenate.

When the supernatant from bulk preparations of pig liver homogenate was assayed for total metabolites it was found that 10-15% of the radioactivity was in the form of metabolites. However, when these metabolites were fractionated by using ion-exchange resin only about 1.5% of the radioactivity applied to the column was eluted with ammonia (metabolite 1), whereas 70% was subsequently eluted with 6M-hydrochloric acid (63% as unchanged guanethidine and 7% as metabolites, mainly metabolite 2). The acid eluate from columns loaded with pig liver supernatant was therefore used for the isolation of metabolite 2 and an alternative incubation method for the production of metabolite 1 was sought.

Rabbit liver homogenates were tested both for total metabolites by using chloroform extraction and specifically for metabolite 1 by using a small ion-exchange column, and were found to give much higher yields of total metabolites and of metabolite 1 than did pig liver. It had been noticed that in the incubation of large-scale pig liver preparations lower guanethidine concentrations gave a higher ratio of metabolite 1 to total metabolites. The results of experiments with different guanethidine

Table 1. *Effect of added cofactors and substrates on the metabolism of guanethidine*

Side-chain-labelled [³H]guanethidine (6mg.) was incubated with 3ml. of pig liver homogenate (equivalent to 1g. of liver) and the cofactors indicated at 37° for 1hr. and the total metabolites were measured as described in the Materials and Methods section. Expts. 1 and 2 were carried out on different batches of liver homogenate.

Expt. no.	NADP ⁺ (mg.)	Additions to homogenate				Guanethidine metabolized (%)
		Glucose 6-phosphate (mg.)	Glucose 6-phosphate dehydrogenase (unit)	Glucose (mg.)	Hexokinase (units)	
1	0.5	5.0	0.2	0.0	0.0	6
	1	5.0	0.2	0.0	0.0	12
	1	10.0	0.2	0.0	0.0	19
2	1	10.0	0.2	0.0	0.0	13
	1	20.0	0.2	0.0	0.0	15
	1	0.0	0.2	100.0	10.0	16

concentrations in rabbit liver are shown in Table 2, and on the basis of these results 9 g. of guanethidine sulphate/kg. of rabbit liver was selected for the production of metabolite 1.

Metabolism of ring-labelled [^3H]guanethidine. The possibility that in the formation of metabolites tritium might be eliminated from [^3H]guanethidine as water, giving rise to unlabelled products and tritiated water, was tested by examining the distillate obtained from rabbit liver homogenates that had been incubated with side-chain- and ring-labelled [^3H]guanethidine. In these experiments 70% of the guanethidine was converted into metabolites that could not be extracted into chloroform. With side-chain-labelled [^3H]guanethidine only 1% of the radioactive metabolic products appeared in the distillate whereas with ring-labelled [^3H]guanethidine 18% of the radioactive metabolic products appeared in the distillate. The result with side-chain-labelled [^3H]guanethidine was confirmed for metabolism in pig liver by the fact that less than 0.1% of the total radioactivity in bulk samples of pig liver supernatant applied to large AG 50 W resin

(X4) columns appeared in the eluate while the sample was being applied and the column washed with water.

Table 2. *Effect of guanethidine concentration on the relative amounts of metabolite 1 and total metabolites formed in rabbit liver homogenate*

The indicated quantities of side-chain-labelled [^3H]guanethidine sulphate were incubated with 3 ml. of rabbit liver homogenate (equivalent to 1 g. of liver) and cofactors as described in the Materials and Methods section. Total metabolites were measured as radioactivity that could not be extracted from alkaline solution. Metabolite 1 was measured as radioactivity eluted from a small column of AG 50 W ion-exchange resin (X4) with aq. NH_3 .

Guanethidine sulphate added (mg.)	Guanethidine sulphate converted into total metabolites (mg.)	Guanethidine sulphate converted into metabolite 1 (mg.)
0.3	0.27	0.18
3	1.65	0.75
9	2.4	0.84

Table 3. *Isotope ratios of metabolites formed in rabbit liver homogenates from a mixture of ring-labelled [^3H]guanethidine and [guanidino- ^{14}C]guanethidine*

Radioactivities (c.p.m.) (red channel) and $^3\text{H}/^{14}\text{C}$ ratios relative to the starting guanethidine are given. (a) High-voltage paper electrophoresis at pH 6.1 of 10 μl . of rabbit liver supernatant. (b) Ammoniacal eluate from a column of AG 50 W resin (X4) loaded with 2 ml. of supernatant. (c) High-voltage electrophoresis of material subsequently eluted from this column with 6M-HCl.

	(a)															
Distance from origin (cm.)	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56
Radioactivity (c.p.m.)—background	29	78	142	151	174	86	45	46	57	69	149	141	181	307	299	73
Relative $^3\text{H}/^{14}\text{C}$ ratio (duplicate counts)			0.65 0.66	0.74 0.74	0.82 0.86						1.03 1.03	0.97 1.05	1.03 1.04	1.10 1.04	1.02 1.02	
Metabolites present			1 and 2								3		Guanethidine			
	(b)															
Fraction no.	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
$10^{-3} \times$ Radio-activity (c.p.m.)	0.06	1.40	1.05	1.25	3.23	5.97	9.55	10.8	10.3	7.96	5.75	3.84	2.25	1.22	0.66	0.36
Relative $^3\text{H}/^{14}\text{C}$ ratio						0.49	0.51	0.52	0.52	0.53	0.54					
Metabolites present						Metabolite 1										
	(c)															
Distance from origin (cm.)	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56
$10^{-3} \times$ Radio-activity (c.p.m.)	0.05	0.29	3.31	3.43	0.40	0.07	0.08	0.07	0.35	2.50	2.04	1.95	8.95	9.90	0.05	0.06
Relative $^3\text{H}/^{14}\text{C}$ ratio			0.97	0.95						1.01	0.96	0.98	1.02	1.04		
Metabolites present			2							3		Guanethidine				

Experiments were carried out with a mixture of [*guanidino*- ^{14}C]guanethidine and ring-labelled [^3H]guanethidine to measure the extent to which loss of hydrogen on the ring carbon atom next to the nitrogen atom occurred in the individual metabolites. Table 3 shows the results obtained when the $^3\text{H}/^{14}\text{C}$ ratio relative to that of starting guanethidine was measured in metabolite 1 eluted from an ion-exchange column with aq. ammonia, and in other metabolites subsequently eluted with 6*M* hydrochloric acid and separated by high-voltage electrophoresis. The formation of metabolite 1 definitely involved the partial loss of tritium. Guanethidine, metabolite 2 and metabolite 3 (a product present in small amounts and poorly resolved from guanethidine) all had $^3\text{H}/^{14}\text{C}$ ratios close to that of the starting material.

The 50% decrease in the $^3\text{H}/^{14}\text{C}$ ratio of metabolite 1 is compatible with the loss of both the hydrogen atoms on one of the ring carbon atoms next to nitrogen. Thus the rate of production of ^3H -labelled metabolite, which could arise only from attack on the untritiated ring carbon atom next to the nitrogen atom, would be exactly half the rate of production of ^{14}C -labelled metabolite, which would arise from attack on either of the two ring carbon atoms next to the nitrogen atom.

Isolation and preliminary identification of metabolite 1. Rabbit liver homogenate was applied to a large bed of AG 50W ion-exchange resin (X4) and eluted with triethylamine to give a crude sample of metabolite 1, which was purified by ion-exchange chromatography as described in the Materials and Methods section, yielding a crystalline sulphate. The purified product gave a single radioactive Sakaguchi-positive spot on electrophoresis at pH 6.1 (pyridine-acetate buffer), indicating that metabolite 1 probably contained a monosubstituted guanidine group, for which the Sakaguchi reaction is considered to be a specific test. The sulphate of metabolite 1 formed a crystalline picrate with an elemental composition suggesting that it was the dipicrate of a product that differed from guanethidine only by the addition of two atoms of oxygen [Found: C, 38.1; H, 4.0; N, 20.2; $\text{C}_{10}\text{H}_{22}\text{N}_4\text{O}_2 \cdot (\text{C}_6\text{H}_3\text{N}_3\text{O}_7)_2$ requires C, 38.4; H, 4.1; N, 20.3%]. The i.r. spectra of the sulphate and the picrate of metabolite 1 had peaks at 1720 cm^{-1} that were not present in the spectra of these salts of guanethidine and could indicate the presence of a carboxyl group. The potentiometric titration curve of the sulphate of metabolite 1 (Fig. 1*a*) showed the presence of two ionizable groups with pK_a values of approx. 4.5 and 9, which could be a carboxylic acid and an aliphatic amino group. On the basis of this evidence, metabolite 1 was tentatively identified as 2-(6-carboxyhexylamino)ethylguanidine (VI in Scheme 2), a product that would be formed by

oxidative attack on one of the ring carbon atoms of guanethidine (V) next to the nitrogen atom and would satisfy the elemental analysis and functional-group requirements observed for metabolite 1.

The synthesis of 2-(6-carboxyhexylamino)ethylguanidine was attempted by allowing aminoethylguanidine to react with 7-bromoheptanoic acid in aqueous solution at pH 10–11. After 3 hr. at 60° a product was isolated from the reaction mixture in good yield (60–70%) by using ion-exchange chromatography as described in the Materials and Methods section. This formed a sulphate and picrate, which gave elemental analyses in agreement with those calculated for the sulphate dihydrate and the dipicrate of 2-(6-carboxyhexylamino)-ethylguanidine.

Fig. 1. Potentiometric titration curves of the following compounds in 5 ml. of water under nitrogen. (a) ----, Sulphate of natural metabolite 1 (3 mg.); —, sulphate of natural metabolite (3 mg.) after 30 min. at pH 11;, water only. (b) —, guanethidine sulphate (5 mg.); ----, guanethidine *N*-oxide sulphate (5.4 mg.).

The melting points and i.r. spectra of the sulphate and picrate of the synthetic product were identical with those obtained for these salts of natural metabolite 1, and the synthetic product and natural metabolite gave Sakaguchi-positive spots of identical mobility on electrophoresis at pH 6.1 (pyridine-acetate buffer), showing that the synthetic product was the same compound as the natural metabolite.

Transformation of metabolite 1. When heated in dilute ammonia, natural metabolite 1 prepared from side-chain-labelled [^3H]guanethidine was transformed into a product that gave a single radioactive peak on electrophoresis at pH 6.1 (pyridine-acetate buffer) with lower mobility than that of metabolite 1. The effect of heating in alkaline solution on the titration curve of metabolite 1 (Fig. 1a) indicated that the transformation involved the loss of the functional group with $\text{p}K_a$ 9. Moistened pH paper indicated the evolution of ammonia or a similar volatile base during the transformation of metabolite 1 in dilute sodium hydroxide.

The transformation products of the natural and synthetic metabolites were not Sakaguchi-positive and formed picrates with closely similar melting points and identical i.r. spectra. The transformation product of synthetic metabolite 1 crystallized from water to give a substance that had an elemental analysis in agreement with that calculated for the dihydrate of a product derived from metabolite 1 by the elimination of ammonia (Found: C, 48.0; H, 9.2; N, 16.9; $\text{C}_{10}\text{H}_{19}\text{N}_3\text{O}_2 \cdot 2\text{H}_2\text{O}$ requires C, 48.2; H, 9.2; N, 16.9%). The picrate of the transformation product gave an analysis in agreement with that calculated for the monopicrate of a product again derived from metabolite 1 by the elimination of ammonia (Found: C, 43.7; H, 5.0; N, 19.2; $\text{C}_{10}\text{H}_{19}\text{N}_3\text{O}_2 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$ requires C, 43.4; H, 5.0; N, 19.0%).

Assuming that metabolite 1 was 2-(6-carboxyhexylamino)ethylguanidine these results suggested that the transformation reaction involved modification of both the guanidine group and the secondary amino group with the elimination of ammonia. A reaction satisfying these conditions would be ring-closure resulting from nucleophilic attack on the guanidine carbon atom by the secondary amino group with the elimination of ammonia derived from one of the unsubstituted guanidine nitrogen atoms, as shown in Scheme 2.

This type of ring-closure has been described for aminoethylguanidine by Adcock *et al.* (1961). To confirm that it was also possible for secondary amines, methylaminoethylguanidine was synthesized. It was found that this compound also underwent transformation on heating in dilute ammonia to a product that was not Sakaguchi-positive. The picrate of this product gave an elemental analysis

consistent with that of 1-methyl-2-iminoimidazoline picrate (Found: C, 36.4; H, 3.5; N, 25.3; $\text{C}_4\text{H}_9\text{N}_3 \cdot \text{C}_6\text{H}_3\text{N}_3\text{O}_7$ requires C, 36.6; H, 3.7; N, 25.6%). Table 4 shows the effect of media of different pH on the rate of transformation of synthetic metabolite 1 and the relative rates of transformation of a number of other guanidine derivatives.

On the basis of these results it was concluded with reasonable certainty that the transformation product of metabolite 1 was 1-(6-carboxyhexyl)-2-iminoimidazolidine (VIII).

Identification of metabolite 1 as 2-(6-carboxyhexylamino)ethylguanidine. The transformation reaction raises the possibility that metabolite 1 might have rearranged as shown in Scheme 2 giving rise to structure (VIII) instead of the proposed structure (VI). Although natural metabolite 1 had been shown to be identical with the product of the reaction of 7-bromoheptanoic acid with aminoethylguanidine, the latter product might also have structure (VIII), which could have been formed if 7-bromoheptanoic acid had alkylated the guanidine group of aminoethylguanidine.

Indirect evidence making this course for the reaction unlikely is provided by the use of similar reaction conditions for the alkylation of an amino group in preference to a guanidine group during the synthesis of octopine from arginine and bromopropionic acid by Akasi (1937a,b,c) and Izumiya *et al.* (1957). Further indirect evidence about the course of the synthetic reaction was obtained by studying the reaction of 7-bromoheptanoic acid with 2-(benzamido)ethylguanidine under conditions identical with those used for the synthesis of metabolite 1. After the reaction, electrophoresis at pH 6.1 (pyridine-acetate buffer) showed the presence of a single Sakaguchi-positive component with the same mobility as the starting material. Quantitative assay for Sakaguchi-positive com-

Table 4. Transformation of metabolite 1 and related compounds

Transformation was measured by applying the Sakaguchi test to samples of solutions before and after heating at 100° for 30 min. The percentage transformation was calculated as the percentage decrease of E_{515} .

Compound	Solvent	Transformation (%)
Synthetic metabolite 1	0.05 M- H_2SO_4	0
	0.1 M-Phosphate buffer, pH 7.4	14
	0.1 M- NH_3	24
	0.1 M- NaOH	100
Methylaminoethylguanidine	0.1 M- NH_3	51
Aminoethylguanidine	0.1 M- NH_3	25
Guanethidine	0.1 M- NH_3	1

pounds showed that this compound accounted for 96% of the starting material. These results showed that benzylation of the primary amino group more or less completely blocked the alkylation of aminoethylguanidine and indicated that, in the synthesis of metabolite 1, alkylation of the guanidine group was unlikely to take place.

Although these results suggested that structure (VI) was likely to be the immediate product of the synthetic reaction, the evidence was indirect and in addition there remained the further possibility that structure (VI) might be formed initially and then rearrange to structure (VIII).

Metabolite 1 was too polar to dissolve in solvents suitable for high-resolution n.m.r. spectroscopy, but a method that would distinguish between these structures was provided by the reaction of metabolite 1 with nitrous acid under conditions where the guanidine group would not be affected (Felix & Müller, 1928; Hunter & Woodward, 1941). After reaction under these conditions a crystalline product was isolated, as described in the Materials and Methods section, which gave an elemental analysis consistent with that of 2-N-(6-carboxyhexyl)-N-nitrosaminoethylguanidine. On treatment of this derivative with strong acid, metabolite 1 was regenerated, which provided further evidence that a nitroso derivative had been formed.

If metabolite 1 had structure (VIII), reaction with nitrous acid under these conditions would have resulted in the irreversible elimination of the primary amino group with the formation of an alcohol or an olefin. The formation of a nitroso derivative under these conditions therefore provided conclusive evidence that metabolite 1 contained a secondary amino group and was 2-(6-carboxyhexylamino)ethylguanidine.

Isolation of metabolite 2 and its identification as guanethidine N-oxide. High-voltage electrophoresis of a sample of the 6M-hydrochloric acid eluate obtained from the large-scale fractionation of acidified protein-free pig liver supernatant showed (Fig. 2) the presence of unchanged guanethidine and a smaller amount of a radioactive Sakaguchi-positive metabolite. Fig. 3 shows the distribution of the radioactivity after countercurrent distribution of a portion of the 6M-hydrochloric acid eluate. Samples of the contents of tubes 62–78 gave a single Sakaguchi-positive spot on low-voltage electrophoresis at pH 6.1 (pyridine-acetate buffer) with mobility 0.69 relative to that of guanethidine. The material in these tubes formed a picrate, m.p. 178–180°, which had an elemental analysis consistent with that of the dipicrate of a product derived from guanethidine by the addition of a single atom of oxygen (Found: C, 38.9; H, 4.1; N, 20.5; $C_{12}H_{22}N_4O \cdot (C_6H_5N_3O_7)_2$ requires C, 39.3; H, 4.2; N, 20.8%).

Guanethidine N-oxide, synthesized by the reaction of hydrogen peroxide with guanethidine in acetic acid, formed a dipicrate, m.p. 178–180°, the i.r. spectrum of which was identical with that of the picrate of metabolite 2. On electrophoresis at pH 6.1 (pyridine-acetate buffer) synthetic guanethidine N-oxide gave a Sakaguchi-positive spot with mobility 0.69 relative to that of guanethidine. The potentiometric titration curves of guanethidine and of the synthetic N-oxide (Fig. 1b) confirmed that in the synthesis conversion of the tertiary amino group in guanethidine with pK_a approx. 4 had taken place.

On the basis of this evidence it was concluded that metabolite 2 was guanethidine N-oxide (IX).

Fig. 2. High-voltage electrophoresis at pH 6.1 of 10 μ l. of the 6M-HCl acid eluate of a 500ml. bed of AG 50W resin (X4) previously loaded with 17l. of supernatant containing guanethidine and metabolites and eluted with aq. NH_3 . The radioactive Sakaguchi-positive components (hatched areas) at 26 cm. and 42 cm. correspond in position to synthetic guanethidine N-oxide and guanethidine respectively.

Fig. 3. Countercurrent distribution after 300 transfers of radioactive guanethidine and metabolites formed in pig liver homogenate and eluted from AG 50W resin (X4) with 6M-HCl. Equal samples from the top and bottom phases of each tube were combined for radioactivity assay.

Scheme 2.

DISCUSSION

The metabolism of guanethidine in liver homogenates requires oxygen (Furst, 1967) and is dependent on NADP⁺. These results suggest that the liver microsomal hydroxylating system originally described by Brodie *et al.* (1955) is directly involved in the formation of the major metabolites of this drug.

The proposed structures of the metabolites are consistent with this suggestion. 2-(6-Carboxyhexylamino)ethylguanidine could be formed as a result of an *N*-dealkylation reaction involving some of the intermediates shown in Scheme 2. Unlike most of the reported *N*-dealkylation reactions in which elimination of the alkyl group as an aldehyde occurs (La Du, Gaudette, Trousof & Brodie, 1955) the metabolism of guanethidine results in ring-opening and the methylene group cleaved from nitrogen is transformed to a carboxylic acid group. Metabolic products of this kind have been reported for nicotine (McKennis, Turnbull & Bowman, 1958; Hucker, Gillette & Brodie, 1960) and for tremorine (Hammer, Holmsted, Karlen, Sjöqvist & Vessman, 1968). These compounds contain an *N*-substituted pyrrolidine ring and in each case one of the products is an *N*-substituted derivative of γ -aminobutyric acid. The metabolic product of nicotine has been shown to undergo spontaneous ring-closure to the corresponding lactam, cotinine, under physiological conditions, but this type of ring-closure would be unlikely to occur with 2-(6-carboxyhexylamino)-ethylguanidine since this would involve the formation of an eight-membered ring. It is possible, however, that the lactam shown as structure (X) in Scheme 2 might be an intermediate in the formation of 2-(6-carboxyhexylamino)ethylguanidine.

The metabolism of guanethidine to guanethidine *N*-oxide is an example of the *N*-oxidation of a tertiary amine, a well-known function of liver microsomes (Gillette, 1966). Although it has been suggested that *N*-oxides may be intermediates in the *N*-dealkylation reaction, Gillette (1966) concluded that this is unlikely in view of experimental evidence showing that the rate of *N*-dealkylation of *N*-oxides is much lower than that of the parent drug.

Further studies (McMartin & Vinter, 1969) have shown that 2-(6-carboxyhexylamino)ethylguanidine, its transformation product, and guanethidine *N*-oxide are the only metabolites excreted in significant quantities in the urine of rats that had been treated with guanethidine.

These findings show that no metabolic attack occurs on the guanidine group or on the carbon atom adjacent to it. Cañas-Rodríguez (1966) came to a similar conclusion about the stability of the guanidine group in guanoxan, which suggests that

when present in foreign compounds the guanidine group may be relatively resistant to metabolism.

The polar guanidine group does, however, have a dominant effect on some of the physicochemical properties of guanethidine, so that its chloroform/water partition coefficient is low at pH 7. The metabolism of this drug *in vitro* and its rapid metabolism *in vivo* (Furst, 1967; Shanker & Morrison, 1965) therefore provide a striking exception to the rule (Gaudette & Brodie, 1959) that only lipid-soluble drugs are readily metabolized by liver microsomes. Other exceptions to this rule have been reported by Mazel & Henderson (1965) and include puromycin aminonucleoside and 1-methylguanosine.

Investigations carried out by Professor Brunner (CIBA, Basle, Switzerland) have shown that 2-(6-carboxyhexylamino)ethylguanidine, 1-(2-carboxyhexyl)-2-iminoimidazolidine and guanethidine *N*-oxide have less than one-tenth of the antihypertensive activity of guanethidine in the renal-hypertensive rat.

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