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Extensive Conjugation of Dopamine (3,4-Dihydroxyphenethylamine) Metabolites in Cultured Human Skin Fibroblasts and Rat Hepatoma Cells

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[G-^{3}H]Dopamine (3,4-dihydroxyphenethylamine) metabolism in human skin fibroblasts and rat hepatoma cells in culture was determined by high-pressure liquid-chromatographic analysis of both cell extract and uptake medium. Conjugated metabolites were selectively hydrolysed by incubation with arylsulphatase or β-glucuronidase before analysis. The principal metabolites of dopamine in fibroblast cells are 3-methoxytyramine 4-*O*-sulphate and 3-methoxytyramine. No significant differences, either in the amounts of these metabolites or in the amount of dopamine metabolism, were observed in fibroblasts from both normal and homocystinuric individuals. In rat hepatoma cells, the major metabolite of dopamine was 3-methoxytyramine 4- or 3-*O*-glucuronide; lower concentrations of dopamine 4- or 3-*O*-glucuronide, 4-hydroxy-3-methoxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and two unidentified glucuronide conjugates were also observed. Significant differences in the relative concentrations of these metabolites in cell and uptake medium were observed in both cell systems.

Cultured cells have become an important tool for the study of genetic, developmental and pharmacological factors involved in neurotransmitter metabolism (Breakefield & Giller, 1976). Cultured fibroblasts can be grown from skin biopsies of patients and control individuals. The expression of enzymes important in catecholamine metabolism in these cells allows a study of possible alterations in their activities, which may occur in association with certain neurological and psychiatric diseases (Roth *et al.*, 1976; Breakefield *et al.*, 1976). In the present study we report on the metabolism of [G-^{3}H]dopamine (3,4-dihydroxyphenethylamine) in cultures of human skin fibroblasts and rat hepatoma cells. Metabolites present both in cells and medium have been identified and quantified by high-pressure liquid chromatography.

Experimental

Materials

Catecholamines and their derivatives, ascorbic acid, saccharo-1,4-lactone, arylsulphatase [type V; EC 3.1.6.1; 11.7 units/mg (one unit will hydrolyse 1.0 μmol of potassium *p*-nitrocatechol sulphate/h at pH 5.0 and 37°C) containing 1.9 Fishman units/mg of β-glucuronidase (one Fishman unit will hydrolyse 1.0 μg of phenolphthalein glucuronide/h at pH 3.8 and 37°C)] from limpets, and β-glucuronidase (type II; EC 3.2.1.31; 48000 Fishman units/g) from *Escherichia coli* were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Reagent-grade phosphoric acid, acetic acid, H_2SO_4, trichloroacetic acid, sodium acetate and neutral alumina were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. NaH_2PO_4 was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ, U.S.A., and the A_{254} of a 1.0M solution was measured to select a batch with the lowest absorption value. Regisil RC-2 [bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane] was purchased from Regis Chemical Co., Morton Grove, IL, U.S.A. Formula 963 scintillation fluid and [G-^{3}H]dopamine (NET-229, sp. radioactivity 6.4Ci/mmol) were obtained from New England Nuclear Co., Boston, MA, U.S.A. Tissue-culture dishes and flasks were obtained from Falcon Plastics, Oxnard, CA, U.S.A.; the Dulbecco–Vogt modification of Eagle's medium (DHEM, no. H-21), 1X Viokase and 0.4% Trypan Blue were from Grand Island Biological Co., NY, U.S.A.; and foetal calf serum was from Flow Laboratories, Rockville, MD, U.S.A. Iso-osmotic phosphate-buffered saline was prepared in our laboratory as previously described (Hawkins & Breakefield, 1978). All chromatographic buffers were filter-sterilized with a 47mm Gelman membrane filter (0.2nm pore size; no. 2220) to remove particulate matter, and degassed *in vacuo* before use. Centrifugations were performed on a Sorvall RC 2B centrifuge at 4°C. Cell numbers were determined with a haemacytometer and percentage viability was monitored by Trypan Blue exclusion.

Confluency of cultures was monitored by phase-contrast microscopic evaluation. Mass spectra were carried out on a Finnegan model 3200 quadrupole mass spectrometer (Finnegan Co., Sunnyvale, CA, U.S.A.) equipped with a Finnegan model 9500 gas-chromatographic inlet system, glass single-jet separator interface.

Cell culture

The following human fibroblast lines were used: HF3 from a healthy 32-year-old male, A2 from a healthy 11-year-old male, 82 from a healthy 30-year-old female, H366 from a 12-year-old male with homocystinuria and H340 from a 13-year-old female with homocystinuria. Lines HF3, 82, H366 and H340 were obtained from the Department of Human Genetics, Yale University School of Medicine; line A2 was obtained from Dr. Samuel Goldstein, McMaster University, Hamilton, Ont., Canada. Fibroblast lines were used between 5 and 20 subcultures after growth as a primary explant. Rat hepatoma line MH_1C_1 (CCL 144) was obtained from the American Type Culture Collection, Rockville, MD, U.S.A. This continuous line was used between 60 to 65 subcultures after establishment of the tumour cells in culture. Viable frozen stocks of all lines were maintained at −70°C.

Cells were grown as monolayers on plastic tissue-culture dishes (100mm) or flasks (75cm^2) in medium supplemented with 10% (v/v) foetal calf serum. Cultures were incubated at 37°C in a humidified atmosphere of CO_2/air (1:19). Fibroblasts were grown in DMEM medium and hepatoma cells in DMEM or F-14 media [F-14 medium was prepared from component chemicals as indicated by Vogel *et al.* (1972)]. Stock cultures were fed at 4 to 6 day intervals and subcultured every 7–14 days in a ratio of 1:5, after resuspension by treatment with 1X Viokase for 5–10min (Viokase was diluted in iso-osmotic phosphate-buffered saline). For labelling experiments, cells were plated on to dishes and used at confluency; at this time there were about 3×10^6 cells (1.5mg of protein, as determined by the method of Lowry *et al.*, 1951, with bovine serum albumin as standard) per dish.

Incubation of cells and preparation of extracts

For analysis of dopamine metabolites, cells were incubated at 37°C for 2–6h in conditioned or fresh growth medium with serum in a 5% CO_2 atmosphere. The incubation medium contained a final concentration of 0.1mM-ascorbic acid and 1–1.6μM-[G-^{3}H]-dopamine (1μCi/100μl). Most incubations were carried out on monolayers with 3ml of medium per dish. In one experiment (Table 2), cells were scraped off the dish gently into 0.5ml of medium containing serum, and incubated at 37°C in a plastic tube.

When metabolites in fibroblasts and medium were examined together, 40μl of 100% (w/v) trichloroacetic acid was added directly to the dish and the mixture was kept at ambient temperature for 30min. The mixture was then transferred to a 15ml plastic centrifuge tube and centrifuged at 27000*g* for 10min. The supernatant was transferred to a second centrifuge tube and sufficient 4.0M-NaOH added to bring the pH to between 7.5 and 8.5; the radioactivity in a small sample (10μl) of this final solution was determined in 3.0ml of Formula 963 scintillation fluid (New England Nuclear). Neutral alumina (1.2g) was added to the solution, the mixture vortex-mixed for 2min, and then centrifuged at 1000*g* for 10min. The supernatant was separated from the alumina pellet and the radioactivity in a small sample of the supernatant was determined as above. The remainder of the supernatant was used for chromatographic studies. When examining hepatoma cells and medium together, ascorbic acid and acetic acid were added to the dish to give final concentrations of 0.10μM and 0.1M respectively. Mixtures were kept on ice for 30min and used directly for chromatographic studies.

In some experiments the growth medium was separated from the cell layer after incubation with [G-^{3}H]dopamine. Cellular metabolites were obtained by draining off the growth medium, rinsing the cell layer quickly with ice-cold iso-osmotic phosphate-buffered saline (0.3ml) and draining briefly (45s). The cell monolayer was then scraped from the surface of the dish in a small, measured volume of buffer (approx. 0.5ml) to which ascorbic acid and acetic acid were added (as above). Samples were centrifuged at 27000*g* for 20min and the supernatant extract was used immediately for chromatographic analysis. The storage of samples of either cell extract or uptake medium on ice for periods greater than 6h, or the freeze-storing and thawing of samples before analysis, caused significant decomposition of the 3-methoxytyramine in the sample, as indicated by appropriate control experiments with non-radioactive 3-methoxytyramine.

Chromatographic examination of [G-^{3}H]dopamine metabolites

T.l.c. analyses of cell extracts and media were carried out on cellulose without fluorescent indicator (Eastman no. 60664) with butan-1-ol/1.0M-acetic acid/95% (v/v) (7:2:2, by vol.) (Johnson & Boukma, 1967). The distribution of radioactivity was determined, as previously described (Breakefield, 1975). High-pressure liquid-chromatographic analysis of cell extracts and media were carried out on Partisil-10 ODS, Partisil-10 SCX and Partisil-10 SAX micro-

particle columns (25cm×0.46cm) (Whatman Inc., Clifton, NJ, U.S.A.) as described previously (Stout *et al.*, 1976). Essential chromatographic operating parameters are to be found in the legends of the Figures. Samples were introduced by a Hamilton syringe (model no. 1805).

Recovery studies

To determine the recovery of radioactive components from high-pressure liquid-chromatography columns, an identical volume of radioactive material as applied to the column was added directly to a blank tube of column effluent generated by the fraction collector. In general, 90–95% of the radioactivity applied to columns was recovered.

Identification of conjugated metabolites of dopamine

For analysis of the unknown metabolite in fibroblast cultures, the following procedure was used. A sample (0.6ml, 2.5×10^5c.p.m./ml) of the cell extract, contained in a 5ml glass culture tube was diluted to 1.5ml with double-distilled water and a small sample was removed for subsequent high-pressure liquid-chromatography analysis. Neutral alumina (0.6g) was added to the remaining diluted extract, the mixture vortex-mixed for 2min and then centrifuged at 1000*g* for 15min. A sample (0.2ml) of the supernatant was carefully transferred to each of four 5ml glass culture tubes. The remaining supernatant was examined by high-pressure liquid-chromatography analysis. Two extract samples were mixed with 0.1M-sodium phosphate buffer, pH6.9 (0.1ml); β-glucuronidase (5mg, 240 Fishman units) was added to one of these samples. The other two samples were mixed with 0.2M-sodium acetate buffer, pH5.0 (0.15ml), containing 0.2% (w/v) NaCl (0.05ml), and arylsulphatase (2mg, 23.4 units) was added to one. Mixtures were incubated for 12–72h at 37°C. In some experiments, samples were removed at various time intervals during the incubation and analysed immediately by high-pressure liquid chromatography; in other experiments fresh samples of enzyme were added during the course of the incubation.

To overcome problems of incomplete enzyme hydrolysis, which might be caused by components in the crude fibroblast extract, an alumina-treated cell extract (0.2ml, 2×10^5c.p.m.) was spotted on to a t.l.c. cellulose sheet alongside a spot containing authentic markers; the sheet was developed as above. The area of the chromatographed cell extract corresponding to the synthetic 3-methoxytyramine 4-*O*-sulphate standard (see below) was cut out into small strips that were gently shaken in 0.1M-acetic acid (1.0ml) for 30min. The mixture was filtered on a scintered-glass filter, the filter was washed with water (2×0.1ml) and the filtrate and washings were freeze-dried overnight. The residue was taken up in either 0.1M-sodium phosphate buffer, pH6.5, or 0.2M-sodium acetate buffer, pH5.0, and incubated with β-glucuronidase or arylsulphatase respectively, as described above.

For hepatoma metabolites a similar procedure was used, except that enzymic treatment was carried out on material from cell extracts or medium after isolation by t.l.c., and incubations with arylsulphatase were carried out in the presence of the β-glucuronidase inhibitor, saccharo-1,4-lactone (2mg/ml). After elution with acetic acid, samples were dried under a stream of N_2.

Synthesis of 3-methoxytyramine 4-O-sulphate

A modification of a procedure described for the synthesis of dopamine 3-*O*-sulphate (Bronaugh *et al.*, 1975) was adopted. 3-Methoxytyramine hydrochloride (0.1g, 0.5mmol) was placed in a 0.5ml glass culture tube at 0°C and H_2SO_4 (98%, 0.3ml, chilled to 0°C) was added. The mixture was vortex-mixed for 30s and left at 0°C for 3min. The reaction was quenched with chilled water (2.5ml) and the resulting solution applied directly to a column (0.9cm×8cm) of Bio-Rad AG 50W X8, 100–200 mesh resin; H^+-form) (Bio-Rad Laboratories, Rockville Center, NY, U.S.A.) that had been washed with water (50ml). The column was eluted with water and 1.0ml fractions were collected. Fractions eluting at above pH3.0 were combined (approx. 50ml) and freeze-dried to yield 3-methoxytyramine 4-*O*-sulphate as a white hygroscopic powder (61.3mg, yield 50.5%) that melted at 270°C with decomposition, and was homogeneous by t.l.c. on cellulose and by high-pressure liquid chromatography on Partisil-10 SCX (Found: C, 41.27; H, 5.50; N, 5.08; S, 12.47. $C_9H_{12}NO_5S$ requires C, 43.72; H, 5.26; N, 5.67; S, 12.95%. $C_9H_{12}NO_5S,\frac{1}{4}H_2O$ requires C, 41.45; H, 5.60; N, 5.37; S, 12.30%).

The synthetic product from the above reaction was hydrolysed and simultaneously converted into the trimethylsilyloxy derivative with bis(trimethylsilyl)-trifluoroacetamide (Regisil) in acetonitrile as previously described (Bronaugh *et al.*, 1975). The derivative was then subjected to gas-chromatographic analysis on a glass U-shaped column (1.2m×4mm internal diam.), packed with 3% OV-17 liquid phase coated on 100–200 mesh Gas Chrom Q (Supelco Inc., Bellefonte, PA, U.S.A.), with helium as carrier gas before mass-spectrometric analysis. The mass spectrum from the above hydrolysed, trimethylsilylated product was identical with that of a trimethylsilylated sample of authentic 3-methoxytyramine (Bronaugh *et al.*, 1975).

Results

Human fibroblasts

A typical thin-layer radiochromatogram of a fibroblast-cell extract after incubation with [G-^{3}H]-dopamine (without alumina treatment) is shown in Fig. 1(*a*) together with the location and size of marker spots of authentic standards. This method of analysis provides poor resolution of radioactive products and poor recovery (50–75%) of radioactive material. The majority of radioactive material in the extract appears to co-migrate with noradrenaline and 3-methoxytyramine. A small amount of acidic material can also be detected. However, this system does not allow a distinction between 4-hydroxy-3-methoxyphenylacetic (homovanillic) acid and 3,4-dihydroxyphenylacetic acid. The difficulties encountered in the above analytical procedure, which are inherent in this chromatographic method (Fairbairn & Ralph, 1968), led us to examine cellular extracts by using high-pressure liquid chromatography. This method of analysis has been shown to be more sensitive and reproducible than t.l.c. (Stout *et al.*, 1976; Molnar & Horvath, 1976) and usually affords quantitative recoveries of radioactive catecholamine materials (Michelot *et al.*, 1977). Our laboratory has developed an analytical procedure for the determination of catecholamines and their metabolites incorporating cation-exchange, anion-exchange and reverse-phase chromatography (Stout *et al.*, 1976). Figs. 2(*a*), 3(*a*) and 4(*a*) illustrate radiochromatograms of fibroblast extracts before alumina treatment containing authentic markers for the substances of interest, together with the corresponding chromatogram obtained with the u.v. detector in these three chromatographic systems. In the cation-exchange radiochromatogram (Fig. 2*a*) the presence of 3-methoxytyramine is confirmed. However, the

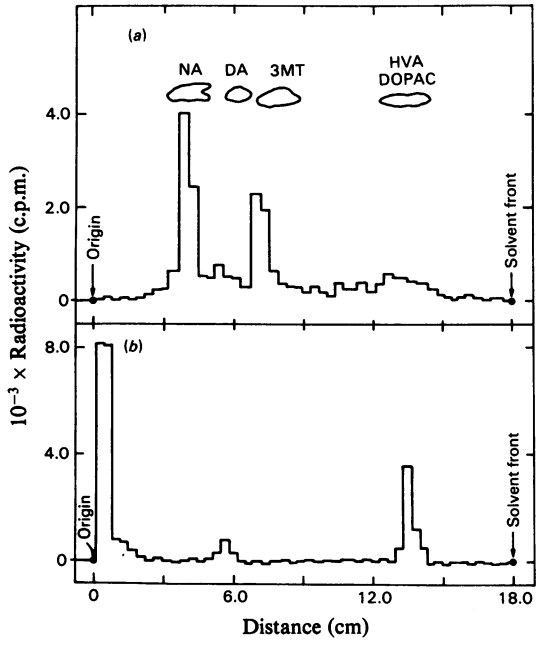

Fig. 1. *Thin-layer radiochromatograms of fibroblast (a) and hepatoma cell (b) extracts after incubation with [G-^{3}H]dopamine on Eastman chromagram cellulose*

Authentic standards were added to cell extracts before analysis. The developing solvent was butan-1-ol/1.0M-acetic acid/95% (v/v) ethanol (7:2:2, by vol.). Abbreviations used: NA, noradrenaline; DA, dopamine; 3MT, 3-methoxytyramine; HVA, homovanillic acid; DOPAC 3,4-dihydroxyphenylacetic acid.

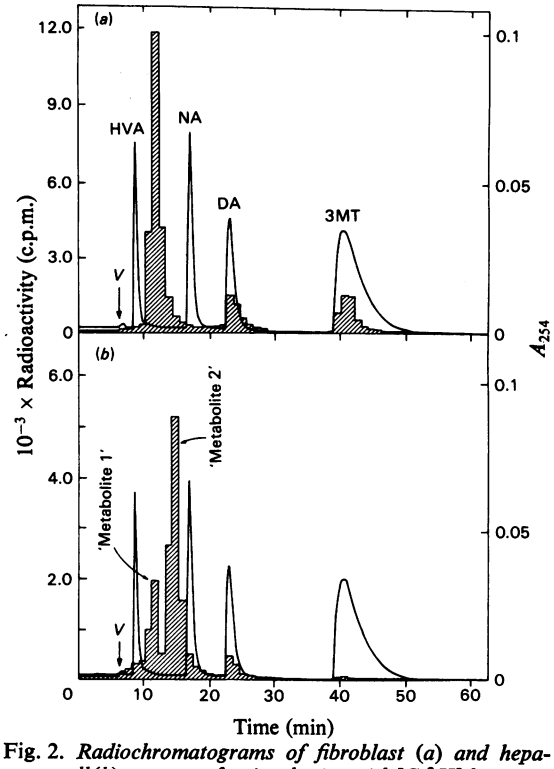

Fig. 2. *Radiochromatograms of fibroblast (a) and hepatoma-cell (b) extracts after incubation with [G-^{3}H]dopamine obtained by microparticulate-silica cation-exchange chromatography*

Authentic standards were added to cell extracts before analysis. 'Metabolite-1' and 'metabolite-2' refer to the major metabolites of [G-^{3}H]dopamine from rat hepatoma cells. The column (25cm × 0.45 cm) contained Partisil-10 SCX; eluent was 0.05M-NaH_2PO_4, pH4.0; flow rate; 0.5ml/min; inlet pressure, 14.1kg/cm^2; temperature, ambient. Abbreviations are defined in the legend to Fig. 1.

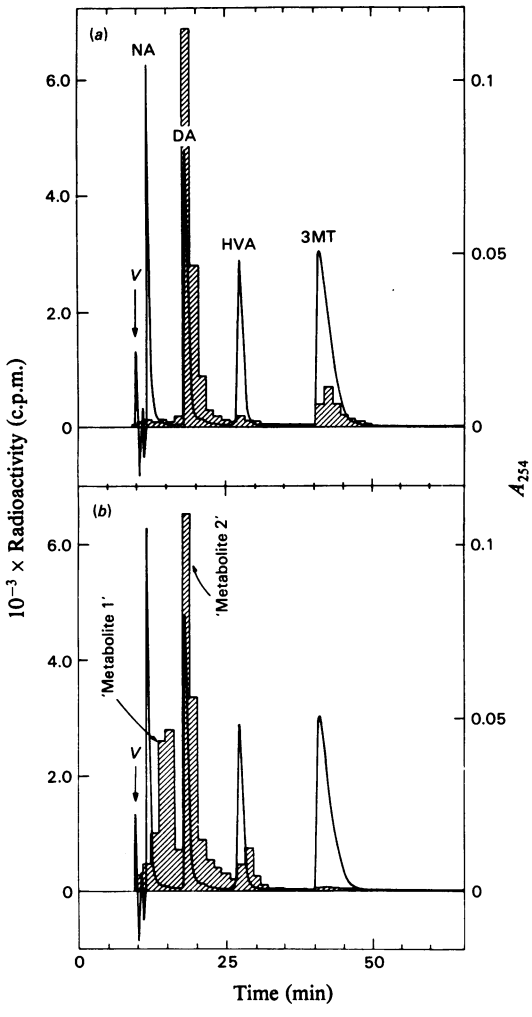

Fig. 3. *Radiochromatograms of fibroblast (a) and hepatoma-cell (b) extracts after incubation with [G-^{3}H]dopamine obtained by microparticulate-silica reverse-phase chromatography*

Authentic standards were added to cell extracts before analysis. The column (25cm×0.45cm) contained Partisil-10 ODS; eluent: 1.0M-sodium acetate buffer, pH4.6; flow rate, 0.4ml/min; inlet pressure, 15.4kg/cm^2; temperature, ambient. Abbreviations are defined in the legend to Fig. 1.

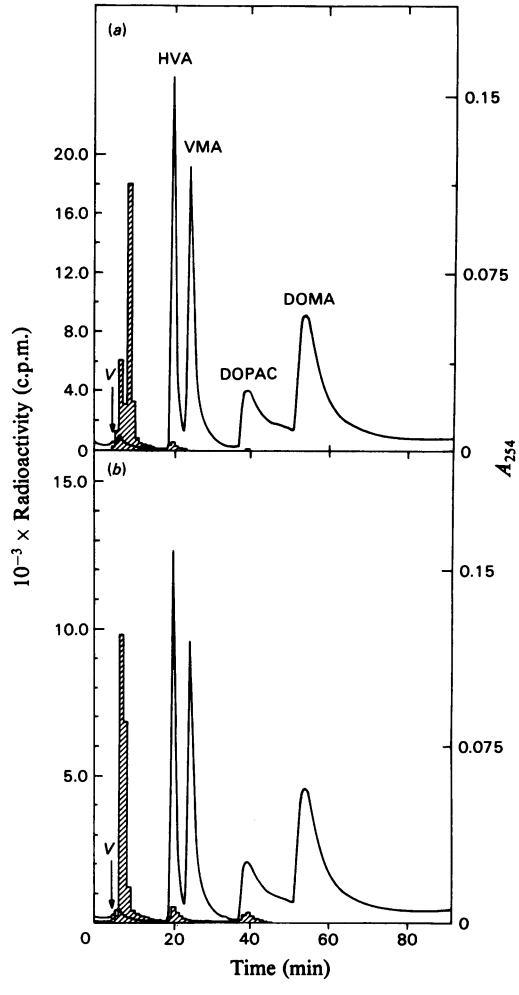

Fig. 4. *Radiochromatograms of fibroblast (a) and hepatoma (b) extracts after incubation with [G-^{3}H]dopamine obtained by microparticulate-silica anion-exchange chromatography*

Authentic standards were added to cell extracts before analysis. The column (25cm×0.45cm) contained Partisil-10 SAX; eluent, 0.1M-sodium acetate buffer, pH4.35; flow rate, 0.9ml/min; inlet pressure, 35.15kg/cm^2; temperature, ambient. Abbreviations; VMA, vanilylmandelic acid; DOMA, 3,4-dihydroxymandelic acid; for other abbreviations see the legend to Fig. 1.

component co-migrating with noradrenaline on the t.l.c. analytical system did not co-elute with this standard or any of the other authentic standards examined. Alumina treatment to remove residual [G-^{3}H]dopamine and its catechol metabolites (Coward *et al.*, 1973) did not decrease the amount of this unknown metabolite. Examination of the cell extract by reverse-phase chromatography (Fig. 3*a*) showed that the above unidentified metabolite co-eluted with dopamine and this was further confirmed on analysis of the alumina-treated (dopamine-free) extract. Analysis of the cell extract (not alumina treated) by anion-exchange chromatography (Fig. 4*a*) indicated the presence of small amounts of both homovanillic acid and 3,4-dihydroxyphenylacetic acid, although the majority (above 97%) of radio-

active material was eluted in the void volume. Table 1 summarizes the results obtained from the analysis of [G-^{3}H]dopamine metabolites in cultured human fibroblasts on these three chromatographic systems.

The apparent polar nature of the unidentified metabolite and its resistance to removal by alumina suggested that it might be an *O*-conjugated product (Michelot *et al.*, 1977). Since the major routes of conjugation of dopamine and its metabolites in man are reported to be via 3-*O*-sulphonation and 3-*O*-glucuronidation (Sharman, 1973; Goodall & Alton, 1968, 1969; Rutledge & Hoehm, 1973; Weil-Malherbe & Van Buren, 1969), a procedure was adopted on the basis of selective enzyme hydrolysis of the unknown conjugate with the enzymes arylsulphatase (EC 3.1.6.1) and β-glucuronidase (EC 3.2.1.31), as reported in a number of papers (Bond & Howlett, 1974; Yeh *et al.*, 1975; Martin *et al.*, 1972; Falck *et al.*, 1977). Fig. 5 shows the results of the high-pressure liquid-chromatography analysis of alumina-treated fibroblast-cell extracts by cation-exchange and reverse-phase chromatography after incubation with arylsulphatase and β-glucuronidase enzymes; the latter enzyme was without effect on the extract, whereas with arylsulphatase the suspected conjugate peak of material decreased from 70 to 30% and was accompanied by a parallel increase in the amount of 3-methoxytyramine, indicating the presence of 3-methoxytyramine 4-*O*-sulphate in the original cell extract. The apparent incomplete hydrolysis of the conjugate could be due to inhibitory components present in the crude cell extract. Previous reports of the enzymic hydrolysis of catechol sulphate conjugates in urine with arylsulphatase have shown that only 80–85% of unconjugated catechol derivatives are released even after extended periods of incubation (Martin *et al.*, 1972; Bertilsson, 1973). To overcome this, the conjugate was isolated from the cell extract by preparative t.l.c. and then incubated with arylsulphatase and β-glucuronidase respectively. Under these conditions 80% conversion of the conjugate into 3-methoxytyramine occurred with arylsulphatase (see Fig. 5); again no change occurred with β-glucuronidase.

To establish further the identity of the conjugate, 3-methoxytyramine 4-*O*-sulphate was synthesized by a modified procedure described for the preparation of dopamine *O*-sulphate isomers (Bronaugh *et al.*, 1975). The synthetic product was characterized by g.l.c.–mass spectrometric and elemental analysis and shown to co-migrate with the radioactive conjugate on both thin-layer and high-pressure liquid-chromatographic systems. Chromatographic examination of the uptake medium showed the presence of both 3-methoxytyramine 4-*O*-sulphate and 3-methoxytyramine; thus these metabolites appear to pass freely through cell membranes. However, the ratio of 3-methoxytyramine 4-*O*-sulphate to 3-methoxytyr-

Table 1. *Analysis of [G-^{3}H]dopamine metabolites in human fibroblast-cell extract*

Experiments were carried out on fibroblast cell line 82 after incubation for 6h at 37°C with [G-^{3}H]-dopamine. For experimental details see the text. Analyses were carried out on Partisil-10 SCX, Partisil-10 SAX and Partisil-10 ODS microparticle columns on non-alumina-treated cell extract. The unidentified metabolite was subsequently identified as 3-methoxytyramine 4-*O*-sulphate.

Metabolite	Percentage composition in cell extract
Dopamine	13.2
Homovanillic acid	3.0
3,4-Dihydroxyphenylacetic acid	0.4
3-Methoxytyramine	16.3
Unidentified metabolite	67.0

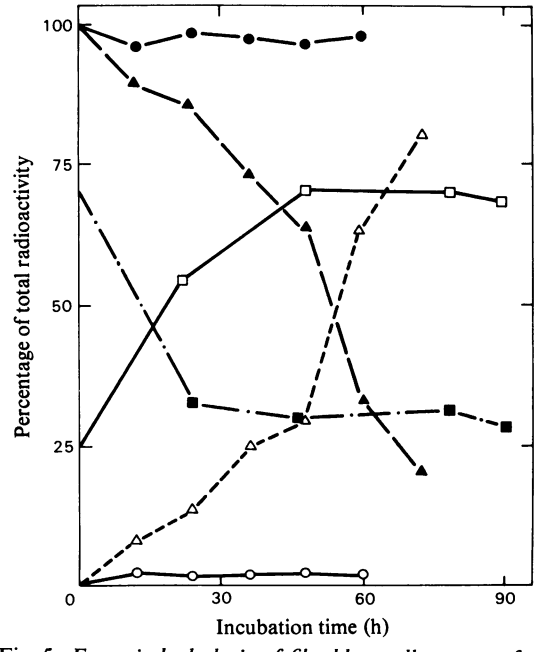

Fig. 5. *Enzymic hydrolysis of fibroblast-cell extract after incubation with [G-^{3}H]dopamine*

Symbols: □ and ■, percentage of 3-methoxytyramine and conjugate respectively in crude cell extract after incubation with arylsulphatase; △ and ▲, percentage of 3-methoxytyramine and conjugate respectively in chromatographed cell extract after incubation with arylsulphatase. Extra enzyme was added at 24 and 48h; ○ and ●, percentage of 3-methoxytyramine and conjugate respectively in chromatographed cell extract after incubation with β-glucuronidase. Extra enzyme was added at 24 and 48h.

Table 2. *3-Methoxytyramine and 3-methoxytyramine 4-O-sulphate concentrations in different fibroblast lines*
Cells were incubated with [G-^{3}H]dopamine for 6h at 37°C. Analyses were carried out on Partisil-10 SCX, Partisil-10 SAX and Partisil-10 ODS microparticulate columns on alumina-treated cell extracts. Dopamine metabolism was determined on the basis of alumina-bound radioactivity. Values refer to single experiments or to means for the numbers of experiments in parentheses (±S.D.) being included for values from four replicates.

Cell line	Type	Metabolism of dopamine (%)	3-Methoxytyramine (%)	3-Methoxytyramine 4-O-sulphate (%)	Sample
82	Normal	—	14.3	85.4	Cell
A2	Normal	—	18.0	85.7	Cell
A2	Normal	—	9.5	90.3	Medium
A2	Normal	20.0±2.43 (4)	24.8±4.81 (4)	71.9±4.45 (4)	Cell and medium
HF_3	Normal	18.15 (2)	25.35 (2)	66.8 (2)	Cell and medium
H366	Homocystinuric	—	26.7	71.5	Cell
H366	Homocystinuric	20.6±2.94 (4)	32.9±4.88 (4)	61.8±4.66 (4)	Cell and medium
H340	Homocystinuric	29.7 (2)	32.15 (2)	64.35 (2)	Cell and medium

amine was greater in the uptake medium than in the cell extract (see Table 2).

The presence of such high concentrations of sulphate conjugate in mammalian cells is surprising in view of the relatively low amounts found in urine (Goodall & Alton, 1968; Bronaugh *et al.*, 1975). However, high concentrations of dopamine 3-*O*-sulphate have been detected in mouse neuroblastoma cells after incubation with dopamine (Michelot *et al.*, 1977). 2-Methoxytyramine 4-*O*-sulphate has been detected as a minor metabolite of L-dopa (3,4-dihydroxyphenylalanine) in the urine of Parkinsonian patients, along with the 3-*O*-sulphate isomer (Bronaugh *et al.*, 1975); this has been interpreted as indicating competitive metabolic inactivation of dopamine by either 3-*O*-sulphate conjugation or 3-*O*-methylation. The absence of sulphate conjugates of dopamine in cultured fibroblasts may indicate that in these cells, methylation precedes conjugation

We have also examined the concentrations of 3-methoxytyramine 4-*O*-sulphate and 3-methoxytyramine in fibroblasts from homocystinuric patients (see Table 2). Homocystinuria is characterized by an aberration of methionine synthesis, owing to either a failure to remethylate homocysteine or a defect in cystathionine synthesis (Mudd *et al.*, 1972; Gerritsen & Waisman. 1972; Griffiths & Tudball, 1976). Failure to metabolize accumulated intracellular homocysteine could result in high cellular concentrations of *S*-adenosylhomocysteine; this compound acts to inhibit transmethylation processes requiring *S*-adenosylmethionine (Borchardt, 1977; Coward *et al.*, 1972). The results in Table 2 show no significant differences between the concentrations of 3-methoxytyramine 4-*O*-sulphate, 3-methoxytyramine and dopamine in the cell extracts or uptake media from cultured fibroblasts of two normal individuals and two homocystinuric patients. Thus the activity of catechol *O*-methyltransferase (EC 2.1.1.6) in fibroblasts from homocystinuric individuals appears to be similar to that in normal fibroblasts, and no accumulation of *S*-adenosylhomocysteine in the former cells is apparent from the present data.

Rat hepatoma

We have also determined the metabolism of [G-^{3}H]dopamine in rat hepatoma-cell culture. In this cell type, extensive conjugation of dopamine metabolites also occurs and dopamine metabolism is rapid and almost complete after a 3h incubation period. Initial examination of the cell extract after incubation, by t.l.c., showed only two main areas of radioactivity, one eluting with the acidic metabolite markers and the other situated at the origin (see Fig. 1*b*). High-pressure liquid-chromatographic analysis of the extract on the three systems described above showed the presence of two unidentified peaks of material eluting with some retention on both cation-exchange and reverse-phase chromatography (see Figs. 2*b* and 3*b*), which accounted for the majority (77.5%) of radioactivity in the sample. Very low concentrations of 3-methoxytyramine were present, but significant amounts of homovanillic acid and 3,4-dihydroxyphenylacetic acid were detected by anion-exchange chromatography (see Fig. 4*b* and Table 3). Examination of the uptake medium showed the presence of the same metabolites. However, there were considerable differences in their relative concentrations compared with those found in the cell (see Table 3). This may reflect the relative abilities of the metabolites to pass through cell membranes. In this respect the acidic products of dopamine metabolism appear to be preferentially released, whereas 'metabolite-2' is retained by the cell.

Isolation of the two unidentified metabolite peaks by preparative t.l.c., incubation with arylsulphatase and with β-glucuronidase, and analysis of the

hydrolysis products by high-pressure liquid chromatography afforded results that are summarized in Table 4. As can be seen, arylsulphatase was without effect, whereas β-glucuronidase produced almost complete hydrolysis of both metabolite peaks within 18h. The results indicate that 'metabolite-2' is probably 3-methoxytyramine 4-*O*-glucuronide whereas 'metabolite-1' appears to be a mixture of dopamine 3-*O*- or dopamine 4-*O*-glucuronide [the diglucuronide is unlikely for steric reasons (Dodgson *et al.*, 1950; Bronaugh *et al.*, 1975)] and two other unidentified glucuronide conjugates, which on hydrolysis with β-glucuronidase release catecholamine metabolites that elute with retention times of 8 and 52min (15.1 and 4.14% respectively) on cation-exchange chromatography, but are not retained on either anion-exchange or reverse-phase chromatography. The identification of these metabolites is at present under investigation. Authentic standards of the glucuronide conjugates described above were unfortunately not available. However, a time-consuming chemical synthesis of dopamine 4-*O*-glucuronide has been reported (Hansson & Rosengren, 1976).

Monoglucuronides of catecholamines have been described (Dodgson & Williams, 1949; Clark *et al.*, 1951; Herrting, 1964), but their identities have not been clearly established. Recently the glucuronides of tyramine and 4-hydroxy-3-methoxyphenylethanol have been detected in a number of mammalian tissues (Wong, 1976*a*,*b*, 1977; Karoum *et al.*, 1971) and a report describing the presence of dopamine 4-*O*-glucuronide and 3-methoxytyramine 4-*O*-glucuronide in a transplantable islet-cell tumour of the golden hamster has been published (Hansson & Rosengren, 1976; Falck *et al.*, 1977). The former glucuronide was present in extremely high concentrations in this tumour, whereas 3-methoxytyramine 4-*O*-glucuronide was present to a much smaller extent.

Table 3. *Analysis of rat hepatoma metabolites of [G-^{3}H]-dopamine*

Cells were incubated with [G-^{3}H]dopamine for 3h at 37°C. The cell extract and uptake medium were analysed on Partisil-10 SCX, Partisil-10 SAX and Partisil-10 ODS microparticulate columns.

	Metabolite (%)	
Metabolite	Cell extract	Uptake medium
Homovanillic acid	7.6	17.7
3,4-Dihydroxyphenylacetic acid	8.5	13.5
Dopamine	8.2	19.5
3-Methoxytyramine	1.9	3.5
'Metabolite-1'	28.1	20.9
'Metabolite-2'	49.4	21.3

Discussion

Degradation of catecholamines in the mammalian body is thought to occur primarily through the

Table 4. *Analysis of unknown hepatoma cell-extract metabolites of [G-^{3}H]dopamine after chromatographic separation and incubation with arylsulphatase and β-glucuronidase enzymes*

For experimental conditions, see the text. 'Metabolite-1' and 'metabolite-2' represent the two major metabolites of [G-^{3}H]dopamine (see Figs. 2*b* and 3*b*). The 3-methoxytyramine observed in the control experiment (−enzyme) with β-glucuronidase probably results from slow hydrolysis of metabolite-2 during analysis.

	Metabolites (%)			
	β-Glucuronidase incubation		Arysulphatase incubation	
Metabolite	−Enzyme	+Enzyme	−Enzyme	+Enzyme
Homovanillic acid	0	0	0	0
3,4-Dihydroxyphenylacetic acid	0	0	0	0
Dopamine	0	7.3	0	0
3-Methoxytyramine	3.3	68.7	0	0
'Metabolite-1'	28.9	0	28.9	30.3
'Metabolite-2'	58.3	3.7	66.3	67.6
Others	5.9*	15.1* 4.1†	3.7‡	1.67‡

* This component eluted with a t_R of 8min on Partisil-10 SCX and in the void volume on Partisil-10 SAX and Partisil-10 ODS columns.

† This component eluted with a t_R of 52min on Partisil-10 SCX and in the void volume on Partisil-10 SAX and Partisil-10 ODS columns.

‡ This component eluted in the void volume on Partisil-10 ODS chromatography.

actions of monoamine oxidase (EC 1.4.3.4) and catechol *O*-methyltransferase (EC 2.1.1.6). We have been surprised to find that in certain cultured-cell types, the predominant metabolites of dopamine occur as sulphate and glucuronide conjugates formed from 3-methoxytyramine. In normal human skin fibroblasts the predominant role of metabolism is first methylation and then formation of a sulphate conjugate. The low degree of deamination in these cells probably reflects their low amounts of monoamine oxidase activity as measured *in vitro* (Breakefield *et al.*, 1976). The fact that methylation precedes conjugation in fibroblasts suggests either intracellular compartmentalization of degradative enzymes or a preferential substrate affinity of the sulphotransferase for 3-methoxytyramine over dopamine. In hepatoma cells, the predominant route of metabolism is methylation followed by conjugation to the glucuronide. Again, the glucuronyl-transferring enzyme (EC 2.4.1.17) appears to prefer 3-methoxytyramine as a substrate. The low extent of deamination in these cells is surprising in view of the high monoamine oxidase activity measured *in vitro* (Hawkins & Breakefield, 1978). A comparison of metabolites in medium compared with cells suggests that deaminated products may be preferentially released, whereas the methylated glucuronide conjugate is retained for a longer period of time.

Analysis of dopamine metabolism in homogeneous populations of living cells in culture allows a detailed description of the intracellular fate of catecholamines in different cell types. Studies in tissues or whole animals provide an average of degradative processes occurring in hundreds of different cell types with varying accessibility to catecholamines and their metabolites. It is impossible to know whether metabolism in skin fibroblasts in culture is exactly the same as *in vivo*, or whether that observed in hepatoma cells accurately reflects degradation in liver parenchymal cells. In culture, both fibroblasts and hepatoma cells show excessive conjugate formation compared with that indicated by other types of studies. These cultured lines provide a model system to study regulation of both methylation and conjugation processes and suggest the need for a re-evaluation of the various routes of catecholamine degradation that occur in different cell types *in vivo*.

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