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Metabolism of Fluorene-9-C^{14} in Rats, Guinea Pigs, and Rabbits*

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The metabolism of fluorene-9-C^{14} in several animal species is reported. The urine and feces contained 57 and 16, 82 and 6, and 39 and 1% of the isotope 48 hours after intraperitoneal injection of 100 mg/kg of fluorene-9-C^{14} into rats, guinea pigs, and rabbits, respectively. The soluble and insoluble liver proteins at 48 hours bound isotope to 155 and 97, 35 and 24, and 67 and 33 mμmoles of compound per gram of dry proteins in the rats, guinea pigs, and rabbits, respectively. Radioactivity was found in all organs analyzed. The liver and kidneys had a relatively high isotopic content. The respired carbon dioxide was void of radioactivity. The urinary radioactivity was composed of 5.6, 1, and 1% free compounds; 39, 17, and 23% sulfuric acid ester fraction; and 48, 79, and 64% glucosiduronic acid fraction in the rat, guinea pig, and rabbit, respectively. The chief metabolites were, in rats, 9-fluorenol glucuronide and 2-fluorenol sulfate; in guinea pigs, 9-fluorenol glucuronide and the 2-fluorenol glucuronide and sulfate; in rabbits, 2- and 9-fluorenol glucuronide and 2-fluorenol sulfate. The 2,9-difluorenol and conjugates were also present in the urine of the three species.

Fluorene is the parent hydrocarbon from which the important carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene) is derived. We have performed studies on the fate of fluorene in various animal species to serve as a model and to gain a better understanding of the metabolism of the more complex carcinogen molecule.

The fate of fluorene in the rabbit has been investigated previously by Neish (1948), who reported that 2-fluorenol and the glucuonide of 2-fluorenol were present in the urine. He also observed that little, if any, fluorene or metabolites were eliminated in the feces. The present studies extended the investigation to rats and guinea pigs as well as to rabbits in view of the species differences known to exist in the metabolism of the acetylamino derivative (Enomoto *et al.*, 1962; Irving, 1962; Miller *et al.*, 1960; Weisburger *et al.*, 1959b), as well as in other polynuclear aromatic hydrocarbons (Williams, 1959).

Fluorene-9-C^{14} was synthesized and used in the metabolism experiments reported here. The isotopic label afforded a more detailed and complete picture of the excretion and distribution of fluorene and metabolites in the animals. The use of this sensitive tool permitted the identification of metabolites which might not have been detected by isolation and color reaction techniques.

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MATERIALS AND METHODS

Synthesis of Fluorene-9-C^{14}.—Fluorene-9-C^{14} was synthesized in 75% yield from $BaC^{14}O_3$ (25.07 mc/mmole) by the procedure of Weisburger and Weisburger (1958). The specific activity of the product was 22 × 10^6 cpm/mg as determined by the wet combustion technique.

From the above material, two products with specific activities of 1.36 × 10^6 and 6.4 × 10^5 cpm/mg were prepared by dilution with pure unlabeled fluorene in a crystallization step. They were used for the biochemical experiments.

Reference Compounds.—2-Fluorenol, 4-fluorenol, 9-fluorenol, and 2,9-difluorenol were supplied by Dr. E. K. Weisburger. The glucuronide of 9-fluorenol was kindly furnished by Dr. W. J. P. Neish, University of Sheffield.

Radioactivity Determinations.—Radioactivity measurements were performed on a windowless gas-flow counter with an efficiency of 46% for C^{14}. The wet combustion technique of Weisburger *et al.* (1952) was used for the conversion of solid samples, feces, tissues, and proteins to barium carbonate, which was plated and the radioactivity determined. The urine and other liquid samples were plated directly in infinitely thin layers. A suitable correction factor was determined for adjusting the counts obtained by the two methods.

Treatment of Animals.—A. Rats. Six 3-month-old female Buffalo-strain rats weighing 150–160 g were injected intraperitoneally with 1 ml of a gum acacia suspension of fluorene-9-C^{14} (1.36 × 10^6 cpm/mg) at a dose level of 100 mg per kilogram body weight. The

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN THE RAT AFTER INTRAPERITONEAL INJECTION OF FLUORENE-9-C^{14}

Data given are the averages of 2 rats per period. Rats weighed 150–154 g and received 15.4 mg (21 × 10^6 cpm) of fluorene-9-C^{14}.

Organs[a]	1 Day			2 Days			8 Days		
	cpm/mg	Total cpm	% of Dose	cpm/mg	Total cpm	% of Dose	cpm/mg	Total cpm	% of Dose
Stomach	40	7,600	0.04	18	3,200	0.02	1.2	200	—
Stomach contents	38	7,500	0.04	74	7,100	0.02	6.0	2,750	0.01
Small intestine	211	429,000	2.00	65	145,000	0.45	3.0	4,600	0.02
Small intestine contents	3,330	627,000	3.00	930	319,000	1.50	18.0	4,600	0.02
Cecum	382	57,500	0.27	202	37,500	0.18	3.4	650	—
Cecum contents	5,300	1,485,000	7.10	3,170	1,495,000	7.10	31.0	16,000	0.76
Large intestine	199	38,300	0.20	150	29,300	0.14	3.0	600	—
Large intestine contents	3,940	265,000	1.80	1,900	947,000	4.50	32.0	6,500	0.03
Kidneys	173	52,700	0.25	82	26,900	0.12	23	6,500	0.03
Liver	78	161,300	0.77	34	66,100	0.31	7	11,600	0.06
Bladder	98	2,830	0.01	35	600	—	5	128	—
Lungs	57	8,000	0.04	22	3,580	—	3	456	—
Spleen	32	2,900	0.01	12	1,180	—	4	380	—
Brain	24	8,080	0.04	4	1,580	—	2	548	—
Heart	34	4,660	0.02	12	1,900	—	2	216	—
Blood[b]	682			287			53		

[a] Dry weight. [b] For blood, 50 μl was plated and the radioactivity determined directly; data are in terms of cpm/50 μl.

rats were placed singly in glass metabolism cages for separate collection of urine and feces. Food and water were available *ad libitum*. Two rats were sacrificed at the end of 1, 2, and 8 days by withdrawal of blood from the abdominal aorta of the anesthetized (ether) animals.

Two additional rats of the same age, sex, strain, and weight were administered 1 ml of a suspension of fluorene-9-C^{14} at the same dose level by stomach tube. One of the rats was placed in a glass metabolism cage permitting also the recovery of the expired carbon dioxide. The rats were kept on the experiment for 8 days.

B. Guinea pigs. Two female National Institutes of Health–bred stock guinea pigs weighing 310–315 g were injected intraperitoneally with fluorene-9-C^{14} (1.36 × 10^6 cpm/kg) at the same dose level given to the rats. The handling of the guinea pigs was as described above except for the diet, which was kale and rabbit chow, and the duration of the experiment, which was 2 days.

C. Rabbits. Two New Zealand White rabbits weighing 2000 g were administered intraperitoneally 200 mg each of fluorene-9-C^{14} (6.4 × 10^5 cpm/mg) in suspension form. The rabbits were kept in wire cages and fed kale and rabbit chow. Separation of urine and feces was poor with the arrangement employed. The rabbits were sacrificed as described above at the end of 48 hours.

Autopsy of Animals.—Upon autopsy of the animals, the liver was perfused with 0.9% saline and an aliquot was homogenized immediately in 0.1 M acetate buffer, *p*H 5. The soluble and insoluble proteins were isolated as described by Weisburger *et al.* (1953). Other selected organs were removed and immediately dried *in vacuo*.

Analysis of Urine.—The urinary metabolites were classified as free compounds and as glucuronide and sulfate conjugates by a combination of ether extractions and enzymic hydrolysis as described by Weisburger *et al.* (1959b). In another approach, the metabolites were separated into the free, sulfate, and glucuronide fractions on an alumina column by the method used for the metabolites of *N*-2-fluorenylacetamide (Weisburger *et al.*, 1961).

Paper Chromatography.—Urines and ether extracts of urines were chromatographed usually on Whatman 1 paper in two solvent systems: (*1*) cyclohexane–water–acetic acid (5:4:2) and (*2*) *sec*-butanol–3% ammonium hydroxide (3:1). The developed chromatograms were exposed to Kodak Royal Blue x-ray film to locate the position of radioactive metabolites.

Identification of metabolites was accomplished by comparison with the corresponding findings for authentic compounds of (*1*) R_F values of metabolites from autoradiographs, (*2*) color tests (Folin and coupling) of phenolic materials on paper chromatograms (Weisburger *et al.*, 1956), (*3*) ultraviolet spectra of ethanol eluates of paper strips (Cary recording spectrophotometer model 14), and (*4*) infrared spectra (Perkin-Elmer recording spectrophotometer, model 21, KBr discs). Inverse carrier isotope dilution procedures with pure unlabeled compounds were applied in a few instances.

RESULTS

Distribution of Radioactivity.—The carbon dioxide expired during the first 48 hours after the oral administration of fluorene-9-C^{14} contained no radioactivity.

Table I shows the distribution of the radioactivity in the different organs of the rats 1, 2, and 8 days after the injection of fluorene-9-C^{14}. The small intestine, cecum, and large intestine and their contents accounted for 5, 7.4, and 2% of the dose at 24 hours and 2, 7.3, and 4.6% at 48 hours. The stomach and contents, however, exhibited only low levels of radioactivity at all periods. The kidneys and the liver had a relatively high isotope content. All other organs analyzed contained carbon-14, but no outstanding concentration of radioactivity was noted.

The specific activity of the soluble and insoluble liver proteins of the rat remained relatively unchanged at 24 and 48 hours (Fig. 1). The soluble proteins, however, showed substantially higher activity than the insoluble proteins at all times except for the 8-day period, where the insoluble proteins were slightly higher. At 2 days the amount of protein-bound carbon-14 in both the soluble and insoluble fractions was largest in the rat, lower in the rabbit, and lowest in the guinea

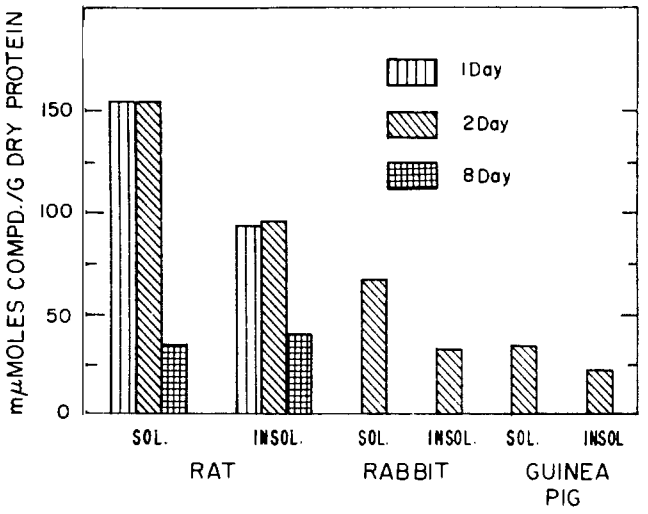

FIG. 1.—Protein-bound C^{14} after intraperitoneal dose (100 mg/kg) of fluorene-9-C^{14}. The specific activities of fluorene-9-C^{14} were 8216 cpm/mμmole for the rat and guinea pig and 3755 cpm/mμmole for the rabbit.

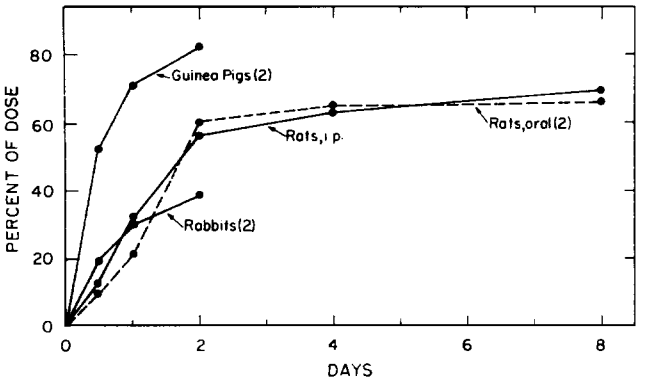

FIG. 2.—Urinary C^{14} after intraperitoneal (100 mg/kg) and oral doses of fluorene-9-C^{14}. Numbers of animals used indicated in parentheses, except for rats injected intraperitoneally where there were 6 rats at 0–24 hrs., 4 at 24–48 hrs., and 2 at 48–196 hrs.

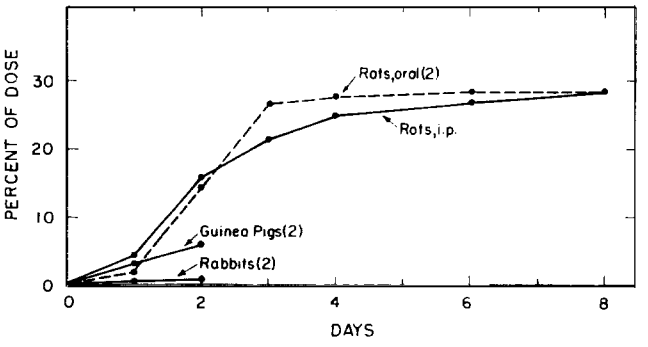

FIG. 3.—Fecal C^{14} in same animals as in Figure 2.

pig. In all three species the soluble proteins had a higher specific activity than the insoluble proteins.

Excretion of Isotope.—The rates of excretion of carbon-14 by rats, guinea pigs, and rabbits after administration of fluorene-9-C^{14} show that in all cases the urine was the major pathway of elimination of the isotope (Fig. 2). Guinea pig urine contained 53% of the dose in the first 12 hours, as compared to only 12 and 20% in rat and rabbit urine. Urinary excretion in a 2-day period accounted for 82, 57, and 39% of the dose in the guinea pig, rat, and rabbit, respectively.

The elimination of radioactivity in the feces was more pronounced in rats than in the other two species (Fig. 3). Thus, the feces contained 16, 6, and 0.7% of the dose in rats, guinea pigs, and rabbits, respectively, in a 2-day experiment. After 8 days, the feces amounted to 28% of the dose in rats.

Total recoveries of radioactivity in the urine and feces for a comparable 48-hour period were 88% in guinea pigs, 73% in rats, and 40% in rabbits. The low total in the rabbits may be related to poor absorption of the injected fluorene. Upon autopsy of the animals of each species, white particles of high radioactivity representing unresorbed material (m.p. 114–115°) were found in the peritoneal cavity. This was particularly noticeable in the rabbits, in which 50% of the dose administered was recovered unchanged from the cavity.

Isolation from Rat Urine and Proof of Structure of Glucuronide of 9-Fluorenol.—Six female Buffalo-strain rats (180–185 g) were administered intraperitoneally a total of 384 mg of unlabeled fluorene, and two other rats were given 109 mg of fluorene-9-C^{14} (1.36×10^6 cpm/mg) at a dose level of 100 mg/kg of body weight in a series of three injections at 48-hour intervals. The pooled urines were treated as described by Neish (1948) for rabbit urine. Fine white needles, 68 mg, m.p. 214–215°, were obtained. The infrared spectrum and the color reactions with concentrated H_2SO_4 and *p*-dimethylaminobenzaldehyde were identical to those of fluorenol glucosiduronic acid. Twenty-nine milligrams of the glucuronide were dissolved in 20 ml of water, buffered at *p*H 6, and incubated with 20 mg of β-glucuronidase at 37° for 18 hours. A white, crystalline product insoluble in the reaction mixture was filtered. The filtrate was extracted with ether (2×10 ml), and the ether extracts were taken to dryness on a steam bath under nitrogen, yielding a white residue. The crystalline product, 9 mg, m.p. 151–154°, and the residue, 4 mg, m.p. 150–153°, were combined and recrystallized twice from aqueous ethanol. The product, 8 mg of white needles, m.p. 153–154°, 216,000 cpm/mg, had an ultraviolet spectrum identical with that of authentic 9-fluorenol.

Attempted Isolation of a Glucuronide of Fluorenol from Rabbit Urine.—The 0-to-48-hour urine of two New Zealand White rabbits which had received 409 mg of fluorene-9-C^{14} (6.4×10^5 cpm/mg) by intraperitoneal injection was treated as described by Neish (1948). An oily product was obtained which after several attempts yielded a few crystals (6.6 mg, m.p. 193–195°).

Several milligrams of this material was dissolved in 2 ml of water, buffered at *p*H 6, and incubated with β-glucuronidase. The reaction mixture was extracted with ether, the extract concentrated, and an aliquot subjected to paper chromatography. Autoradiographs showed spots with R_F values corresponding to those of 2-fluorenol and 9-fluorenol. Ultraviolet spectra of the material eluted from the respective paper strips were identical with those of the authentic compounds.

Ether-Extractable Urinary Radioactivity of the Rat, Guinea Pig, and Rabbit.—For each species, relatively small amounts of the radioactivity were transferable to the organic phase from the urine buffered at *p*H 6 (Table II). Thus, the bulk of a dose was excreted as water-soluble metabolites. After treatment of the urine with β-glucuronidase, a substantial fraction of the isotope was ether-extractable in all cases. This presumably depicts compounds present as glucosiduronic acids. In the 0-to-12-hour sample, 85% of the carbon-14 in guinea pig urine was due to such conjugates, while they amounted to 76 and 46% in rabbit and rat urine, respectively. This type of metabolite appeared in

TABLE II
PERCENTAGE OF URINARY C^{14} ETHER EXTRACTABLE AFTER INTRAPERITONEAL INJECTION OF FLUORENE-9-C^{14}

	0–12 hr.			12–48 hr.		
	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit
Free[a]	3.8	1.0	1.5	2.3	1.0	2.9
Glucuronide[b]	46	85	76	40	68	61
Sulfate[c]	11	3.8	3.9	12	12	5.9

[a] The urine was buffered at *p*H 6 and extracted five times with freshly distilled ether. [b] Aqueous phase from *a*, after removal of ether, was incubated with bacterial β-glucuronidase at 37° for 18 hours and extracted 5 times with ether. [c] Aqueous phase from *b*, incubated with Taka-Diastase and extracted five times with ether.

TABLE III
CHROMATOGRAPHY OF ETHER-EXTRACTABLE METABOLITES[a]

	R_F values × 100[b]								
	Free[c]			Glucuronide[d]			Sulfates[e]		
Compound	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit
9-Fluorenol	40–66 (27)	39–67 (1)	37–65 (4)	37–63 (79)	39–67 (21)	36–60 (31)	40–68 (2)	38–61 (7)	36–63 (6)
2-Fluorenol	9–29 (4)	10–30 (1)	11–29 (19)	9–26 (5)	10–28 (32)	11–29 (39)	11.5–29 (35)	11–30 (45)	9–32 (55)
Unknown	2–5 (6)	2–4 (5)	—	2–4.5 (2.3)	2–5 (5)	—	2–5 (10)	2.5–5 (10)	—
2,9-Difluorenol and unknown	0–2 (51)	0–2 (88)	0–2 (72)	0–2 (13)	0–2 (29)	0–2 (17)	0–2 (47)	0–2 (33)	0–2 (22)

[a] Chromatograms were run on Whatman No. 1 paper in cyclohexane–water–acetic acid (5:4:2) and the developed chromatograms exposed to Kodak Royal Blue x-ray film. [b] Range (front to back of spot); R_F values of authentic compounds: 2,9-difluorenol, 0–2; 2-fluorenol, 10–30; 9-fluorenol, 38–65; 4-fluorenol, 34–52. Figures in parentheses are percentages of total radioactivity per strip. [c] Ether extract of urine buffered at *p*H 6. [d] Ether extract of aqueous phase of *d* after incubation with β-glucuronidase. [e] Ether extract of aqueous phase of *e* after incubation with Taka-Diastase.

somewhat lower amounts in the 12-to-48-hour period, particularly in rabbit and guinea pig urine.

The sulfuric acid conjugates of fluorene, measured by the amount of radioactivity which was ether extractable after hydrolysis by Taka-Diastase, formed a larger fraction of rat urine than of guinea pig or rabbit urine in the first 12 hours. During the 12-to-48-hour period, the proportion of these conjugates increased substantially in guinea pig urine, although it showed but slight changes in rat urine. An elevation was observed also in the rabbit. It must be noted that other studies from this laboratory demonstrated incomplete hydrolysis of certain sulfates by Taka-Diastase (Weisburger *et al.*, 1961). Thus, the values reported may be somewhat low. Figure 4 gives the separation of the urinary metabolites into the free, sulfate, and glucuronide fractions by the alumina column technique. These data confirm those obtained by specific enzyme procedures (Table II). The rat excreted more free and sulfuric acid conjugates than the other species. The guinea pig showed most conjugation with glucuronic acid, the rat showed least, and the rabbit was intermediate.

Identification and Quantitative Determination of Metabolites.

A. PAPER CHROMATOGRAPHY.—Chromatography of the ether-extractable metabolites of fluorene-9-C^{14} in the cyclohexane solvent gave four distinct radioactive spots with rat and guinea pig urine and three with rabbit urine (Table III). Two of the components have been identified as 2-fluorenol (R_F 0.09–0.30) and 9-fluorenol (R_F 0.38–0.68) by comparison with the authentic compounds and ultraviolet spectroscopy of the eluates. The unknown (R_F 0.02–0.05) present in the rat and guinea pig urine seemingly is missing in the rabbit urine.

The spot with a mobility of 0.0–0.02 (cyclohexane system) was shown to consist of 2,9-difluorenol and unknown material(s) by chromatography in benzene–acetic acid–water (10:3:7), as seen in Table IV. In the benzene system the 2- and 9-fluorenol move as a single spot with R_F 0.88–0.92. The unknown with a mobility of 0.35–0.75, although a very broad spot, may be the same unknown appearing with R_F 0.02–0.05 in the cyclohexane system. The ultraviolet spectrum of the ethanol eluate of the spot with R_F 0.14–0.27 was in agreement with that of the ethanol eluate of the reference compound, 2,9-difluorenol.

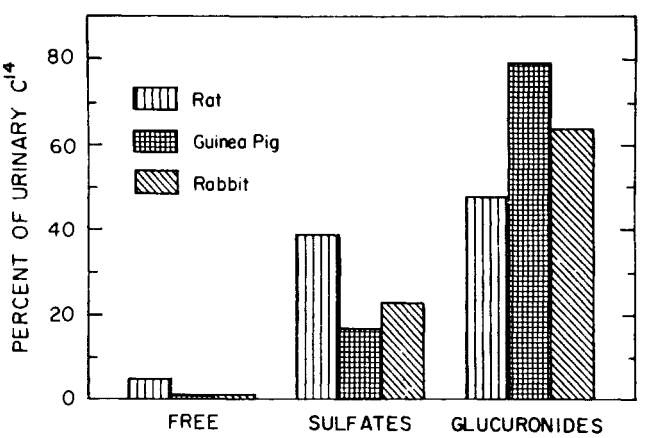

FIG. 4.—Separation of urinary metabolites of the rat, guinea pig, and rabbit into free, sulfate, and glucuronide fractions on alumina column. 0–48 hour urine used.

The R_F values of the aqueous metabolites in *sec*-butanol–3% NH_4OH (3:1) are given in Table V. The chromatograms of rat urine exhibited four major spots, with R_F values of 0.51–0.58, 0.73–0.79, 0.83–0.88, and 0.93–0.96. These spots represent 9-fluorenylglucosiduronic acid (0.51–0.58), 2-fluorenyl sulfate (0.83–0.88), and free metabolites (0.93–0.96). Spot 0.73–0.79 is the sulfate ester of 2,9-difluorenol, conjugation probably being on the 2-hydroxy group.

TABLE IV

CHROMATOGRAPHY OF ETHER-EXTRACTABLE METABOLITES[a] IN BENZENE–ACETIC ACID–WATER (10:3:7)

Identity	R_F Values × 100[b] Metabolites	Reference Compounds
Unknown	0–2 (11)	
2,9-Difluorenol	14–27 (16)	15–26
Unknown	35–75 (9)	
2-Fluorenol, 9-Fluorenol	88–92 (63)	87–94

[a] From β-glucuronidase-hydrolyzed guinea pig urine. [b] Range (front to back of spot). Figures in parentheses are percentages of total radioactivity per strip.

TABLE V

CHROMATOGRAPHY OF URINARY METABOLITES[a] OF FLUORENE-9-C^{14}

Metabolites were identified as follows: the spots were eluted with water and the eluates treated with β-glucuronidase or acid (0.2 N HCl). The reaction mixtures were ether extracted and the extracts chromatographed in the cyclohexane solvent system. The R_F values were computed and compared with those of authentic reference compounds. Ultraviolet spectra of ethanol eluates were also determined.

Spot No.	R_F Values[b] × 100 Rat	Guinea Pig	Rabbit	Identity
1	0–11	0–15	0–15	
2	24–27	—	—	
3	29–34	28–33	—	
4	—	*36–41*	37–40	
5	—	—	43–45	
6	47–49	—	—	
7	*51–58*	*54–57*	*52–56*	9-Fluorenylglucosiduronic acid
8	59–65	*58–63*	*57–62*	2-Fluorenylglucosiduronic acid
9	68–71	66–70	68–71	
10	*73–79*	—	73–75	9-Hydroxy-2-fluorenyl sulfate
11	—	77–81	77–81	
12	*83–88*	*86–91*	*84–87*	2-Fluorenyl sulfate
13	*93–96*	—	*92–96*	Free compounds

[a] Chromatogram was run on Whatman No. 1 paper in *sec*-butanol–3% ammonium hydroxide (3:1, v/v). The developed chromatogram was exposed to Kodak Royal Blue x-ray film. [b] Range (front to back of spot). Italics indicate major spots.

Four major spots were also observed from chromatography of guinea pig urine. Three spots were glucuronides: R_F 0.36–0.41, unknown (perhaps 2,9-difluorenol-glucosiduronic acid) and absent in rat urine; 0.54–0.57, 9-fluorenylglucosiduronic acid; and 0.58–0.63, 2-fluorenylglucosiduronic acid, which showed only weakly in rat urine. The fourth spot, R_F 0.86–0.91, was 2-fluorenyl sulfate. Rabbit urine gave a similar picture with four major spots: R_F 0.51–0.58 and 0.59–0.65, 9- and 2-fluorenylglucosiduronic acids, respectively, and 0.84–0.87 and 0.92–0.96, corresponding to 2-fluorenyl sulfate and the free metabolites.

B. QUANTITATIVE DETERMINATIONS.—The quantitative determination of the metabolites of rat urine by the inverse isotope dilution technique is shown in Table VI. Fluorene was not detected in the urine under the conditions shown. Therefore, complete metabolic conversion apparently took place. 2-Fluorenol was present to the extent of 1.4% of the dose as free metabolites and 0.3% as glucuronide and 4% as sulfate conjugates. 9-Fluorenol accounted for 1% of the dose as free, 21% as glucuronide, and only trace amounts as sulfate conjugates. Evidence obtained by the same technique suggested the presence of 2,9-difluorenol as a metabolite to the extent of 6% as the free and conjugated forms. However, this value may be in error by as much as 50% owing to the difficulty in reaching constant actiivity.

Quantitative data for all species (Table VII) were also derived from paper chromatograms of ether-extractable metabolites classified as free metabolites, glucuronides, and sulfates by the alumina column technique. The values obtained for the rat by both methods are in good agreement.

The guinea pig urine showed values of 19 and 15% of the dose for glucuronides of 2-fluorenol and 9-fluorenol, respectively. Sulfuric acid conjugation accounted for 2.9 and 0.4% of the dose for 2- and 9-fluorenol, respectively. The free compounds were present only in trace amounts.

The metabolites of rabbit urine were similar to those of the guinea pig. Glucuronide acid conjugation with 2- and 9-fluorenol amounted to 10 and 8% of the dose, respectively, while sulfuric acid conjugation was 1% with 2-fluorenol and 0.1% (trace) with 9-fluorenol. Here again the free metabolites were present in only trace amounts.

DISCUSSION

The absence of radioactivity in the respired carbon dioxide in the rat is in agreement with the observations made previously by Weisburger *et al.* (1951), who demonstrated the inability of the rat to oxidize significant amounts of the 9-carbon atom of the fluorene nucleus of the acetylamino derivative to carbon dioxide.

Radioactivity was distributed in all organs examined (Table I). However, it is of interest that binding should have occurred to the soluble and insoluble proteins of the liver in view of the relationship established between protein binding of metabolites and carcinogenicity of certain chemicals (Weisburger *et al.*, 1958, 1959a). Perhaps some form of binding is a detoxication reaction, whereas another is more directly related to the carcinogenic process. The use of noncarcinogenic fluorene in comparison to its carcinogenic 2-acetylamino derivative may permit discrimination between these two biochemical processes.

The mode of administration of fluorene-9-C^{14}, intraperitoneal injection or oral intake, seemingly had little effect on the total amounts of radioactivity excreted into the urine and feces of rats. There was a difference, however, in the rate of excretion. Slow absorption from the peritoneal cavity no doubt controlled the rate after intraperitoneal injection and accounted for the appreciable amounts of radioactivity appearing in the urine and feces of rats as late as 144 hours after injection.

The polynuclear hydrocarbon, fluorene, was apparently metabolized completely by rats, guinea pigs, and rabbits, as evidenced by its absence from the urine.

The rat differs from the guinea pig and the rabbit in the hydroxylation and subsequent conjugation of fluorene (see Table VII). The principal site of hydroxylation by the rat appeared at the 9-position, which in turn was conjugated almost exclusively with glucuronic acid. Hydroxylation at the 2-position occurred to a lesser degree; and the 2-fluorenol conjugate was that of the sulfuric acid ester.

The guinea pig and rabbit excreted 2-fluorenol and 9-fluorenol in about equal amounts as the glucuronide. The 2-fluorenol was also conjugated with sulfuric acid,

TABLE VI
URINARY METABOLITES OF FLUORENE-9-C^{14} IN THE RAT[a]

Compound	% of Dose[b]			
	Free	Glucuronide	Sulfate	Total
Fluorene	— (3000; 0; 0)	— (2000; 0; 0)	— (1440; 0; 0)	—
2-Fluorenol	1.4 (39,400; 29,100; 26,900; 28,700)	0.3 (27,800; 19,100; 18,200; 19,100)	4.0 (63,500; 43,100; 43,400; 42,500)	5.7
9-Fluorenol	1.1 (14,500; 8460; 8550; 8730)	21 (140,000; 112,000; 114,000; 113,000)	Trace (22,306; 455; 419; 460)	22
2,9-Difluorenol[c]	—	—	—	6 (8400; 5110; 5150)

[a] 0–48 hour urine was used. [b] Values were obtained by inverse isotope dilution experiments. Figures in parentheses are first and last two or three counts expressed in cpm/mmole. [c] A sample of urine after sequential hydrolysis by β-glucuronidase and 0.2 N HCl was used.

TABLE VII
URINARY METABOLITES OF FLUORENE-9-C^{14} IN VARIOUS SPECIES[a]

Compound	% of Dose[b]		
	Rat	Guinea Pig	Rabbit
2-Fluorenol	0.4	Trace	Trace
2-Fluorenyl sulfate	2.5	2.9	1.1
2-Fluorenylglucosiduronic acid	1.2	19	10
9-Fluorenol	1.0	Trace	Trace
9-Fluorenyl sulfate	0.1	0.4	0.1
9-Fluorenylglucosiduronic acid	19	15	8.3

[a] 0–48 hour urine was used. [b] The data were obtained by cutting the paper chromatograms of ether extracts (see text) according to the spot revealed by radioautography. The isotope content of spots was obtained after elution into ethanol by direct plating of aliquots. Values shown are products of the % of spot, % ether-extractable C^{14}, and % of urinary C^{14}.

while 9-fluorenol, as in the rat, was present only as the glucuronide.

The dihydroxylated derivative, 2,9-difluorenol, was shown to be a metabolite also. This metabolite appears to be present as both a glucuronide and a sulfate conjugate. Thus, fluorene was hydroxylated at the most reactive 9-position, as well as the secondary center of reactivity, the 2-position, by rats, guinea pigs, and rabbits. The data support the concept presented earlier (Weisburger *et al.*, 1958; *cf.* Williams, 1959) that biochemical hydroxylation of aromatic ring systems involves several distinct enzyme systems.

In addition to the unknown metabolites demonstrated by paper chromatography, an unidentified volatile ether-extractable substance has been noted after β-glucuronidase hydrolysis of urine. It was discovered when pronounced increases in background counts of the gas-flow counter were noted after the direct plates of these extracts were processed. The material may represent a dihydro-mono-ol conjugate. This type of compound has been shown to be a metabolite of other polynuclear hydrocarbons such as napthalene, anthracene, and phenanthrene (Williams, 1959). Another unknown metabolite with R_f 0.02–0.05 in the cyclohexane system may possess a dihydrodiol-type structure.

In this study the rabbit was shown to excrete both the 2- and the 9-fluorenol as glucuronides in about equal amounts. However, Neish (1948) reported isolation of what he thought was the glucuronide of 2-fluorenol. Dr. Neish generously supplied us with his compound, and we were able to prove by melting point, infrared spectrum, and color reaction that his compound and the glucuronide of 9-fluorenol isolated from rat urine were identical. The unsuccessful attempts by Neish to isolate 2-fluorenol after acid hydrolysis of his glucuronide further substantiate the view that his material was the 9-fluorenol conjugate, inasmuch as the 9-fluorenol liberated would not have been detected by the procedure used.

Dewhurst (1962) recently reported that fluorene was hydroxylated chiefly in the 2-position in the rabbit, and that in the rat 70–80% of the hydroxylation took place at the 4-position and 20–30% at the 2-position. Because of Dewhurst's findings, a portion of urine from rats which had received fluorene (1%) incorporated into the diet (ground Purina rat pellets) for a period of 7 days was treated with β-glucuronidase and ether extracted, and the solvent was removed. The residue was crystallized from dilute ethanol to give a solid, 6.8 mg, m.p. 148–152° with sintering at 137°, with the ultraviolet spectrum of 9-fluorenol. The mother liquor was taken to dryness and the residue in ether chromatographed on a 2 × 15 cm alumina column by Dewhurst's procedure.

Collection of ten 50-ml fractions gave fractions 2 and 3, with the spectrum of 9-fluorenol (24 mg on the basis of the molar absorbancy); fractions 4 and 5, representing mixtures of 9- and 4-fluorenol; fraction 6, containing 4-fluorenol (0.25 mg); and subsequent fractions, mixtures of 2- and 4-fluorenol (0.05 mg). Thus, the material isolated by crystallization and by chromatography was chiefly 9-fluorenol. The phenolic metabolites were present in much smaller amounts, confirming the isotope data in respect to the 2-fluorenol. Probably one of the *minor* metabolites on the chromatograms is the glucuronide of the 4-fluorenol. However, in view of the small relative amount of this material produced under our conditions this compound was not noted during chromatography of the metabolites after enzymic release from the conjugates (the reference 4-fluorenol showed R_f 34–52, cyclohexane system). Dewhurst's failure to find 9-fluorenol may have been due to his purification scheme, which involved partition between ether and sodium hydroxide solution.

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Functional Tyrosyl Residues in the Active Center of Bovine Pancreatic Carboxypeptidase A*

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Acetylimidazole, a new agent for the acetylation of proteins, when reacted with carboxypeptidase A increases esterase activity and abolishes peptidase activity. These changes can be prevented by β-phenylpropionate and can be reversed by deacetylation with hydroxylamine. The alterations in enzymatic activities correlate exactly with the acetylation and deacetylation of two tyrosyl residues and are accompanied by spectral shifts which are attributable to the characteristic spectral properties of *O*-acetyltyrosine. Difference spectra and hydroxamate formation have been employed for a quantitative analysis of these changes.

The search for functionally active amino acid residues in the active center of carboxypeptidase A has recently received new direction by the results of chemical modifications of the enzyme. Acylation with monocarboxylic anhydrides, iodination, and photooxidation of the enzyme increase esterase and decrease peptidase activity (Riordan and Vallee, 1962; Vallee *et al.*, 1963). Extensions of these investigations using acetic anhydride (Riordan and Vallee, 1963; and in preparation), and iodine (Simpson and Vallee, 1963) have supported the view previously advanced (Vallee *et al.*, 1963), that tyrosyl or histidyl residues, or both, are involved in the catalytic mechanism of carboxypeptidase.

In the course of efforts to identify these groups, it has been found that acetylation of the enzyme with acetylimidazole also abolishes peptidase activity completely while esterase activity increases even more than with acetic anhydride. These alterations in specificity correlate closely to the acetylation of two tyrosyl residues, presumably located close to the catalytically active zinc binding site (Vallee *et al.*, 1960). The results with acetylimidazole show promise that this mild acetylating agent may prove more selective than monocarboxylic acid anhydrides. Acetylimidazole does not seem to have been employed previously as an agent for the acetylation of proteins.

Experimental

Materials.—Five times recrystallized carboxypeptidase A of bovine pancreas, [(CPD)Zn], was prepared by the method of Allan *et al.*[1] Enzyme prepared by the method of Anson (1937) was obtained from the Worthington Biochemical Corp., Freehold, New Jersey. The zinc-to-protein ratio of both preparations was between 0.98 and 1.03 g atoms/mole based on a molecular weight of 34,300 (Smith and Stockell, 1954; Vallee and Neurath, 1955; Brown *et al.*, 1961). *N*-acetylimidazole was prepared by the method of Boyer (1952), and when recrystallized from isopropenyl acetate had a melting range of 100–101°. *N*-acetyltyrosine and *N,O*-diacetyltyrosine were obtained from the Cyclo Chemical Corp., Los Angeles, and used without further purification. Precautions to prevent contamination by adventitious metal ions were taken throughout these studies (Thiers, 1957; Coleman and Vallee, 1960).

Methods.—*Peptidase activity* was determined using the synthetic substrate carbobenzoxyglycyl-L-phenylalanine (Mann Research Laboratories) (Coleman and Vallee, 1960). Activity is expressed as an apparent proteolytic coefficient, *C*, defined as $\log a_0/a$ per minute per μmole of enzyme, where a_0 and a represent the concentration of substrate at time zero and time t, respectively. The assays were carried out at 0° in 0.02 M sodium Veronal—1.0 M NaCl buffer, *p*H 7.5. *C* was calculated from the linear portion of the first-order reaction plots before hydrolysis exceeded 15%. *Esterase activity* was determined by *p*H titration (Snoke, *et al.*, 1948) with 0.1 M NaOH of the hydrogen ions released on hydrolysis using a *p*H-stat (Radiometer, Copenhagen) and recorder (Ole Dich, Copenhagen). Assays were performed at 25° with 5 ml of 0.01 M

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[1] B. J. Allan, P. J. Keller, K. A. Walsh and H. Neurath, in preparation.