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SEPARATION OF THE URINARY METABOLITES OF *N*-2-FLUORENYLACETAMIDE ON COLUMNS OF DEAE-CELLULOSE ANION EXCHANGER*

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Abstract—A method was developed for the resolution of the water-soluble urinary metabolites of the carcinogen *N*-2-fluorenylacetamide and related compounds. The separation was achieved by means of column chromatography on DEAE-cellulose anion exchanger in the formate form. The conjugated metabolites were eluted with an increasing gradient to 0·5 M ammonium formate buffer, pH 3·5, in a nine-chamber Varigrad system (0, 0, 0·1, 0, 0·35, 0, 0·6, 0·8, 1·0). In this scheme, twenty individual peaks were obtained containing glucuronic and sulfuric acid conjugates as well as unknown water-soluble metabolites. Thus the major metabolites of *N*-2-fluorenylacetamide, namely the 3-, 5-, 7-, and *N*-hydroxy derivatives, were demonstrated in the form of glucuronides, as well as the sulfuric acid ester of *N*-(7-hydroxy-2-fluorenyl)acetamide. In addition, evidence is given for new metabolites, namely the sulfuric acid esters of *N*-(5-hydroxy-2-fluorenyl)acetamide and possibly of the 3- and 1-hydroxy isomers. Takadiastase, often used to hydrolyze sulfuric acid esters, splits quantitatively the sulfuric acid conjugate of *N*-(7-hydroxy-2-fluorenyl)acetamide, but none of the other sulfates, which are hydrolyzed by mild acid treatment. Further metabolites have been separated but not yet identified. The new chromatographic technique described may be useful for the water-soluble metabolites of other drugs for which the polystyrene type of ion-exchangers fail, owing to irreversible adsorption.

STUDIES of the metabolism of *N*-2-fluorenylacetamide (FAA)† and related compounds have usually involved enzymatic or acid hydrolysis of conjugates of glucuronic acid and sulfuric acid prior to the identification of the metabolites.[1–3] Thus, conjugates of a number of ring- and nitrogen-hydroxylated metabolites were found as detoxification and activation products of the carcinogen.[3–5] However, the proof of structure rested chiefly on specific means of hydrolysis of the conjugates followed by analyses of the split products. Also, there was consistent evidence that there were water-soluble metabolites which were neither glucuronides nor sulfuric acid esters. Moreover, it was suspected that there might be small amounts of materials that were not within the capability of the techniques used heretofore.

This report presents a novel method for the separation and direct determination of carcinogen metabolites based on the degree of ionization of the compounds, specifically

* A preliminary summary of this study was listed in the Abstracts of the Sixth International Congress of Biochemistry, New York, 1964, V, 408. Irving and associates (*Fedn Proc.* **25**, 743, 1966; and *Proc. Am. Ass. Cancer Res.* **7**, 76, 1966) have presented papers on the application of the methods detailed herein.

† *Abbreviations used:* PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; DEAE, diethylaminoethyl; FAA, *N*-2-fluorenylacetamide (or 2-acetylaminofluorene); N-OH-FAA, *N*-hydroxy-*N*-2-fluorenylacetamide (or *N*-hydroxy-2-acetylaminofluorene); 1-OH-FAA, the 1-hydroxy derivative; etc.

of the conjugates. The technique is suitable for application to the study of the metabolism of other drugs. The method for the fractionation of the urinary metabolites of FAA and derivatives depends on the use of DEAE-cellulose ion exchanger under specified conditions. This procedure afforded immediate evidence for metabolites as water-soluble conjugates, and might provide the potential tools for actual isolation of these materials.

METHODS

Samples of urinary metabolites. Four male Fischer rats, 5 months old, 298 g, were injected i.p. with a suspension of ^{14}C-labeled *N*-2-fluorenylacetamide ($4{\cdot}68 \times 10^6$ counts/min mg or 0·75 mc/mmole) in a 1% gum acacia solution. The dose level was 100 mg/kg body weight. The animals were housed in stainless steel metabolism cages and the urines were collected in ice-cold containers for a 24-hr period.

Column chromatography. DEAE-cellulose anion exchanger, 0·9 mg/g, medium mesh (Sigma Chemical Co.) was prepared as described by Peterson and Sober.[5] The filter cake was finally suspended in 5% formic acid and then washed with water until free of acid. The column (1·3 × 30 cm) was prepared by pouring this material, in distilled water, into the column in several increments, and packing with increasing nitrogen pressure, reaching finally 9 lbs. A filter paper disk was placed on top of the cellulose to prevent disturbance of the surface during loading of sample and elution.

Samples of urine (2–10 ml) were diluted with an equal volume of water and applied to the column under 1–2 lbs of nitrogen pressure to facilitate loading. The column was washed with 200 ml water and further developed by gradient elution with 0·5 M formic acid–ammonium formate buffer, pH 3·5, as the limiting buffer. A nine-chamber Varigrad with 200 ml per chamber was programmed as follows: 0, 0, 0·1, 0, 0·35, 0, 0·6, 0·8, and 1. A flow rate of 60 ml/hr was maintained by means of a constant-flow pump (Buchler Instruments Inc., New York). Fractions of 6 ml were collected, starting with the water wash.

An earlier scheme involved two separate gradients with smaller Varigrad chambers of 1 liter total capacity. The first schedule was 0, 0, 0·03, 0, 0·13, 0, 0·13, 0·26, 0·3, with 100 ml in each of the nine chambers. Immediately following was the second schedule, 0·5, 0, 0·6, 0, 0·7, 0, 0·8, 1·0, 1·0, also 100 ml per chamber. The limiting buffer for both schedules was 0·5 M formic acid–ammonium formate, pH 3·5.

Determination of radioactivity. Aliquots (0·1 ml) of the fractions were pipetted into counting vials with 15 ml scintillation counting mixture containing 3 g PPO, 0·1 g POPOP, 700 ml toluene, and 300 ml methanol. Counting was performed in a Packard liquid scintillation spectrometer.

Identification of individual metabolites. Fractions corresponding to each peak of the elution curves, measured by ^{14}C from the carcinogen, were combined and taken to dryness *in vacuo*. The ammonium formate was removed by sublimation (40–50°) and the residue dissolved in water. The aqueous solutions were buffered with 1 M acetate, pH 6, and subjected sequentially to ether extraction (five times, equal volumes), bacterial β-glucuronidase hydrolysis followed by ether extraction, and finally the remaining aqueous phase was hydrolyzed (sulfuric acid esters) under mild acid condition (0·2 N HCl) by refluxing for 15 min. After adjustment to pH 6 the mixture was extracted with ether.

The radioactive fractions from the DEAE-cellulose columns were chromatographed on Whatman 3 MM paper in solvent system *A*, *sec.*-butanol:ammonium hydroxide (3:1 v/v). The ether-extractable metabolites were chromatographed in solvent system *B*, cyclohexane:*tert.*-butanol:acetic acid:water (16:4:2:1). Thin-layer chromatography was performed on "chromogram" sheet (20 × 20 cm) of silica gel (Distillation Products Industries) with petroleum ether:acetone, 7:3 v/v as solvent. Authentic compounds (when available) were run simultaneously as references. In this paper, R_f values are measured from leading to trailing edge of each spot. The developed chromatograms were exposed to X-ray film (Kodak Royal Blue) to locate ^{14}C-labeled metabolites. Quantitative data on the individual metabolites were obtained by cutting out the radioactive spots and counting them. Also, appropriate color tests and spectroscopy aided in identifying the radioactive products.[1,4,6]

RESULTS

In Table 1 is shown the distribution of urinary-^{14}C into the various classes of metabolites. The free, unconjugated metabolites accounted for 5·2 per cent; the values for conjugated compounds were 44·3 and 40·0 per cent for the glucuronides and sulfate esters respectively.

TABLE 1. CLASSIFICATION OF URINARY METABOLITES OF *N*-2-FLUORENYLACETAMIDE

Class	Percentage of Urinary-^{14}C
(1) Free compounds	5·2
(2) Glucosiduronates	44·3
(3) Sulfates	40·0
(4) Other water-soluble metabolites	10·5

The fractionation was based on the ether solubility of radioactive metabolites in urine at pH 6 (1); after treatment with bacterial β-glucuronidase (2); and after mild acid hydrolysis (3). Forty-two per cent of the dose was excreted in the urine in 24 hr.

The separation of the metabolites of the same urine by DEAE-cellulose chromatography is shown in Fig. 1. The ^{14}C-elution curve of the complete urine has been delineated into twenty peaks (A-T) by the dotted lines. Portions of each peak, after removal of the ammonium formate (see Methods), were buffered at pH 6 and subjected to ether extraction, incubation with β-glucuronidase, and mild acid hydrolysis. Table 2 lists the percentage of urinary isotope in the various fractions as well as the results of the ether extraction, β-glucuronidase, and acid treatment of the individual peaks.

The free metabolites were the first to emerge from the column. They appeared during the water wash very early after the start of the gradient. The glucuronides, predominantly in fractions E-L, were held more firmly and eluted as the salt concentration increased. Finally, the sulfuric acid esters appeared when the molarity of the gradient was appreciable.

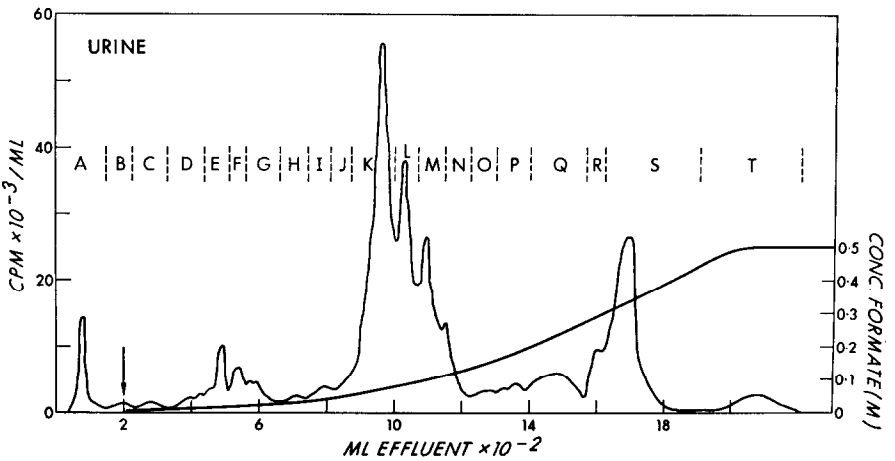

FIG. 1. Chromatography of DEAE-cellulose column of the urinary metabolites of *N*-2-fluorene-9-^{14}C-ylacetamide. The sample consisted of 2·5 ml of urine diluted 1 : 1 with H_2O and contained 10×10^6 counts/min. Recovery of the isotope was 92 per cent. The arrow indicates the start of the gradient.

TABLE 2. DEAE-CELLULOSE COLUMN SEPARATION OF URINARY METABOLITES OF *N*-2-FLUORENYLACETAMIDE

Fraction	Percentage of Urine-^{14}C			
	Total	Free	Glucuronides	Sulfates
A	2·8	1·8	0·2	0·1
B	0·5	0·4		
C	0·7	0·7		
D	1·5	1·3		
E	2·4	0·6	0·8	0·1
F	1·8	0·5	1·1	
G	2·3	1·0	0·9	
H	1·3	0·5	0·3	
I	1·8	0·5	0·8	0·1
J	1·9	0·3	1·2	0·1
K	25·3	0·8	23·0	
L	13·0	2·2	10·4	
M	9·0	0·2	1·1	6·3
N	4·8	0·2	0·4	3·2
O	1·1		0·2	0·5
P	2·6		0·4	1·4
Q	6·1	0·3	2·4	2·1
R	4·0	0·2	0·2	3·2
S	13·6			12·4
T	3·3			1·7
Total	99·8	11·6	43·3	31·2

Aliquots from each fraction were buffered at pH 6 and extracted 5 times with ether (Free). The aqueous layer was incubated in β-glucuronidase and extracted 5 times with ether (Glucuronides). The remaining aqueous layer was subjected to mild acid hydrolysis (0·2 N HCl) for 15 min, cooled, neutralized with solid $NaHCO_3$, and extracted 5 times with ether (Sulfates).

Nature of Metabolites in Each Fraction

Complete urine

Peak A. Paper chromatography of this peak in solvent *A* yielded four spots with R_f values of 0·44–0·47, 0·50–0·64, 0·76–0·86 and 0·91–0·99, which were present to the extent of 9, 21, 35, and 36 per cent of the fraction respectively. Sixty-five per cent of the isotope was ether-extractable, which denoted free metabolites. Spots with R_f 0·76–0·86 and 0·91–0·99 corresponded to values reported for free metabolites of *N*-2-fluorenylacetamide in solvent *A*.[1] Chromatography of the ether extract in solvent *B* yielded three major spots with R_f 0·40–0·50, 0·73–0·84 (1-OH-FAA), and 0·86–0·98. The spots accounted for 50, 30, and 17 per cent of the extract respectively.

Peak B. The radioactivity of this fraction was ether-soluble in an amount of 85 per cent. Paper chromatograms revealed two distinct spots, R_f 0·76–0·86 and 0·92–0·98, which also were in agreement with those reported for free metabolites in solvent system *A*. Chromatography in solvent system *B* showed spots with mobilities of 0·01–0·06, 0·58–0·74 (3-OH-FAA), 0·78–0·85 and 0·90–0·97, which amounted to 54, 19, 17, and 10 per cent, respectively, of the ether-extractable metabolites.

Peak C. This fraction consisted also essentially of free metabolites. Ninety-seven per cent of the isotope was ether-soluble. Paper chromatograms in solvent *B* revealed major spots with R_f values of 0·0–0·04 and 0·18–0·26. The latter mobility was the same as that of authentic *N*-(5-hydroxy-2-fluorenyl)acetamide, which accounted for 68 per cent of the extract.

Peak D. Only one distinct spot, R_f 0·78–0·89, was present in this fraction. The ^{14}C was ether-extractable to the extent of 86 per cent, and upon chromatography had an R_f value of 0·08–0·16, which was the same as that of the authentic 7-OH-FAA in the cyclohexane system, solvent *B*. The ether extracts of fractions E-L also showed the presence of small amounts of the 5- and 7-hydroxy derivatives.

Peak E. Paper chromatograms of the fraction revealed three distinct spots with R_f values of 0·0–0·03, 0·07–0·14, and 0·79–0·87 (solvent *A*) which represented 28, 38, and 34 per cent of the isotope respectively. Twenty-four per cent of the fraction was ether-extractable and, by paper chromatography in the cyclohexane system, solvent *B*, showed the presence of free metabolites, of which the 7-hydroxy derivative was the chief component.

Peak F. Radioactive areas with R_f values of 0·19–0·22, 0·28–0·34, 0·37–0·47, 0·79–0·90, and 0·92–0·99 (solvent *A*) were observed in this fraction. The amounts of isotope in each fraction were 4, 8, 37, 21, and 21 per cent respectively. The latter two spots had mobilities like that of the free metabolites, and, indeed, spots corresponding to the 5-, 7-, and *N*-hydroxy derivatives of *N*-2-fluorenylacetamide were present on the paper chromatograms of the ether extracts in solvent system *B*.

The spot with R_f 0·37–0·47 corresponded to that reported for N-OH-FAA glucuronide.[4,7] Treatment of the fraction with bacterial β-glucuronidase did reveal a spot with R_f 0·75–0·87 in the cyclohexane system, which was identical with that of the authentic *N*-hydroxy derivative. The enzyme also liberated a metabolite with R_f 0·28–0·39 which agreed with that of standard aminofluorene. One of the spots, R_f 0·19–0·22 or 0·28–0·34, observed in the butanol system *A*, may thus represent a *N*-glucuronide of aminofluorene, or a glucuronide of an unknown metabolite that yielded aminofluorene upon hydrolysis.

Peak G. Paper chromatography of fraction G showed the same spots that appeared in peak F. However, the spot with a mobility of 0·28–0·34 was missing.

Peak H. There were four major spots in this fraction, R_f 0·04–0·07, 0·08–0·12, 0·78–0·89, and 0·90–0·98. The first two represented unidentified metabolites; the latter two had mobilities like those of free metabolites. Major spots corresponding to the 5- and 7-hydroxy derivatives, as well as trace amount of other free metabolites, were observed in the paper chromatograms of the ether extracts.

Peak I. Paper chromatography of this fraction in the butanol system revealed four areas of radioactivity. The values were 0·02–0·08, 0·11–0·18, 0·42–0·51, and 0·77–0·86. The spot with R_f 0·77–0·86 contained free metabolites. Treatment of the fraction with β-glucuronidase and ether extraction gave three spots with values of 0·00–0·04, 0·16–0·23, and 0·27–0·35 in the cyclohexane system. The metabolite (0·16–0·23) showed a slightly different mobility from that of the authentic *N*-(5-hydroxy-2-fluorenyl)acetamide (0·18–0·26). The unknown metabolite with R_f value of 0·27–0·35 has been observed previously by us and other investigators.[2,4]

Peak J. This fraction also exhibited four distinct spots on paper with values of 0·0–0·07, 0·10–0·15, 0·42–0·50, and 0·77–0·84. The ether extract chromatographed in the cyclohexane system showed the presence of the 5- and 7-hydroxy derivatives. The extract after β-glucuronidase hydrolysis revealed two spots. The major metabolite had an R_f value of 0·0–0·05 which was unidentified. The lesser one, with a value of 0·08–0·12, was 7-OH-FAA.

Peak K. Virtually all the metabolites appeared to be glucosiduronic acids. Chromatography of this fraction in the butanol solvent system showed three distinct spots. The major spot which accounted for 74 per cent of the fraction had a value of 0·08–0·17. The second spot, R_f 0·26–0·33, represented 14 per cent of the fraction. Both areas have been described previously,[6,8] the former as the glucuronides of *N*-(5- and 7-hydroxy-fluorenyl)acetamide, and the latter as that of *N*-(3-hydroxyfluorenyl)acetamide. Spot 0·08–0·17 was shown to be predominately the 7-hydroxy derivative by β-glucuronidase hydrolysis of fraction K, and subsequent paper chromatograms of the ether extracts in the cyclohexane solvent system. The third spot (R_f 0·69–0·78) remains unknown.

Peak L. In this fraction also glucosiduronic acid predominanted. There were four spots with R_f values 0·09–0·17, 0·26–0·32, 0·48–0·53, and 0·74–0·88, and relative amounts of 72, 3, 2, and 18 per cent respectively. The first two spots were identical with those of fraction K, namely 5- and 7-hydroxy glucuronides and the 3-hydroxy glucuronide. The spot 0·09–0·17, however, in this fraction was chiefly the glucuronide of *N*-(5-hydroxyfluorenyl)acetamide. The third spot (R_f 0·48–0·53) which was possibly the glucuronide of *N*-(1-hydroxyfluorenyl)acetamide,[6,8] however, could not be unequivocally identified because the amount present was small. The fourth spot (R_f 0·74–0·88) was due to a free ether-extractable metabolite.

Peak M. The major part of this and the next peak had the properties of sulfuric acid esters. Chromatograms of fraction M revealed four spots. There were two weak areas corresponding to R_f of 0·10–0·14 and 0·49–0·53, which were 6 and 3 per cent of the fraction respectively. These spots have been identified as small amounts of the 5-hydroxy-FAA and possibly the 1-hydroxy-FAA glucuronides as described for the previous fraction. The major spots, R_f 0·60–0·63 and R_f 0·70–0·80, constituted 22 and 65 per cent of the fraction respectively. The spot 0·60–0·63 was unidentified. The identity of the spot with a mobility of 0·70–0·80 will be described below.

Peak N. Two major spots with R_f values of 0·69–0·72 and 0·74–0·78 were detected in this fraction after paper chromatography; they constituted 26 and 58 per cent of the isotope respectively. The first spot was unidentified and the second will be discussed below. There were also three minor unknown spots with values of 0·0–0·05, 0·07–0·11, and 0·34–0·43.

Peak O. Chromatography of this fraction in the butanol solvent system yielded four radioactive spots with R_f values of 0·00–0·06, 0·41–0·45, 0·53–0·58, and 0·78–0·83. The percentages of the fraction represented by each spot were 14, 31, 16, and 35 respectively. These metabolites were unidentified.

Peak P. Chromatography of the metabolites in this fraction yielded four spots with R_f values of 0·0–0·02, 0·05–0·08, 0·63–0·68, and 0·78–0·82. The identity of these spots has not been established.

Peak Q. Chromatography in the butanol system showed the presence of four major spots in this fraction. The R_f values were 0·0–0·02, 0·05–0·09, 0·32–0·36, and 0·60–0·66. These spots accounted for 13, 37, 12, and 29 per cent of the isotope respectively. The first three metabolites were unidentified and were probably glucuronides, on the basis of their mobilities. The metabolite (R_f 0·60–0·66) will be discussed under peak R.

Peak R. Two distinct metabolites were discernible in the fraction. The metabolite with value of 0·55–0·58 represented 64 per cent of the total ^{14}C, and was also present in peak S. The lesser metabolite, 27 per cent, R_f 0·60–0·66, was identified as the *N*-(5-hydroxyfluorenyl)acetamide conjugate of sulfuric acid, which has been mentioned as a possible metabolite previously.[8]

Peak S. This fraction revealed one spot, R_f 0·55–0·58, in the butanol system; it has been reported previously to be the sulfuric acid ester of *N*-(7-hydroxy-2-fluorenyl)acetamide.[8,9] The fraction was hydrolyzed to the extent of 91 per cent with 0·2 N HCl and the ether-extractable metabolite when chromatographed on the cyclohexane system showed a spot corresponding to that of authentic *N*-(7-hydroxy-2-fluorenyl)acetamide.

Peak T. Paper chromatograms of this fraction revealed two distinct spots. The major spot had a R_f of 0·36–0·44 and accounted for 92 per cent of the ^{14}C. The lesser spot had a mobility of 0·21–0·28. Both spots were unidentified.

Urine after removal of free compounds and glucosiduronic acids

The ^{14}C-elution curve of urine free of glucuronic acid conjugates is shown in Fig. 2. The peaks corresponding to the glucuronic acid conjugates (Fig. 1), namely F, J. K, and L, have been deleted. Peaks A′ and E′ (all peaks remaining are now designated by a prime symbol) are more prominent. The major peaks M′, N′, R′ and S′ were isolated and treated as described in Table 3. It is noteworthy that peaks M′ and N′ were essentially unaffected by Takadiastase,[8] whereas the isotope of peak S′ was thus rendered ether-extractable to the extent of 95 per cent. However, when subjected to mild acid hydrolysis, the radioactivity of peaks M′ and N′ were transferred to ether in appreciable amounts, 85 and 77 per cent respectively. Under the same condition, 98 per cent of the ^{14}C of peak S′ was ether-soluble. Radioactivity in peak R′ was rendered ether-extractable to the extent of 18 per cent by Takadiastase and 60 per cent by acid hydrolysis. Chromatography of the ether-extractable metabolites hydrolyzed by enzyme showed the presence of 7-OH-FAA (R_f 0·08–0·16) and a metabolite with

a value of 0·0–0·04. The presence of these metabolites is explained as due to overlapping of peak R′ by peaks Q′ and S′ (Fig. 2). However, chromatography of the ether extract obtained after acid hydrolysis revealed the presence of 5-OH-FAA (R_f 0·18–0·26). Thus, it appeared that the sulfuric acid ester of 5-OH-FAA was split by mild

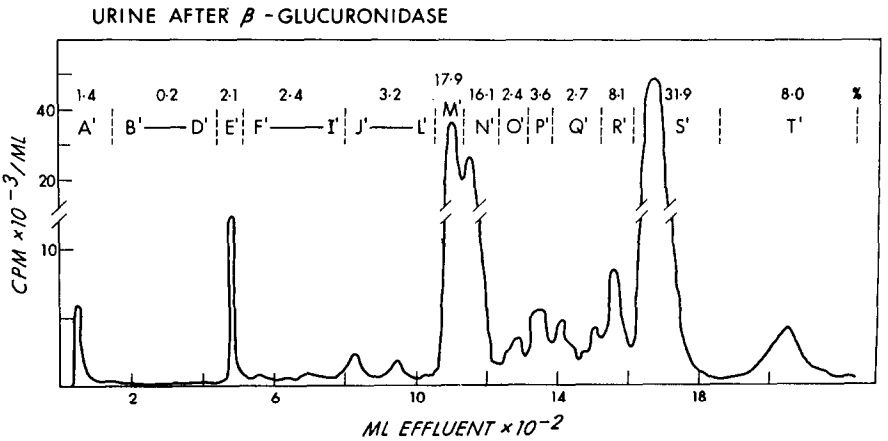

FIG. 2. Chromatography of β-glucuronidase-hydrolyzed urine (9 ml, 6·3 × 10^6 counts/min). Eighty-three per cent of the isotope applied was recovered.

acid but not by Takadiastase. Peak S′ has been identified as the sulfuric acid ester of *N*-(7-hydroxy-2-fluorenyl)acetamide. These data are in agreement with observations previously reported by this laboratory, which demonstrated that Takadiastase did not hydrolyze all the sulfates quantitatively.[8]

TABLE 3. TREATMENT OF FRACTIONS FROM DEAE-CELLULOSE COLUMN OF β-GLUCURONIDASE-HYDROLYZED URINE

Treatment	Fractions			
	M′	N′	R′	S′
(1) Buffered at pH 6	1·2	2·9	2·3	
(2) β-Glucuronidase				
(3) Takadiastase	0·7	2·4	18	95·0
(4) Acid hydrolysis (0·2 N HCl)	85	77	60	98·0

Peaks M′, N′ and R′ were unaffected by acid phosphatase incubation at pH 5, as described by Troll *et al.*[19] for the hydrolysis of bis(2-amino-l-naphthyl)phosphate.

Paper chromatography of peak M′ in solvent system *A* revealed two radioactive spots with R_f values of 0·46–0·53 and 0·72–0·80, while N′ showed spots R_f 0·67–0·76 and 0·78–0·84. Peak S′ yielded a single spot with a value of 0·49–0·60.

Paper chromatograms of the ether extracts after acid hydrolysis of peaks M′ and N′, revealed two spots, R_f 0·49–0·65 and 0·84–0·95, in solvent *B* which were present in both peaks. After acetylation, there were also two spots in both, one of which had the same R_f value as that of the ether extract—0·46–0·63. However, the other spot showed

a decrease in mobility from R_f 0·84–0·95 to 0·73–0·83. Thin-layer chromatography of the same extracts after acetylation showed that there were also two spots in both M′ and N′. One with R_f 0·54 was present in both fractions. Peak M′ also possessed a metabolite with a value of 0·40, identical with the mobility of authentic 1-OH-FAA, while N′ showed a metabolite (0·28) which agreed with that of 3-OH-FAA. Additional data are necessary for the positive identification of these metabolites. Nevertheless, this is the first evidence for the presence of sulfuric acid conjugates of 1-OH- and 3-OH-FAA and, like the 5-OH-FAA conjugate, they are not hydrolyzed by the sulfatase present in Takadiastase but are split by 0·2 N HCl at 100° in 15 min.

Peaks O′ to T′ showed the same spots upon paper chromatography in solvent *A* as were present in the respective peaks of the untreated urine (Fig. 1), with the exception of the spots of low mobility, R_f 0·00–0·02 and 0·05–0·09, which were present in peaks P′, Q′, and R′. These metabolites were probably glucuronic acid esters which were hydrolyzed by the action of β-glucuronidase and subsequently removed by ether extractions.

Urine after removal of all known conjugates

The elution curve in Fig. 3 represents the separation of the metabolites that remained in the aqueous phase after sequential β-glucuronidase and acid hydrolysis. These

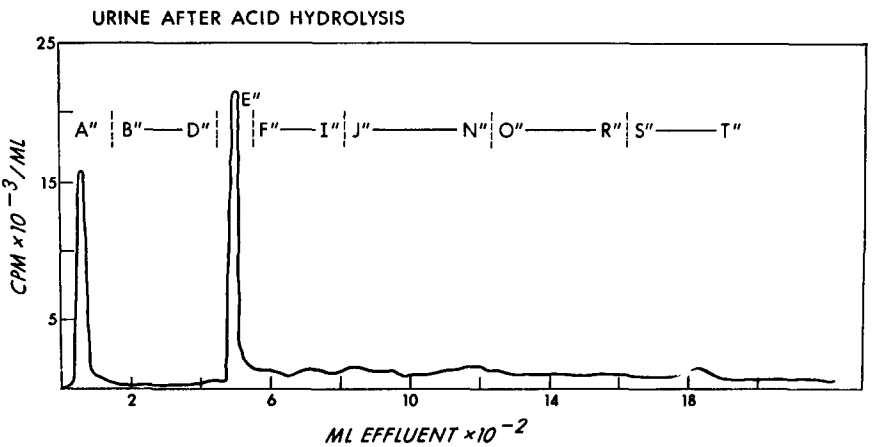

FIG. 3. Chromatography of acid-hydrolyzed urine (30 ml, 2·6 × 10^6 counts/min). The recovery of ^{14}C was 67 per cent. Additional ^{14}C-material was removed from the DEAE-cellulose by washing the exchanger with 0·5 N NaOH.

metabolites accounted for 10–15 per cent of the total urinary isotope. The recovery of applied ^{14}C was 67 per cent. Again the peaks which were acid-hydrolyzable have been eliminated. There were two prominent peaks, A″ and E″, remaining which accounted for 18 and 17 per cent of the recovered radioactivity in these water-soluble compounds. Paper chromatography of peak A″ showed two spots with R_f values of 0·44–0·47 and 0·50–0·64 in solvent *A*. Peak E″ also revealed two spots, with R_f values of 0·00–0·03 and 0·07–0·14, in the same solvent system. Small amounts of the isotope were not localized but appeared in every tube. The identities of peaks A″ and E″ are under investigation.

DISCUSSION

Many drugs, particularly those containing aromatic or heterocyclic rings in their structure, are often metabolized by animals and man to hydroxylated and other related derivatives which appear in tissues and in urine as water-soluble conjugates such as glucosiduronic acids and sulfuric acid esters. Identification of these metabolites was often made after hydrolysis by specific enzymes such as β-glucuronidase, or by appropriate conditions of acidity and temperature and subsequent examination of the split products. Isolation of the major metabolites, as with glucosiduronic acids, has sometimes been feasible via precipitation of the insoluble lead salts, followed by suitable treatment to give the free acid which could then be analyzed. Also, some procedures permitted the resolution of metabolites into classes of compounds, namely free sulfuric acid esters, and glucosiduronic acids.[8,10–15] Additional efforts were required to further separate and establish the nature of the metabolites. These techniques are somewhat cumbersome and indirect.

A new method was therefore desirable, which would obviate some of the lengthy steps and at the same time permit better quantitation. In a few cases, ion-exchange separation on styrene polymers such as Dowex gave reasonable results. However, it has been our experience that drugs containing heterocyclic and, even more so, polynuclear aromatic rings, were irreversibly adsorbed by resins of this type. We have therefore developed a new technique, chromatography on DEAE-cellulose with a formate buffer gradient at pH 3·5, obviating this difficulty, which is eminently suitable for the fractionation of the water-soluble metabolites of *N*-2-fluorenylacetamide and related compounds (Table 4). We have also applied it to a study of the metabolism of

TABLE 4. IDENTITY OF MAIN METABOLITES IN THE MAJOR FRACTIONS

Fraction	Identity
F	N-OH-FAA Glucuronide
K	7-OH-FAA Glucuronide
	3-OH-FAA Glucuronide
L	5-OH-FAA Glucuronide
	1-OH-FAA Glucuronide
M	1-OH-FAA Sulfate
N	3-OH-FAA Sulfate
R	5-OH-FAA Sulfate
S	7-OH-FAA Sulfate

N-hydroxy-*N*-2-fluorenylacetamide, of diethylstilbestrol, and of *N*-2-fluorenylacetamide in a number of species and under a variety of conditions. On the basis of our experience to date it appears that this procedure may be useful with some minor modification of the experimental procedures, such as operating pH, for the separation of other water-soluble complex drug metabolites.

As described here, the method served to resolve the total urinary components, including the free unconjugated metabolites. While certain of these conjugated metabolites develop as sharp peaks during the early part of the elution schedule, some others seem poorly resolved. It may be advisable, therefore, to extract free metabolites, for example, with ether, to avoid complications in the elution pattern of the conjugates, which in any case form the major part of most drug metabolites. The

fractionation system on DEAE-cellulose depended on the pK of the drug metabolites. The specific values of pK for the conjugates of *N*-2-fluorenylacetamide derivatives have not been determined, Such a study would no doubt make a significant contribution to an overall understanding of the behavior of these metabolites *in vivo*, especially in regard to their transport to various target organs, as well as in respect to the permeability of cellular membranes to them.[16]

The glucosiduronic acid conjugate of *N*-hydroxy-*N*-2-fluorenylacetamide was eluted earlier than the ring-hydroxy conjugates, suggesting that this substance, so important in the carcinogenic behavior of this type of compound, has a lower charge than the latter. As may be expected, the sulfuric acid conjugates are more highly ionized and therefore are most retarded. In this case again the sulfuric acid esters of the *ortho*-hydroxylated derivatives, namely the 5-, 1-, and 3-hydroxy compounds, appear earlier than the extended *para*-derivative, the 7-hydroxy compound. Sulfuric acid esters of the *ortho*-hydroxy derivatives occur in small amounts, and it was only by this highly sensitive and quantitative technique that their existence could be demonstrated. Also, while earlier data from this laboratory indicated that there were sulfuric acid esters which Takadiastase could not hydrolyze, there was no good way to identify them specifically. With the present technique this has now been done. However, in view of the failure of the gentle Takadiastase procedure to affect them, we have resorted to mild acid to split these conjugates. It could be, therefore, that there is a sulfuric acid ester also of *N*-hydroxy-2-fluorenylacetamide, which may rearrange to a ring-hydroxylated compound during acid hydrolysis. Booth and Boyland have presented data showing that 2-naphthylhydroxylamine can be conjugated with sulfuric acid and possess such properties.[17]

One of the main reasons for developing this chromatographic system was to gather direct evidence for the existence of metabolites of *N*-2-fluorenylacetamide of the mercapturic acid type. Indirectly, there seemed to be indication for sulfur-containing metabolites.[18] While several of the peaks on the DEAE-cellulose elution pattern are still unknown, there is no evidence as yet that they repesent mercapturic acids. Indeed, administration of sulfur-labeled methionine or cysteine together with or prior to *N*-2-fluorenylacetamide yielded no peaks in the elution pattern of the urines that could be ascribed to such derivatives. Two of the unknown peaks, A″ and E″, which are not glucuronic or sulfuric acid esters, are not highly ionized, considering their position in the eluate. This property serves as a tool to develop information on their identity.

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