Effect of 2,4-D on Incorporation of ^{14}C into Urinary Metabolites from Rats Receiving ^{14}C-Labeled Acetate

The small amount of radioactivity recovered in the rat urine from oral-administered ^{14}C-labeled acetate consisted of several different metabolites, one of which was identified as urea. The relative abundance of labeled metabolites was dependent upon the sex of the rat and ^{14}C-labeled acetate. A single oral dose of 2,4-D at 100 mg. per rat altered the pattern of radioactive metabolites found in the urine. In females, the effect of 2,4-D was greater on the metabolism of the methyl carbon than on the carboxyl carbon of acetate.

The effect of 2,4-D on the elimination pattern of respiratory $^{14}CO_2$ from labeled acetate has been reported (Philleo and Fang, 1967). This paper reports a study on the labeling patterns of urinary metabolites from control and 2,4-D–treated rats receiving labeled acetate.

EXPERIMENTAL

Adult, white rats of an inbred Oregon State Wistar strain, 5 to 9 months old, were used. The weights of the females ranged from 250 to 280 grams, while the males weighed 350 to 400 grams.

Both 2,4-D (100 mg. per rat) and acetate-^{14}C were orally administered to the rats by means of a stomach tube as described by Philleo and Fang (1967). After the acetate-^{14}C was administered, the rat was placed in a Delmar metabolism cage with an excess of food and water. For the first 12 hours, a urine sample was collected and centrifuged at low speed to remove particles of food. The urine obtained was chromatographed either one-dimensionally or two-dimensionally on Whatman No. 1 chromatography paper. The chromatograms were developed in BAW (1-butanol–acetic acid–water, 12:3:5) or EAW (95% ethanol–ammonium hydroxide–water, 18:1:1). For the two-dimensional chromatograms, the BAW solvent was used first, followed by the EAW solvent.

Radioautograms of the two-dimensional chromatograms were prepared. A Photovolt electric densitometer was used to estimate the relative percentage of the various radioactive metabolites in the urine from the radioautograms. Ten readings were taken for each spot on the radioautogram, and the values were averaged. The outline of each radioactive spot was traced on a paper and cut out, and the paper weighed on an analytical balance to determine the area of the spot. The relative amount of radioactivity in each spot was calculated by multiplying the average density of each spot by its area and comparing with the sum of all spots. This method gave results within 5% error.

Urine samples also were analyzed for radioactivity in the proteins, ketone body, and volatile compounds.

In an attempt to identify or characterize the radioactive metabolites found in the urine, various colorimetric reactions were carried out on the paper chromatograms, after the chromatograms had been steamed in an autoclave for 30 minutes to remove any residual solvent that might interfere with the tests. An acid-base indicator and the Altman reagent were used for detecting organic acids; Ehrlich reagent for urea and compounds containing the indole moiety; aniline-diphenylamine reagent for sugars; copper acetate–dithio-oximide reagent for fatty acids and cholesterol; and ninhydrin for amines, amino acids, and proteins (Smith, 1960).

RESULTS AND DISCUSSION

In an attempt to determine if any urinary radioactivity was present in the form of volatile components, the urine was made acidic or basic and then steam-distilled. Neither the acidic nor the basic distillate of urine samples from control and 2,4-D–treated rats receiving acetate-1-^{14}C or acetate-2-^{14}C was radioactive.

Trichloroacetic acid and heat treatment were used to precipitate the urinary proteins. There was very little precipitate and the radioactivity was about 0.03 to 0.04% of the total urine radioactivity. The radioactivity of proteins precipitated from 2,4-D–treated urine was very small and no different from those of control animals.

The method of Van Slyke (1917) was used to precipitate the acetone bodies in the urine. A known amount of acetone was added to the urine to ensure an accurate determination. No radioactivity was found from all samples. Acetone bodies are not found in normal healthy rats; however, MacKay *et al.* (1940) found an increase in acetone bodies in the urine of fasted rats given acetic acid in doses exceeding tracer amounts.

Table I summarizes the chromatographic separations o. urine samples from rats receiving ^{14}C-labeled acetate.

The R_f values and the percentage of the total urine radioactivity of each spot were averages from several chromatograms. Normal females given acetate-1-^{14}C showed four radioactive spots, plus the origin. Spots I and II constituted some 96% of the urinary radioactivity for normal adult females receiving acetate-1-^{14}C or -2-^{14}C. Normal adult male rats given acetate-1-^{14}C showed only two radioactive spots from the 12-hour urine. Spots I and II make up about 98.5% of the urine radioactivity. Spot I comprised a far greater percentage and spot II a smaller percentage of the total urine radioactivity for the males than for the females.

The results indicate that both C-1 and C-2 of acetate yield two similar major metabolites in normal rats; the amounts of the minor metabolites varied, depending upon the position of the ^{14}C label. The female rats had several minor metabolites which were not present in the urine of males. The major metabolites were present under all circumstances, though the relative percentages varied.

In addition to the R_f values (Table I), various colorimetric reactions were carried out in a further attempt to identify or characterize the radioactive urinary products.

Spot I gave a strong yellow color reaction with the Ehrlich reagent, and it chromatographed like unlabeled urea, in the two solvent systems. When unlabeled urea and spot I were cocrystallized, after one recrystallization a constant specific activity was obtained which was not altered by further recrystallizations. This strongly indicated that spot I was urea.

Many different colorimetric reactions were carried out on spot II. A weakly positive reaction was given with the acid-base indicator. This reaction was observed from chromatograms developed from either acidic or basic solvents. The Altman test for organic acids, especially those from the Kreb's citric acid cycle and phenolic acids, gave a very weak reaction only on chromatograms run in the EAW solvent. Spot II remains close to the origin in EAW, but moves some distance in BAW (Table I). Spot II gave negative colorimetric tests for sugars and fatty acids, and positive tests with Ehrlich and ninhydrin reagents.

A further attempt, made by repeated extracting of spot II in acidic or basic aqueous solution with various solvents (Table II), indicated that spot II is preferentially water-soluble, possibly because of some acidic character. It also may have aromatic characteristics, as indicated by solubility in acidic ethyl ether. In the EAW solvent, a weakly positive reaction was obtained with the Altman reagent, which is positive for phenolic acids.

Allantoin, an oxidative product of uric acid, chromatographed like spot II, and since allantoin was known to be present in rather large quantities in rat urine, it was suspected that spot II might be allantoin. However, when an attempt was made to cocrystallize spot II with allantoin, uric acid, creatine, or creatinine separately, the radioactivity was lost during the first recrystallization. Thus, spot II is not allantoin, uric acid, creatine, or creatinine and remains unidentified.

Very little work has been published on the urinary metabolites of acetate-^{14}C. Gordon (1963), in his work with citrate metabolism, found that citrate was labeled in the urine of rats treated with acetate-1-^{14}C. In the work presented here, the presence of citrate in the urine was detected by Altman reaction on the paper chromatogram; however, its location did not correspond to that of any radioactive spots.

Table II. Extraction of Radioactive Spot II from Aqueous Solution by Organic Solvents

Solvent	Volume Used, Ml.	pH	Radioactivity Extracted, C.P.M.
Ethyl ether	10	Acidic	600[a]
		Basic	0
tert-Amyl alcohol	2	Acidic	74
		Basic	294
Ethyl acetate	10	Acidic	0
		Basic	60
CCl_4	10	Acidic	0
		Basic	0

[a] Initial radioactivity in 2 ml. of aqueous solution before extraction with each solvent = 2490 c.p.m., extraction repeated twice with each solvent.

Table I. R_f Values and Relative ^{14}C Labeling of Urinary Metabolites from Control and 2,4-D–Treated Rats Receiving ^{14}C-Labeled Acetate

Spot No.	R_f Values BAW	R_f Values EAW	Females: Acetate-1-^{14}C, No 2,4-D	Females: Acetate-1-^{14}C, 2,4-D	Females: Acetate-2-^{14}C, No 2,4-D	Females: Acetate-2-^{14}C, 2,4-D	Males: Acetate-1-^{14}C, No 2,4-D	Males: Acetate-1-^{14}C, 2,4-D
I (urea)	0.56	0.51	74.1%	78.5%	72.1%	44.5%	91.5%	71.4%
II	0.28	0.01	21.8	14.0	23.3	30.8	7.1	23.1
III	0.86	0.73	...	2.2	2.0	24.3	...	...
IV	0.48	0.25	0.8	...	1.2	...	...	...
V	0.54	0.07	...	...	0.8	...	...	...
VI	0.47	0.03	...	2.9	...	...	...	3.9
VII	0.37	0.04	...	2.0	...	...	...	...
VIII	0.81	0.44	2.7	...	...	...	...	...
IX	0.00	0.00	0.6	0.4	0.6	0.4	1.4	1.6

Animals given oral dose of 100 mg. of 2,4-D 1 hour prior to administration of ^{14}C-labeled acetate (5×10^7 d.p.m./14.5 μmoles). Two animals were used in each group. Urine samples (0 to 12 hours) were chromatographed two dimensionally in 1-butanol–acetic acid–water, 12:3:5 and ethanol–ammonium hydroxide–water, 18:1:1. Radioactive spots were detected by radioautography and measured by a densitometer. Values were average of two or four chromatograms.

The urine from females given 2,4-D and acetate-1-^{14}C contained five radioactive spots, plus the origin, while the urine from female rats given 2,4-D and acetate-2-^{14}C contained only three spots, plus the origin. The data (Table I) indicate that some of the radioactive spots present in the 2,4-D–treated rats were not present in the controls. Also, some of the radioactive spots present in the controls were absent from the urine of 2,4-D–treated rats.

Besides comparing the fluctuation of the minor metabolites, it is also of special interest to compare the 2,4-D–treated rats with the controls in other respects. In 2,4-D–treated female rats given acetate-1-^{14}C, the percentage of labeling in the urine actually increased over that of the controls (Philleo and Fang, 1967). This increase of some 4.5% is thought to be significant, because the values were taken from work with several different rats and many experiments, and in every case this slight increase was noted. Spot II decreased by about 30% under the influence of 2,4-D. Spots III, VI, and VII appeared under 2,4-D stress, while spot IV of the control was lost.

Female rats given 2,4-D and acetate-2-^{14}C also differed from the controls. Urea decreased almost 40% in the 2,4-D–treated rats, and spot II increased by over 30%. The greatest increase for any single spot was noted for III, which increased 12 times over that found in the control. Spots IV and V of the control were not found in the 2,4-D–treated rats.

In comparing the urinary metabolites of acetate-1-^{14}C and -2-^{14}C, the two carbons of acetate were metabolized in part by the same pathways and in part by separate pathways. In the acetate-1-^{14}C and -2-^{14}C controls, urea constituted approximately the same percentage of the radioactivity found in the urine. This was also true for spot II. In acetate-1-^{14}C–fed rats under the stress of 2,4-D, the percentage of total urinary radioactivity found as urea increased, while the percentage of radioactivity as spot II decreased. Since urea has only one carbon, the evidence indicated that the two carbons of acetate were split, possibly involving a decarboxylation reaction, and that the presence of 2,4-D had a greater effect of the further metabolism on the methyl carbon than on the carboxyl carbon. Of special interest is the observation that 2,4-D increased the amount of spot III, which was entirely absent from the acetate-1-^{14}C control. However, under the influence of 2,4-D, it was present, even though only as a minor metabolite. Spot III was present in the acetate-2-^{14}C control as a minor metabolite, and under the influence of 2,4-D it increased enough to be considered a major metabolite, along with urea and spot II.

In the male rats, urea decreased under the influence of 2,4-D. Spot II increased about three times. Spot VI, present with the 2,4-D–treated acetate-1-^{14}C females, was again present under the same conditions with the males. Spot III, which showed such interesting results with the females, was not found in the male urine.

CONCLUSIONS

Clearly, these data showed that acetate was metabolized differently by the two sexes, and that the carbons of acetate did not follow identical pathways in their metabolism. The oral-administered 2,4-D greatly affected the urinary metabolites of acetate-^{14}C.

As with the control animals, the only identified urinary metabolite of acetate was urea. Spot II for the controls showed the same colorimetric characteristics as spot II for the 2,4-D–treated animals. Other than the determination of the R_f values listed in Table I, there has been no further characterization of the metabolites.

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