Biochemical Pharmacology, Vol. 20, pp. 3447–3462. Pergamon Press, 1971. Printed in Great Britain

METABOLISM OF 3,4-DIHYDROXYPHENYLALANINE BY ISOLATED PERFUSED RAT LIVER

GERTRUDE M. TYCE

Mayo Clinic and Mayo Foundation, Department of Biochemistry, Rochester, Minn. 55901, U.S.A.

(*Received* 15 *March* 1971; *accepted* 21 *May* 1971)

Abstract—The metabolism of L-3,4-dihydroxyphenylalanine (L-dopa) and DL-dopa by the isolated perfused rat liver is described. After injection of 5 μc (192 μg) L-dopa-3-^{14}C into the portal vein, the liver took up approximately one-third of the dose within 30 min. Blood cells of the perfusate (chiefly erythrocytes) took up one-fourth of the dose within 5 min; subsequently, most of this radioactivity was released back into plasma. L-Dopa disappeared from plasma rapidly (half-life, approximately 30 min); after 2 hr of perfusion, less than 2 per cent of the dose could be recovered unmetabolized. Metabolites of dopa released from liver into bile accounted for up to 48 per cent of the dose and were chiefly glucuronides of *N*-acetyldopamine and *N*-acetyl-3-methoxydopamine. Major metabolites released into plasma were the glucuronides of 3-methoxy-4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, the sulfate of *N*-acetyl-3-methoxydopamine, and 3-methoxy-4-hydroxyphenylalanine. Amines in plasma represented a minor fraction (less than 1 per cent of the dose) at all times during the perfusion. Norepinephrine and its metabolites were never detected. After injection of 5 μc DL-dopa-2-^{14}C (32 or 192 μg), less ^{14}C was taken up by liver than when L-dopa was used; similar amounts of ^{14}C were taken up by the blood cells but were retained for the 5 hr of perfusion. DL-Dopa disappeared more slowly from plasma than L-dopa; after 2 hr, 16 per cent of the dose was recovered unmetabolized. The metabolites of DL-dopa in bile (22 per cent of the dose) and in plasma were similar to those found when L-dopa was substrate.

THE CONCENTRATION of the neurotransmitter 3,4-dihydroxyphenylethylamine (dopamine) is decreased in certain areas of the brain in patients with Parkinson's disease.[1] Attempts to remedy this deficiency by the oral administration of large doses of the immediate precursor, 3,4-dihydroxyphenylalanine (dopa), have been successful in alleviating several of the symptoms of this disease.[2,3] When dopa is given by mouth it is absorbed into the portal circulation and is immediately available for uptake and metabolism by liver. It seemed important, therefore, to study the hepatic metabolism of dopa, and the present investigation used the isolated perfused rat liver to determine: (1) how quickly dopa was cleared from the perfusate by the liver, and (2) the nature and disposition of the metabolites.

In most of these experiments the metabolism of L-dopa was studied; in a few, DL-dopa was used. Early attempts to treat Parkinson's disease with DL-dopa were largely unsuccessful because of the high incidence of side-effects.[2] Therefore, it was of interest to compare the metabolism of DL-dopa and L-dopa.

Preliminary reports of part of this work have been made.[4,5]

METHODS

Materials. The following compounds were purchased: L-dopa from Mann Laboratories, 3-methoxy-4-hydroxyphenylethanol from Aldrich Chemical Company, 3-methoxy-4-hydroxyphenylethylamine and 3-methoxy-4-hydroxyphenylglycol from

Calbiochem, and DL-dopa-2-^{14}C (sp. act., 31·0 mc/m-mole), L-dopa-3-^{14}C (5·2 mc/m-mole), and dopamine-2-^{14}C (55 mc/m-mole) from Amersham/Searle Corp. DL-Norepinephrine-7-^{14}C (48 mc/m-mole) was obtained from New England Nuclear Corp. 3-Methoxy-4-hydroxyphenylalanine was a gift from Dr. A. Pletscher, Hoffman–La Roche, Basel, Switzerland. *N*-Acetyl-3,4-dihydroxyphenylethylamine monohydrate, *N*-acetyl-3-methoxy-4-hydroxyphenylethylamine piperazine salt, and 3,4-dihydroxyphenylethanol 1,4-diazobicyclo (2.2.2.) octane salt were gifts from Dr. A Manian, Psychopharmacology Research Branch, National Institute of Mental Health.

Perfusion of isolated rat liver. Male Sprague–Dawley rats weighing 325–405 g were used. The method of perfusion was that described by Brauer *et al.*[6] as modified by Flock and Owen.[7] A rat liver was perfused with a mixture of 100 ml rat blood and 20 ml isotonic saline for 30–40 min to remove extraneous pressor substances from the perfusate. This liver was then discarded and a second (experimental) liver was connected to the system. Perfusion of this liver was continued for 5 hr. The mean weight of the livers was 10·92 g (S.E. 0·91).

In a number of experiments, ascorbic acid was added to the perfusate because it was thought that this would prevent auto-oxidation of dopa to melanin intermediates. A primer dose of 50 mg ascorbic acid in 2 ml isotonic saline was injected over 2 min followed by continuous infusion (1·25 ml/hr) of 2×10^{-2} M.

In all experiments, 5 μc dopa was injected in a volume of 0·3 ml directly into the portal vein of the experimental liver; this was equivalent to 192 μg L-dopa and 32 μg DL-dopa. In two experiments, carrier DL-dopa was added so that the injected dose was 192 μg. The purity of radioactive dopa was checked by co-chromatography with a reference standard in *n*-butanol–acetic acid–water (12:3:5) during each perfusion.

Studies were also made of the disposition and metabolism of dopa-^{14}C in the perfusate alone, without a liver in the system.

Sampling and measurement of total radioactivity. After the injection of dopa-^{14}C, samples of perfusate were collected at 5, 15, 45 and 60 min, and then hourly. Ascorbic acid (1 per cent solution in saline) was immediately added to each aliquot (0·15 ml/ml of perfusate). Bile was collected continuously for two half-hour intervals and then hourly. After 5 hr of perfusion the liver was frozen in powdered Dry Ice and kept at $-18°$ until it was analyzed.

Total radioactivity was measured by adding aliquots of whole blood, plasma or bile to a 2:1 mixture of toluene–PPO–DMPOPOP* and Triton X-100[8] and counting in a liquid scintillation counter. In some experiments, radioactivity was measured separately in platelet-rich plasma (prepared by centrifugation at 500 rev/min for 30 min) and in platelet-poor plasma (prepared by centrifugation at 2500 rev/min for 30 min). In a few experiments, radioactivity in erythrocytes was measured; aliquots of perfusate were centrifuged, plasma and buffy coat were removed, aliquots of erythrocytes were laked by addition to water, and the radioactivity was measured in samples of the laked erythrocytes.

The experimental liver was homogenized in 5 vol. of 0·4 M perchloric acid and radioactivity was measured in aliquots of the homogenate and in the supernatant after centrifugation. The total ^{14}C in liver at intermediate times during the perfusion

* PPO = 2,5-diphenyloxazole; DMPOPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene.

was calculated by subtracting the sum of the ^{14}C in bile, whole blood, and samples removed for analysis from the total ^{14}C added to the system.

Isolation and identification of metabolites. Methanol–acetone extracts of bile and of plasma[9] were applied to Whatman 3MM paper and chromatographed by descent in *n*-butanol–acetic acid–water (12:3:5) or in *n*-butanol–pyridine–water (1:1:1). Two-dimensional chromatograms of extracts of bile were also prepared (butanol–pyridine–water for 20 hr followed by butanol–acetic acid–water for 8 hr). Paper chromatography was done in a nitrogen atmosphere.

Distribution of radioactivity on strip chromatograms was determined with a 4π gas-flow strip scanner equipped with a continuous recording system. Autoradiographs were made by exposing X-ray film (Kodak no-screen) to the chromatograms for 21 days.

Radioactive areas of chromatograms of bile and of plasma were cut out and eluted with 0·1 N acetic acid. These eluates were dried *in vacuo*. The residues were dissolved in small volumes of appropriate buffers and incubated at 37° for 18 hr with bacterial β-glucuronidase (Sigma Chemical Company, St. Louis; 3.2.1.31 β-D-glucuronide glucuronohydrolase) at pH 6·5 (0·067 M phosphate buffer), with Glusulase (Endo Products, Inc., Richmond Hill, N.Y.), which contains β-glucuronidase and aryl sulfatase (3.1.6.1 arylsulfate sulfohydrolase), at pH 5·4 (0·1 M citrate buffer), or with buffer alone. Both enzymes were added to give final concentrations of 1000 units of β-glucuronidase activity/ml. After incubation, protein was precipitated by the addition of 4 vol. of methanol–acetone (1:1) and the mixture was centrifuged. The supernatant was concentrated under a stream of nitrogen and the products of enzyme hydrolysis were identified by co-chromatography with reference standards in the above solvents, in isopropanol–water–ammonia (20:1:2) or in toluene–methanol–ethyl acetate–water (1:1:1:1; Bush "C").[10] After radioactivity was located on these chromatograms, phenolic compounds were detected by spraying with diazotized *p*-nitroaniline.

Metabolites of dopa in bile, erythrocytes, plasma and liver also were separated by a combination of ion-exchange and adsorption chromatography, as outlined by Bartholini and Pletscher.[11] Bile (1·0 ml), plasma (0·5 to 4·0 ml) or erythrocytes (1·0 ml) were mixed with 0·1 vol. of a 1% solution of tetrasodium ethylenediaminetetraacetate, and proteins were precipitated by addition of 4 vol. (8 vol. for erythrocytes) of 0·4 N perchloric acid and centrifugation. Liver was homogenized in 5 vol. of 0·4 N perchloric acid and centrifuged. Perchlorate was removed by adjustment to pH 5 with KOH and centrifugation. After addition, as carriers, of 50 μg dopa, dopamine, 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid), 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethanol, 3-methoxy-4-hydroxyphenylethanol, *N*-acetyldopamine and *N*-acetyl-3-methoxydopamine, glucuronidase or Glusulase was added to some of these extracts (with appropriate buffers; final concentration 500 units glucuronidase/ml) and the mixtures were incubated overnight; protein (of the enzyme) and perchlorate were again precipitated.

Extracts were adjusted to pH 2 and added to Dowex 50-X4 columns (diameter, 0·5 cm; height, 5 cm; Na^+ form, 200–400 mesh, cycled according to Hirs *et al.*[12]). Amino acids were eluted from these columns with 20 ml of 0·5 M potassium acetate buffer (pH 6·5); amines were eluted with 20 ml of 2 N HCl. Acidic deaminated metabolites were separated from neutral and conjugated compounds in the effluent from the Dowex column by acidification to pH 2 with 3 N HCl and three extractions into

equal volumes of ethyl acetate. In amino acid and amine fractions, catechols were separated from 3-*O*-methyl compounds by adsorption on alumina (500 mg) at pH 8·4 and elution with 0·2 N HCl.[11]

Radioactivity in aliquots of each fraction was measured in the liquid scintillation counter. Aliquots of each fraction were reduced in volume and chromatographed in the above solvents or in 0·2 M ammonium acetate buffer (pH 6·0)–isopropanol (2:1).

The mean recovery of dopa-^{14}C through the Dowex column (0·1 to 1·0 μg added to 3–4 ml of rat plasma) was 86·1 per cent (S.E. 1·6) in 16 experiments; through alumina, the mean recovery was 86·0 per cent (S.E. 2·7) in six experiments. Recovery of 0·1 μg dopamine-^{14}C added to 3–4 ml of rat plasma was 70·0 to 88·0 per cent in four experiments; recovery of norepinephrine-^{14}C was 62·0 to 70·0 per cent. No corrections were made for the recoveries of these compounds.

Considerable ^{14}C (up to 22 per cent of that added) was detected in the amino acid fraction eluted from the Dowex column when norepinephrine-^{14}C was added to plasma, so chromatographic studies of this fraction were necessary to ascertain whether norepinephrine was present. The solvent, 0·2 M ammonium acetate buffer (pH 6·0)–isopropanol (2:1), was useful to separate dopa (R_f 0·69) from norepinephrine (R_f 0·82).

RESULTS

Disposition of dopa-^{14}C in perfusate without liver

Five min after injection of L-dopa-^{14}C into blood circulating in the perfusion system (without a liver, ascorbate absent from perfusate), 97 per cent of the radioactivity was in whole blood and 56 per cent was in plasma (Fig. 1). Throughout the 5 hr, ^{14}C in whole blood accounted for about 88 per cent of the dose. ^{14}C in plasma decreased in the first hour to about 48 per cent of the dose and thereafter remained fairly constant.

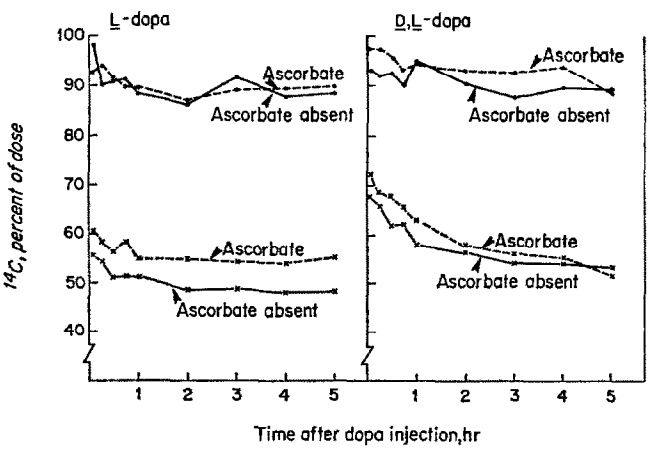

FIG. 1. Disposition of ^{14}C in perfusate (perfusion system without liver) after addition of 5 μc L-dopa-^{14}C (192 μg) or DL-dopa-^{14}C (32 μg). Values are means of two experiments. Ascorbate (1·25 ml/hr, 2×10^{-2} M in saline) was infused in some experiments. No corrections have been made for the 9–11 per cent of ^{14}C removed from the system in sampling. Circles = whole blood; X = plasma.

Five min after dopa injection, the difference between ^{14}C in whole blood and ^{14}C in plasma was 41·7 and 43·5 per cent (two experiments) of the dose; most of this difference was accounted for as ^{14}C in erythrocytes (40·7 and 42·9 per cent of the dose). Likewise, at 5 hr after dopa injection, ^{14}C in erythrocytes was 32·6 and 40·2 per cent of the dose, and the difference between ^{14}C in whole blood and in plasma was 37·3 and 38·5 per cent of the dose. At either time, no difference could be detected between ^{14}C in platelet-rich plasma and in platelet-poor plasma.

The addition of ascorbic acid to the perfusate decreased the amount of ^{14}C in erythrocytes both at 5 min (21·3 and 33·1 per cent of the dose in two experiments) and at 5 hr (25·6 and 28·8 per cent of the dose) after dopa injection; however, further experiments would be necessary to establish the significance of this effect.

At 5 min after the injection of DL-dopa-^{14}C, 93 per cent of the dose was in whole blood and 68 per cent was in plasma. During the 5 hr of perfusion, ^{14}C in whole blood decreased to 89 per cent of the dose; ^{14}C in plasma decreased steadily to 53 per cent of the dose. ^{14}C in erythrocytes increased from 25·8 per cent of the dose at 5 min to 35·8 per cent at 5 hr after dopa injection. The addition of ascorbate did not appear to affect the disposition of ^{14}C in the perfusate after injection of DL-dopa-^{14}C.

Disposition of ^{14}C after injection of dopa-^{14}C in liver perfusions

After the injection of 5 μc L-dopa-^{14}C into the liver, ^{14}C in whole blood decreased to about 45 per cent of the dose in the first hour and thereafter remained constant (Fig. 2). When ascorbate was added to the perfusate, ^{14}C in whole blood started to increase after the first hour, reaching a final value of 55 per cent of the dose. When DL-dopa was injected (ascorbate added to perfusate), ^{14}C in whole blood decreased to 70 per cent of the dose in the first hour and then remained constant.

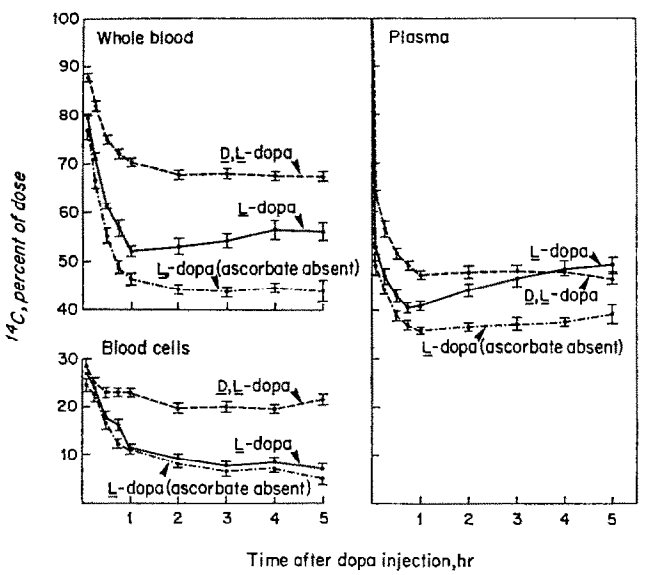

FIG. 2. Total ^{14}C in whole blood, plasma and blood cells at different times after injection of 5 μc L-dopa-^{14}C (192 μg) or DL-dopa-^{14}C (32 μg) into perfusate with liver in system. Ascorbate was infused into perfusate unless otherwise indicated. Data represent means (± S.E.) of 11 experiments using DL-dopa-^{14}C, 9 experiments with L-dopa-^{14}C, and 3 experiments with L-dopa-^{14}C without ascorbate.

^{14}C in plasma decreased to 36 per cent of the dose in the first hour and then increased slightly, to 39 per cent of the dose, when L-dopa-^{14}C was injected. With the addition of ascorbate to the perfusate, ^{14}C in plasma decreased less rapidly in the first hour, to 40 per cent of the dose, and then increased to a final value of 48 per cent of the dose. After injection of DL-dopa, ^{14}C in plasma decreased to 47 per cent of the dose in the first hour and then remained constant.

^{14}C in cellular elements (^{14}C in whole blood minus ^{14}C in plasma) accounted for 25–28 per cent of the dose at 5 min after injection of L-dopa-^{14}C; this decreased sharply and finally accounted for 4–7 per cent of the dose. No difference was noted when ascorbate was added. When DL-dopa-^{14}C was added, ^{14}C in cellular elements accounted for 25 per cent after 5 min, decreased to 20 per cent after 2 hr, and then remained constant. In a number of experiments, ^{14}C in erythrocytes measured at 5 min and at 5 hr accounted for 80–90 per cent of the ^{14}C in cellular elements.

^{14}C in liver increased to 35–40 per cent of the dose in the first hour after injection of L-dopa and then decreased to 6 per cent of the dose (Fig. 3). The addition of ascorbate did not affect the amount of ^{14}C in liver. When DL-dopa was injected, ^{14}C in liver increased to 21 per cent of the dose after 30 min and then decreased to 6 per cent of the dose after 5 hr.

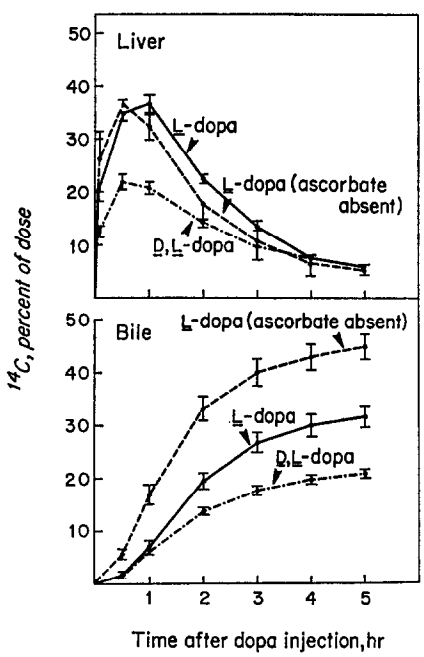

FIG. 3. Total ^{14}C in liver and bile at different times after injection of 5 μc L-dopa-^{14}C (192 μg) or DL-dopa-^{14}C (32 μg) into perfusate. Data are from the same experiments as in Fig. 2.

The excretion of ^{14}C in bile followed a sigmoid curve. When L-dopa was the substrate, the cumulative excretion of ^{14}C was 48 per cent of the dose without ascorbate and 32 per cent with ascorbate added to the perfusate. The cumulative excretion of ^{14}C after injection of DL-dopa-^{14}C was 21 per cent of the dose.

No differences were noted in the disposition of ^{14}C from DL-dopa-^{14}C when the amount of nonradioactive dopa was 32 or 192 μg.

Metabolites in perfusate during liver perfusions

L-*Dopa*-^{14}C *as substrate.* At 5 min after injection, L-dopa-^{14}C accounted for 32 per cent of the dose when ascorbate was added to the perfusate (Fig. 4). Between 5 and 120 min after injection, L-dopa-^{14}C disappearance was exponential; a line of best fit (drawn by the method of least squares) had the equation $\log_e Y = -0{\cdot}02417X + 3{\cdot}568$. During this interval, L-dopa had a half-life of 29 min in the perfusate. At 2 hr after injection, only 2·2 per cent of the dose was recovered as L-dopa-^{14}C and after 2 hr the decrease was much slower (Fig. 5). At the end of the experiment, only 0·5 per cent of the dose remained as unmetabolized L-dopa-^{14}C.

The ^{14}C-labeled amine fraction was very minor in these experiments. At no time did this fraction account for more than 1 per cent of the dose, but it was more abundant during the first 30 min than at later times. The amount of *O*-methyldopa increased during the perfusion to a final value of 4·2 per cent of the dose. ^{14}C-labeled organic acids accounted for 4 per cent of the dose early in the perfusion, but for only 1·5 per cent after 5 hr. The most abundant fraction in the perfusate was the conjugate fraction, which increased during the perfusion to a final value of 35 per cent of the dose.

When ascorbate was not added to the perfusate, L-dopa-^{14}C accounted for 26 per cent (mean) of the dose at 5 min after injection (Fig. 4). Its disappearance from 5 min to 2 hr after injection was exponential; the line of best fit had the equation $\log_e Y = -0{\cdot}02578X + 3{\cdot}338$. Its half-life in plasma was 27 min. The amount of L-dopa-^{14}C in the perfusate was significantly less ($P < 0{\cdot}05$) at 5, 15 and 30 min than when ascorbate was added to the perfusate. However, there was no difference in the decay constant in the presence or absence of ascorbate in the perfusate. The distribution of ^{14}C in the fractions studied was similar throughout the perfusion in the presence or absence of ascorbate. The only significant difference was that the conjugate fraction accounted for only 25 per cent of the dose at 5 hr after injection when ascorbate was absent.

DL-*Dopa*-^{14}C *as substrate.* At 5 min after the injection of DL-dopa-^{14}C (ascorbate added to perfusate), 43 per cent of the dose was recovered unchanged (Fig. 6). Unlike perfusions with L-dopa, the decrease of DL-dopa-^{14}C between 5 and 120 min did not show a single exponential component. DL-Dopa-^{14}C decreased to a final value of 16 per cent of the dose. The amine fraction was minor, accounting for 1·3 per cent of the dose at 5 min and for 0·2 per cent at 5 hr. *O*-Methyldopa accounted for more radioactivity when DL-dopa was substrate compared to L-dopa. ^{14}C-labeled organic acids accounted for 1·8 per cent of the dose at 1 hr after injection and decreased to 1·2 per cent after 5 hr. Conjugated metabolites accounted for 16 per cent of the dose at the end of the perfusion.

Metabolites in perfusate with no liver in system

Both in the presence and in the absence of ascorbate in the perfusate, at 5 and 300 min after injection of L-dopa-^{14}C, the recovery of ^{14}C in perchloric acid extracts was much less from erythrocytes than from plasma (Table 1). In erythrocytes, the amounts of ^{14}C in other fractions were large relative to the dopa fraction. In plasma, most of the ^{14}C was in the dopa fraction.

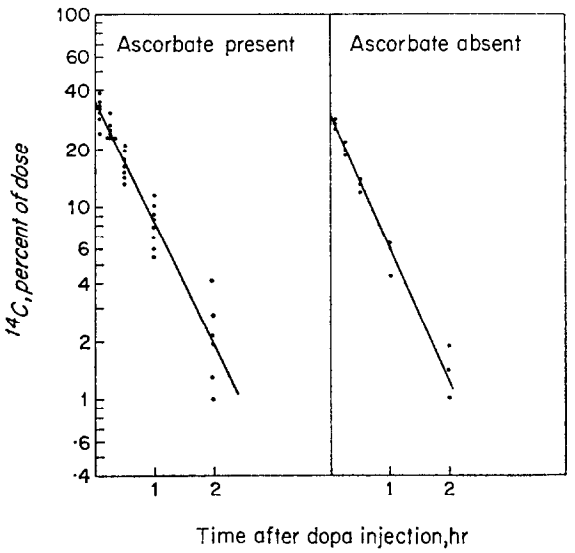

FIG. 4. L-Dopa-^{14}C in plasma at different times after injection of 5 μc L-dopa-^{14}C in isolated rat liver perfusions. Lines of best fit were drawn by the method of least squares.

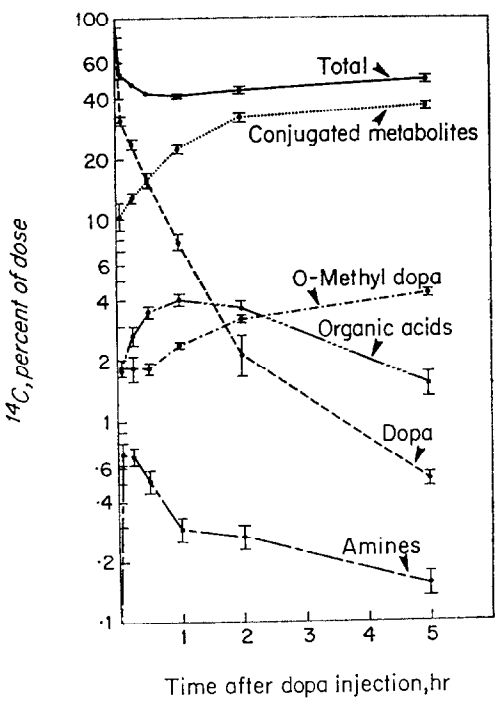

FIG. 5. Metabolites of L-dopa-^{14}C in plasma at different times after injection of 5 μc (192 μg) into isolated perfused rat liver system. Means (± S.E.) of nine perfusions are shown.

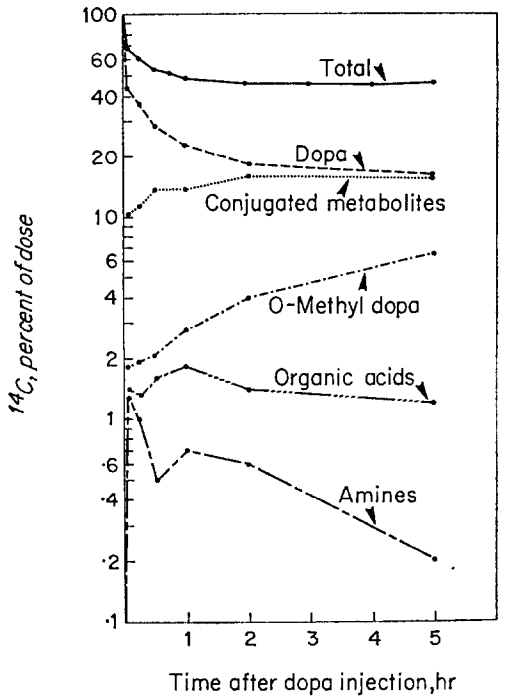

FIG. 6. Metabolites of DL-dopa-^{14}C in plasma at different times after injection of 5 μc (192 μg) into isolated perfused rat liver system. Means of two experiments are shown.

TABLE 1. DISPOSITION OF ^{14}C IN PERFUSATE WITHOUT LIVER

Perfusate fraction	Ascorbate in perfusate	^{14}C (% dose)* Total	PCA extract	Dowex effluent	Amines	*O*-methyl-dopa	Dopa
At 5 min after L-dopa-^{14}C injection†							
Erythrocyte	—	40·7	18·8	1·5	0·2	2·8	7·3
		42·9	17·0	2·6	0·1	2·9	1·7
	+	21·3	17·9	5·6	0·2	3·0	2·3
		33·1	21·2	2·7	0·1	1·4	2·8
Plasma	—	55·8	55·6	9·6	1·0	1·6	38·5
		55·0	53·2	13·4	0·2	1·2	30·3
	+	61·4	61·8	4·8	0·4	1·7	43·5
		59·6	63·4	16·4	0·4	1·9	32·9
At 300 min after L-dopa-^{14}C injection†							
Erythrocyte	—	32·6	17·7	2·6	0·3	3·4	2·5
		40·2	10·8	1·5	0·04	2·0	1·8
	+	25·6	17·5	1·6	0·2	2·4	4·9
		28·8	18·8	3·9	0·3	3·6	4·4
Plasma	—	46·8	43·9	6·6	0·1	3·7	32·2
		46·8	39·5	5·5	0·2	3·6	26·0
	+	57·7	50·8	10·4	0·3	3·8	31·1
		54·2	52·2	4·3	0·1	3·4	38·4

* Duplicate experiments were done; the results of each are shown.
† Dose = 5 μc (192 μg).

With both plasma and erythrocytes, radioactivity was not extracted from acidified aliquots of the Dowex effluent into ethyl acetate; therefore, organic acids were not detected. After incubation with Glusulase or glucuronidase, the disposition of ^{14}C in the Dowex effluent was not changed and organic acids were again not detected.

Metabolites of dopa-^{14}C in bile

Paper chromatograms of methanol–acetone extracts of bile from liver perfusions with DL-dopa-^{14}C or L-dopa-^{14}C as substrate showed two major and three minor peaks of radioactivity (Fig. 7). The following compounds were not detected by either paper or column chromatography: dopa, dopamine, norepinephrine, free alcohols and free deaminated acids. The two major peaks accounted for 85–95 per cent of the radioactivity in bile. These two compounds were not easily separated on one-dimensional chromatograms of bile (Figs. 7, 8) and were eluted together for enzymatic hydrolysis. Both of the compounds were completely hydrolyzed by β-glucuronidase (Fig. 8).

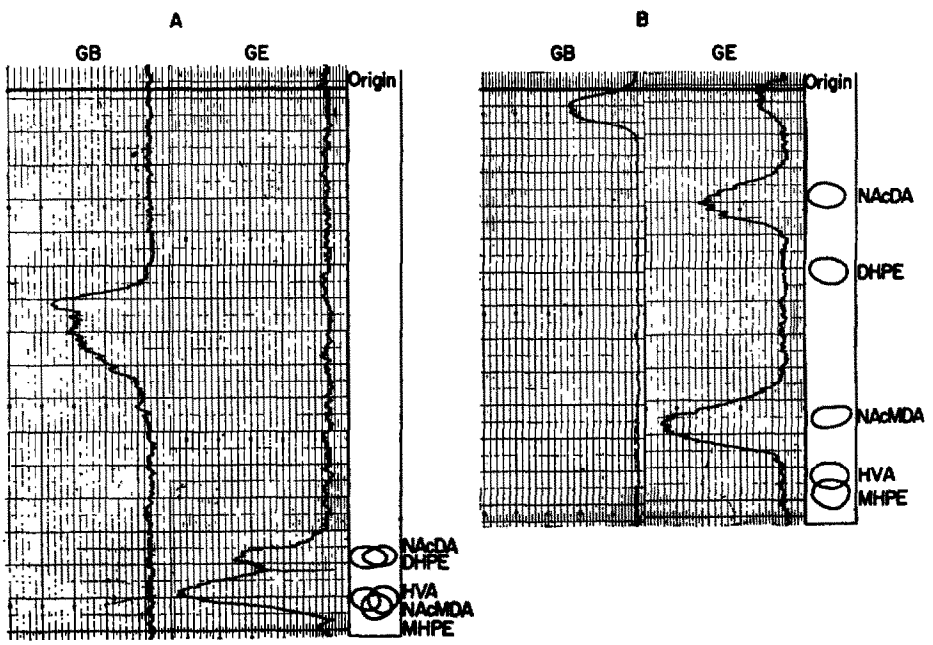

FIG. 8. Scans of radioactivity on one-dimensional chromatograms of products of glucuronidase hydrolysis of major radioactive compound in bile (eluted from chromatograms of methanol–acetone extracts of bile; see Fig. 7) after injection of 5 μc L-dopa-^{14}C in isolated perfused rat liver system. Incubation was with β-glucuronidase in 0·067 M phosphate buffer, pH 6·5 (GE) or with buffer alone (GB). Products of hydrolysis were chromatographed in *n*-butanol–acetic acid–water (A) or Bush "C" solvent (B). In B, the scale of radioactivity on chromatogram of GB is three times less sensitive than for GE.

Co-chromatography with reference standards identified three products in the enzyme hydrolysate: *N*-acetyl-3-methoxydopamine, *N*-acetyldopamine, and an unidentified compound which migrated with *N*-acetyldopamine in *n*-butanol–acetic acid–water but stayed on the origin in Bush "C" solvent. On hydrolysis of the radioactive *N*-acetyl-3-methoxydopamine at pH 1 for 6 hr, a compound was recovered with the chromatographic properties, in *n*-butanol–acetic acid–water, of 3-methoxydopamine.

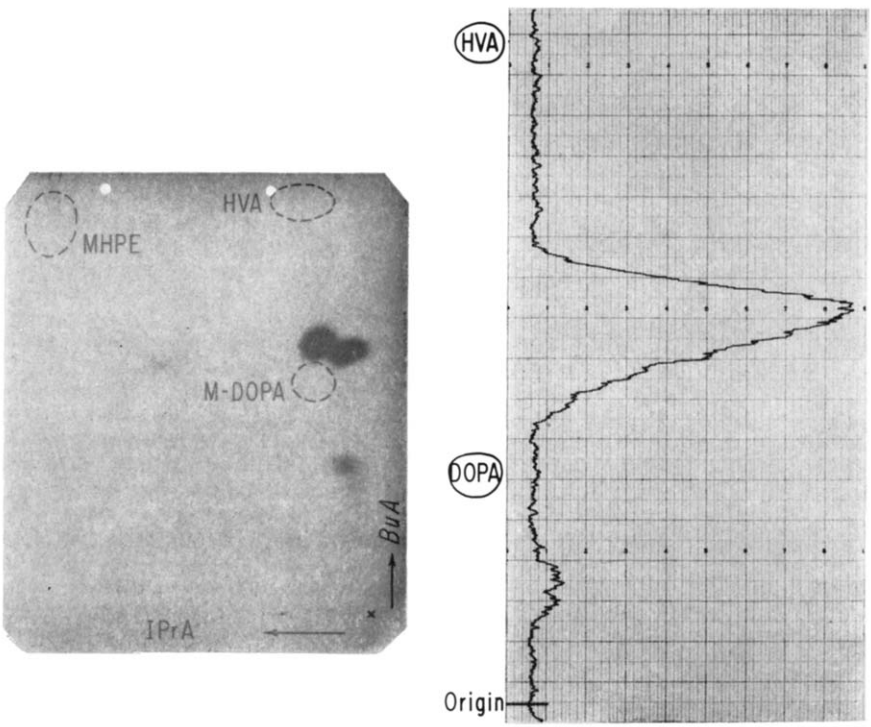

FIG. 7. Metabolites of L-dopa-^{14}C in bile. Left, autoradiograph of two-dimensional chromatogram of methanol–acetone extract of bile, run in *n*-butanol–acetic acid–water (BuA) and isopropanol–ammonia–water (IPrA). Right, scan of radioactivity on one-dimensional chromatogram (*n*-butanol–acetic acid–water). Standards: HVA, 3-methoxy-4-hydroxyphenylacetic acid; MHPE, 3-methoxy-4-hydroxyphenylethanol; M-DOPA, 3-methoxy-4-hydroxyphenylalanine.

B.P.—*facing page* 3456

The unknown compound (together with *N*-acetyl-3-methoxydopamine) passed into the effluent of an alumina column at pH 8·4, indicating that it was not a catechol.

N-Acetyl-3-methoxydopamine glucuronide in bile accounted for 21·0 per cent of the dose of L-dopa-^{14}C in the absence of ascorbate and 17·7 per cent when ascorbate was present; it accounted for 11·6 per cent of the dose when DL-dopa-^{14}C was substrate (Table 2). The glucuronide of *N*-acetyldopamine accounted for 12·2 and 5·8 per cent of the dose of L-dopa-^{14}C in the absence and presence of ascorbate, respectively, and 5·6 per cent of the dose of DL-dopa-^{14}C. The unknown glucuronide accounted for 5–7 per cent of the dose of L-dopa-^{14}C and 2·5 per cent of the dose of DL-dopa-^{14}C.

Four minor peaks were found in bile, but not all were present in each perfusion. Each accounted for 2·5 per cent or less of the injected ^{14}C. These compounds were identified, by enzymatic hydrolyses and co-chromatography with reference standards, as the glucuronides of dopamine, 3-methoxydopamine and 3-methoxy-4-hydroxyphenylacetic acid (HVA), and the sulfate of 3,4-dihydroxyphenylacetic acid (DOPAC).

TABLE 2. METABOLITES OF DOPA-^{14}C IN BILE FROM ISOLATED PERFUSED RAT LIVER*

Fraction	L-Dopa		DL-Dopa
	No ascorbate (N = 3)	Ascorbate (N = 8)	Ascorbate (N = 10)
Total	44·4 ± 2·3	31·8 ± 2·0	21·6 ± 0·7
3-Methoxy-4-hydroxyphenylacetic acid glucuronide	2·5 ± 1·2	0·7 ± 0·1	0·8 ± 0·2
3,4-Dihydroxyphenylacetic acid sulfate		0·6 ± 0·3	0·2; 0·9
3-Methoxydopamine glucuronide	1·0 ± 0·6	0·9 ± 0·2	1·3 ± 0·2
Dopamine glucuronide	0·5; 1·9		0·2; 0·7
N-Acetyl-3-methoxydopamine glucuronide	21·0 ± 2·0	17·7 ± 1·1	11·6 ± 0·6 (3)†
N-Acetyldopamine glucuronide	12·2 ± 1·3	5·8 ± 0·8	5·6 ± 0·9 (3)†
Unknown glucuronide	6·6 ± 0·6	4·8 ± 1·0	2·5 ± 0·9 (3)†

* Bile was collected for 5 hr after injection of dopa-^{14}C. Data are shown as means (±S.E.) of per cent of dose with numbers of experiments in parentheses.

† It was not realized that the major radioactive peak eluted from chromatograms of methanol–acetone extracts of bile run in *n*-butanol–acetic acid–water comprised more than one compound in the early experiments, and only three separations were done.

Metabolites of dopa-^{14}C in plasma after 5 hr of perfusion

At the end of 5 hr of perfusion, more extensive separations were undertaken of those fractions, separated by column chromatography, which could contain more than one compound (the amine, organic acids and conjugate fractions).

In all perfusions, the amine fraction accounted for only 0·2 per cent of the dose of ^{14}C in the final plasma and contained almost equal amounts of methoxydopamine and dopamine. No norepinephrine was detected. When aliquots of the amine fraction were chromatographed on alumina columns at pH 8·4, approximately equal amounts were found in the effluent and in the eluate. Paper chromatography showed similar amounts of HVA and DOPAC (Table 3). Alcohols were not detected in significant amounts.

TABLE 3. METABOLITES OF DOPA-^{14}C IN PLASMA IN ISOLATED PERFUSED RAT LIVER SYSTEM*

Fraction	L-Dopa		DL-Dopa
	No ascorbate (N = 3)	Ascorbate (N = 9)	Ascorbate (N = 7)
Total	38·6 ± 1·8	48·4 ± 1·7	46·8 ± 1·1
Protein-free extract	33·8 ± 1·0	45·7 ± 1·5	44·4 ± 1·0
Dopa	0·6 ± 0·07	0·5 ± 0·04	15·6 ± 0·2
O-Methyldopa	5·9 ± 0·4	4·3 ± 0·1	6·5 ± 0·4
Amines	0·2 ± 0·07	0·2 ± 0·02	0·2 ± 0·02
3-Methoxy-4-hydroxyphenylacetic acid:			
Free	0·8 ± 0·2	0·6 ± 0·1	0·9 ± 0·4
Glucuronide	5·8 ± 0·3	12·3 ± 1·1	5·0 ± 1·7
3,4-Dihydroxyphenylacetic acid:			
Free	0·5 ± 0·1	0·8 ± 0·1	0·7 ± 0·1
Glucuronide	4·5 ± 0·6	11·7 ± 0·7	5·4 ± 1·6
N-Acetyl-3-methoxydopamine sulfate	0·8 ± 0·2	8·8 ± 1·5	0·3; 0·5
3-Methoxy-4-hydroxyphenylethanol sulfate	1·6; 1·9		3·8 ± 1·3

* Plasma sampled 5 hr after injection of 5 μc dopa-^{14}C. Data are shown as means (± S.E) of per cent of dose with numbers of experiments in parentheses.

After hydrolysis with glucuronidase or Glusulase and column and paper chromatography of the hydrolysates, the major metabolites in the conjugate fraction were shown to be the glucuronides of HVA and DOPAC and the sulfate of *N*-acetyl-3-methoxydopamine. When ascorbate was not added to the perfusate, less ^{14}C was found in these metabolites (Table 3), and small amounts of several unidentified compounds were detected by paper chromatography of the enzyme hydrolysates in Bush "C".

From 88 to 95 per cent of the total radioactivity in plasma was extracted into perchloric acid in all perfusions. Thus, there was no evidence that radioactivity was incorporated into protein.

Metabolites of dopa-^{14}C in liver after 5 hr of perfusion

Most of the ^{14}C in perchloric acid homogenates of liver was in the protein-free supernatant. Most of the ^{14}C in liver was found in the conjugate fraction; because this represented only a very small part of the dose (2·5 per cent or less), no attempt was made to separate this further. *O*-Methyldopa-^{14}C was present in relatively large amounts. Minor amounts of radioactive dopa and radioactive amines also were detected (Table 4).

Disposition of D-*dopa-^{14}C*

It was assumed that DL-dopa-^{14}C was a mixture of equal amounts of D- and L-dopa and that in a given fraction or metabolite in the liver perfusion: (^{14}C from L-dopa-^{14}C) + (^{14}C from D-dopa-^{14}C) = 2(^{14}C from DL-dopa-^{14}C). Approximate calculations could then be made to describe the disposition and metabolism of D-dopa-^{14}C by liver based on perfusions in which DL- and L-dopa-^{14}C were substrates (ascorbate added in both cases).

More ^{14}C was calculated to be present in whole blood after 5 hr of perfusion when

TABLE 4. METABOLITES IN PERFUSED ISOLATED LIVER*

Fraction	L-Dopa		DL-Dopa
	No ascorbate (N = 3)	Ascorbate (N = 9)	Ascorbate (N = 7)
Total	4·7 ± 0·4	5·8 ± 0·2	5·5 ± 0·3
Protein-free extract	4·4 ± 0·5	5·2 ± 0·2	4·4 ± 0·3
Dopa	0·07 ± 0·03	0·1 ± 0·02	0·6 ± 0·1
O-Methyldopa	1·1 ± 0·2	1·5 ± 0·1	1·3 ± 0·2
Amines	0·2 ± 0·1	0·2 ± 0·02	0·08 ± 0·01
Conjugates	2·5 ± 0·2	2·4 ± 0·4	1·7 ± 0·2

* Liver sampled 5 hr after injection of 5 μc dopa-^{14}C. Data are shown as means (± S.E) of per cent of dose with numbers of experiments in parentheses.

D-dopa-^{14}C was substrate than when L-dopa-^{14}C was substrate (Table 5); similar amounts of ^{14}C were present in plasma. At 5 min after injection, 23 per cent of the dose of D-dopa-^{14}C was in cells of the perfusate; this increased during the 5 hr to 36 per cent. When L-dopa-^{14}C was injected, the percentage of the dose in blood cells decreased during the perfusion. The maximal uptake of ^{14}C by liver occurred between 30 and 60 min after injection of dopa (Fig. 3); this accounted for 32·0 per cent of the dose of L-dopa, but only 10·0 per cent of the dose of D-dopa. Excretion into the bile accounted for 31·8 per cent of the dose of L-dopa-^{14}C, but only 11·4 per cent of the dose of D-dopa-^{14}C.

After 5 hr of perfusion, 30·7 per cent of the dose of D-dopa-^{14}C was unmetabolized in plasma, but only 0·5 per cent of the dose of L-dopa-^{14}C. More D-dopa-^{14}C than

TABLE 5. DISPOSITION AND METABOLISM OF D-DOPA-^{14}C CALCULATED FROM LIVER PERFUSIONS WITH L-DOPA-^{14}C OR DL-DOPA-^{14}C AS SUBSTRATE

Fraction	Minutes after dopa injection	^{14}C (% of dose)		
		L-Dopa-^{14}C	DL-Dopa-^{14}C	D-Dopa-^{14}C*
Whole blood	300	56·1	67·6	79·1
Plasma	300	48·4	46·8	45·2
Blood cells	5	27·0	25·0	23·0
Blood cells	300	7·0	21·5	36·0
Liver	30–60†	32·0	21·0	10·0
Liver	300	5·8	5·5	5·2
Bile	300	31·8	21·6	11·4
Dopa in plasma	300	0·5	15·6	30·7
O-Methyldopa in plasma	300	4·3	6·5	8·7
Cmpd. 1‡ in bile	300	17·7	11·6	5·5
Cmpd. 2§ in bile	300	5·8	5·6	5·4

* Calculated; see text.
† At time of maximal uptake of ^{14}C by liver.
‡ Cmpd. 1, *N*-acetyl-3-methoxydopamine glucuronide.
§ Cmpd. 2, *N*-acetyldopamine glucuronide.

L-dopa-^{14}C was metabolized to 3-*O*-methyldopa. The sum of the ^{14}C in dopa and in 3-*O*- methyldopa accounted for a major portion (87 per cent) of the ^{14}C in plasma when D-dopa-^{14}C was the substrate.

Most of the ^{14}C in bile could be accounted for as the glucuronides of *N*-acetyldopamine and *N*-acetyl-3-methoxydopamine, whether D- or L-dopa-^{14}C was substrate.

DISCUSSION

At 5 min after the injection of L-dopa-^{14}C into the isolated perfused rat liver system, only a quarter to a third of the dose remained in plasma, due largely to uptake by blood cells and by the liver. After this, the decrease of dopa-^{14}C in plasma followed two phases. Between 5 min and 2 hr after injection, the half-life of dopa in plasma was 24 min in the absence of ascorbate (29 min when ascorbate was present). This disappearance was due to further uptake and metabolism by liver; concurrently, metabolites were being released into bile and into plasma. After 2 hr, only small amounts of dopa-^{14}C were present in plasma, and the disappearance then occurred at a slower rate. At this time, it was possible that end-product inhibition was operative or that the small amounts of dopa were so diluted in the large volume of perfusate that uptake by the liver was slow.

The uptake of dopa from the plasma into blood cells was chiefly, but perhaps not exclusively, by the erythrocytes. This uptake was inhibited to a certain extent by the presence of ascorbate in the perfusate. D-Dopa was taken up by erythrocytes more slowly than L-dopa. However, once in the cells, D-dopa was retained longer than L-dopa. The poor recovery of ^{14}C after precipitation of protein from erythrocytes could be due to nonspecific adsorption of the dopa to protein or to binding to enzyme sites. The former is suggested by the fact that L-dopa-^{14}C left the blood cells when a liver was present in the system and when the concentration of dopa in plasma was rapidly decreasing. The latter is suggested by the observations that, without a liver in the system, the ratio of metabolites to dopa was greater in erythrocytes than in plasma, possibly because metabolism of dopa was occurring in the cells and metabolites were being released into plasma. Cohn *et al.*[13] recently demonstrated S-adenosylmethionine: catechol-*O*-methyl transferase (EC 2.1.1.6) activity in human erythrocytes.

Only small amounts of amines were detected in the plasma at any time during the perfusion. Apparently, when formed, dopamine was metabolized rapidly by liver; this was suggested by the shape of the curve of amine-^{14}C in the perfusate. Norepinephrine was not detected in bile, plasma, or liver in these experiments. The major metabolites of norepinephrine in isolated perfused rat liver have been found, in this laboratory, to be the glucuronide of normetanephrine and the sulfate of 3-methoxy-4-hydroxyphenylglycol.[14] These compounds also were not detected after the injection of dopa, although they would have been easily distinguishable from other conjugates in bile and plasma by their slow movement on chromatography in butanol–acetic acid–water.[14] Wurtman *et al.*[15] previously found no free norepinephrine-^{14}C in homogenates of whole mice after injection of DL-dopa-^{14}C.

Small amounts of the alcohol metabolite of dopamine, 3-methoxy-4-hydroxyphenylethanol (MHPE), were present as a sulfate conjugate in plasma. The formation of MHPE and 3,4-dihydroxyphenylethanol (DHPE) from dopamine-^{14}C by rat liver

homogenates was demonstrated by Goldstein *et al.*[16] In experiments *in vivo* in which dopamine-^{14}C was injected into rats, MHPE-^{14}C was found in urine; DHPE-^{14}C was found in urine only when the rats had been treated with inhibitors of catechol-*O*-methyl transferase or aldehyde dehydrogenase (aldehyde:NAD oxidoreductase; EC 1.2.1.3). In the present experiments, no free or conjugated DHPE was detected.

Large amounts of *N*-acetylated compounds were produced by the liver, *N*-acetyldopamine glucuronide and *N*-acetyl-3-methoxydopamine being the major metabolites in bile. Goldstein and Musacchio[17] previously identified *N*-acetyldopamine and *N*-acetyl-3-methoxydopamine in urine of rats after the administration of dopamine-^{14}C. Further work is necessary, using perfusions of livers of other species, to determine whether *N*-acetylation as a route of metabolism is more prominent in the rat than in other animals. However, *N*-acetyldopamine and *N*-acetyl-3-methoxydopamine have been detected in the urine of patients with Parkinson's disease receiving L-dopa,[18] and *N*-acetylnormetanephrine and *N*-acetylmetanephrine have been demonstrated in the urine of patients with neuroblastoma.[19]

D-Dopa was metabolized more slowly than L-dopa. More D- than L-dopa was metabolized via catechol-*O*-methyl transferase; it has been shown previously[20] that this enzyme shows no stereospecificity with respect to the D- and L-isomers of epinephrine. L-Aromatic amino acid decarboxylase (EC 4.1.1.26) was shown by Holtz[21] not to act on D-dopa. Nevertheless, 10 per cent of the dose of D-dopa-^{14}C was excreted into bile as *N*-acetylated metabolites of dopamine and 3-methoxydopamine, and 5·6 per cent of the dose was in plasma in compounds other than dopa or 3-*O*-methyldopa. It was assumed, therefore, that about 15·6 per cent of the dose of D-dopa-^{14}C was acted on by D-amino acid oxidase to form 3,4-dihydroxyphenylpyruvic acid, which was then transaminated to L-dopa. The oxidase and transaminase reactions occurred much more slowly than the subsequent decarboxylation of L-dopa. However, caution should be exercised in extrapolation from data with DL-dopa-^{14}C as substrate to conclusions regarding the metabolism of D-dopa. It is possible that D-dopa may have inhibited the metabolism of L-dopa or that decreased uptake of D-dopa by liver accounted for the apparent decrease in metabolism.

The addition of ascorbate to the perfusate appeared to inhibit uptake of dopa by blood cells; the recovery of ^{14}C in perchloric acid extracts of erythrocytes was also improved. Ascorbate in the perfusate also caused a decrease in the excretion of ^{14}C into bile. This decrease was chiefly in the *N*-acetylated compounds. However, in the presence of ascorbate, increased amounts of *N*-acetylated compounds were present in plasma so that overall *N*-acetylation was not decreased.

The rapid clearance of dopa by the liver and its uptake by erythrocytes explain why very large doses are needed in the treatment of patients with Parkinson's disease. Further studies on the metabolism of dopa in the presence of inhibitors of enzymes are necessary to determine to what extent it is possible to delay the clearance of dopa from plasma, or whether, when one metabolic pathway is inhibited, compensatory increases occur in metabolism by alternate pathways. Such was found to be the case in studies on the metabolism of 5-hydroxytryptamine by the isolated perfused rat liver when enzyme inhibitors were added.[22]

Acknowledgements—The author thanks Drs. E. V. Flock and C. A. Owen, Jr., for advice and criticism. The investigation was supported in part by Grant NS-9143 from the National Institutes of Health, Public Health Service, and by a donation from Mrs. Charles S. Lips. The author is grateful to Eaton Laboratories for a generous gift of the L-dopa-^{14}C.

REFERENCES

1. O. HORNYKIEWICZ, *Wien. klin. Wschr.* **75,** 309 (1963).
2. G. C. COTZIAS, M. H. VAN WOERT and L. M. SCHIFFER, *New Engl. J. Med.* **276,** 374 (1967).
3. M. D. YAHR, in *L-Dopa and Parkinsonism* (Eds. A. BARBEAU and F. H. MCDOWELL), p. 5. F. A. Davis, Philadelphia (1970).
4. G. M. TYCE, E. V. FLOCK and C. A. OWEN, JR., *Fedn Proc.* **28,** 905 (1969).
5. G. M. TYCE, *Fedn Proc.* **29,** 935 (1970).
6. R. W. BRAUER, R. L. PESSOTTI and P. PIZZOLATO, *Proc. Soc. exp. Biol. Med.* **78,** 174 (1951).
7. E. V. FLOCK and C. A. OWEN, JR., *Am. J. Physiol.* **209,** 1039 (1965).
8. R. C. MEADE and R. A. STIGLITZ, *Int. J. appl. Radiat. Isotopes* **13,** 11 (1962).
9. G. M. TYCE, E. V. FLOCK and C. A. OWEN, JR., *Am. J. Physiol.* **215,** 611 (1968).
10. I. E. BUSH, *Biochem. J.* **50,** 370 (1952).
11. G. BARTHOLINI and A. PLETSCHER, *J. Pharmac. exp. Ther.* **161,** 14 (1968).
12. C. H. W. HIRS, S. MOORE and W. H. STEIN, *J. biol. Chem.* **200,** 493 (1953).
13. C. K. COHN, D. L. DUNNER and J. AXELROD, *Science, N.Y.* **170,** 1323 (1970).
14. R. P. ZIMON, G. M. TYCE, E. V. FLOCK, S. G. SHEPS and C. A. OWEN, JR., *J. Pharmac. exp. Ther.* **157,** 89 (1967).
15. R. J. WURTMAN, C. CHOU and C. ROSE, *J. Pharmac. exp. Ther.* **174,** 351 (1970).
16. M. GOLDSTEIN, A. J. FRIEDHOFF, S. POMERANTZ and J. F. CONTRERA, *J. biol. Chem.* **236,** 1816 (1961).
17. M. GOLDSTEIN and J. M. MUSACCHIO, *Biochim. biophys. Acta* **58,** 607 (1962).
18. L. P. O'GORMAN, O. BORUD, I. A. KHAN and L. R. GJESSING, *Clinica chim. Acta* **29,** 111 (1970).
19. O. BORUD and L. R. GJESSING, *Clinica chim. Acta* **27,** 552 (1970).
20. J. AXELROD and R. TOMCHICK, *J. biol. Chem.* **233,** 702 (1958).
21. P. HOLTZ, *Naturwissenschaften* **27,** 724 (1939).
22. G. M. TYCE, E. V. FLOCK and C. A. OWEN, JR., *Biochem. Pharmac.* **17,** 1543 (1968).