ethanol filtrate was treated with 2,4-dinitrophenylhydrazine and a hydrazone was obtained. It was recrystallized from acetone and had m.p. 306–307° dec. Reported for benzyl methyl ketone 2,4-dinitrophenylhydrazone, m.p. 156°.[17]

(c) **In Aqueous Buffered Solution.**—A solution of 2.0 g. (0.012 mole) of amphetamine hydrochloride in 500 ml. of 0.0625 *M* potassium dihydrogen phosphate was adjusted to pH 7.0 by the addition of potassium hydroxide solution, 1.0 g. (0.0048 mole) of dehydroascorbic acid–methanol complex in 20 ml. of buffer was added, and the pH was again brought up to 7.0. The solution was then kept at room temperature for 36 hr., during which time it turned dark amber. Then 25 ml. of concd. sulfuric acid was added and the solution was distilled into a saturated aqueous hydrochloric acid solution of 2,4-dinitrophenylhydrazine. No hydrazone derivative could be isolated.

(17) S. M. McElvain, "The Characterization of Organic Compounds," The Macmillan Co., New York, N. Y., 1953, p. 256.

Acetylhydrazine as an Intermediate in the Metabolism of Aroylhydrazines[1,2]

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The oral administration of benzhydrazide or 1-acetyl-2-benzoylhydrazine leads in the rabbit to the urinary excretion of 1,2-diacetylhydrazine. 1-Acetyl-C^{14}-2-benzoylhydrazine, prepared from benzhydrazide and acetic anhydride-1-C^{14}, in the rat similarly led to the urinary excretion of 1,2-diacetylhydrazine, which had a specific activity of approximately 80% of the administered compound. 1,2-Diacetylhydrazine-C^{14} itself was converted only slightly (0.1–0.3%) to respiratory carbon dioxide. Acetylhydrazine in the dog was converted in part to hydrazine. These data are consistent with previous experiments on isoniazid and its metabolite acetylisoniazid. They suggest that one of the routes for the metabolism of certain aroylhydrazines in man, the rabbit, and the rat is: aroylhydrazine → 1-acetyl-2-aroylhydrazine → acetylhydrazine → 1,2-diacetylhydrazine.

Previous studies[3,4] were designed to test a hypothetical route[5] in which the metabolism of isonicotinic acid hydrazide leads to the

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(2) Aided by a research grant (RG-5337) from the National Institutes of Health, U. S. Public Health Service.

(3) A. S. Yard and H. McKennis, Jr., *Federation Proc.*, **17**, 421 (1958).

(4) A. S. Yard and H. McKennis, Jr., *J. Med. Pharm. Chem.*, **5**, 196 (1962).

(5) H. McKennis, Jr., A. S. Yard, J. H. Weatherby, and J. A. Hagy, *J. Pharmacol. Exptl. Therap.*, **126**, 109 (1959).

formation of 1,2-diacetylhydrazine by way of the intermediates 1-acetyl-2-isonicotinylhydrazine[6] and acetylhydrazine. This led to the demonstration that the anesthetized dog after administration of acetylisoniazid excreted acetylhydrazine in the urine. It was shown[5] also in the dog, by means of the iodate reaction,[7] that after the administration of acetylhydrazine or hydrazine under comparable conditions the urine was devoid or essentially devoid of 1,2-diacetylhydrazine. Following a single oral dose of 1-acetyl-2-isonicotinylhydrazine to the human, 1,2-diacetylhydrazine was isolated from the urine.

The foregoing and related experimental data support the view that acetylhydrazine participates in some species as an intermediate in the metabolism of isoniazid and tend to support the general equations for the metabolism of aroylhydrazines: $RCONHNH_2 \rightarrow RCONHNHCOCH_3 \rightarrow CH_3CONHNH_2 \rightarrow CH_3CONHNHCOCH_3$.

The present study provides new data on the metabolism of benzhydrazide, acetylbenzhydrazide (1-acetyl-2-benzoylhydrazine), and acetylhydrazine in the dog, rat, and rabbit. It was of particular interest to study benzhydrazide as an analog of isoniazid since the metabolism of benzhydrazide is uncomplicated by the possibilities of amine oxide and of quaternary ammonium compound formation, which may contribute significantly to the metabolism of isoniazid by way of methyl acceptance at the pyridine nitrogen[8] or by way of phosphonucleotide formation.[9,10] An inquiry into aspects of the metabolism of benzhydrazide also assumed particular importance since El Masri, Smith, and Williams[11] concluded from their studies in the rabbit that the major route in the metabolism of benzhydrazide, *p*-chlorobenzhydrazide and *p*-methylbenzhydrazide could be represented by $RC_6H_4CONHNH_2 \rightarrow NH_2NH_2 + RC_6H_4CO_2H \rightarrow RC_6H_4CONHCH_2CO_2H$, where R = H, Cl or CH_3. Following administration of benzhydrazide to the rabbit, these investigators found hydrazine (isolated by conversion to benzalazine) in the urine to the extent of 5% of the administered dose.

In our present series with New Zealand White female rabbits, intraperitoneal administration of benzhydrazide (102 mg./kg.) gave rise to urinary excretion of 1,2-diacylhydrazine as indicated by the

(6) H. B. Hughes, *ibid.*, **109**, 444 (1953).
(7) H. McKennis, Jr., J. H. Weatherby, and E. P. Dellis, *Anal. Chem.*, **30**, 499 (1958).
(8) H. McKennis, Jr., A. S. Yard, and E. V. Pahnelas, *Am. Rev. Tuberc. Pulmonary Diseases*, **73**, 956 (1956).
(9) L. J. Zatman, N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, *J. Am. Chem. Soc.*, **75**, 3293 (1953).
(10) D. S. Goldman, *ibid.*, **76**, 2841 (1954).
(11) A. M. El Masri, J. N. Smith, and R. T. Williams, *Biochem. J.*, **68**, 587 (1958).

TABLE I

METABOLISM OF 1-ACETYL-C^{14}-2-BENZOYLHYDRAZINE IN THE MALE ALBINO RAT[a]

	Rat No. 1[b], 102 g.				Rat No. 2, 135 g.				Rat No. 3, 145 g.			
	Per cent of radioactivity recovered											
Day	Urine	Respiratory CO_2	Feces	Total	Urine	Respiratory CO_2	Feces	Total	Urine	Respiratory CO_2	Feces	Total
1	44.3	33.9	↓	↓	41.8	32.1	↓	↓	43.8	27.1	↓	↓
2	0.9	3.1	↓	↓	0.2	1.7	↓	↓	0.2	1.4	↓	↓
3	0.2	0.9	↓	↓	↓	1.0	↓	↓	↓	0.7	↓	↓
4	↓	0.5	↓	↓	↓	0.3	↓	↓	↓	0.4	↓	↓
5	0.1	0.3	↓	↓	0.3	0.3	↓	↓	0.3	0.2	↓	↓
Total	45.5	38.7	6.7	90.9	42.3	35.4	6.4	84.1	44.3	29.8	7.6	81.7

[a] All animals received 11.2 mg. of 1-acetyl-C^{14}-2-benzoylhydrazine orally in 2 ml. of water (7.64×10^5 c.p.m. at infinite thickness). [b] The urine from this animal was filtered through Celite and then concentrated to dryness. The residue was triturated with hot absolute alcohol. The ethanolic solution was diluted with water and was placed upon Dowex 50 (H^+) which retained 41% of the activity in the solution. The activity was removed by elution with 2 *M* ammonium hydroxide. Upon paper chromatography radioactivity was found at the origin and distributed unevenly throughout the chromatogram. The fraction comprising the eluate and water washings showed upon paper chromatography two radioactive zones, R_f 0.53 and R_f 0.79, corresponding to 1,2-diacetylhydrazine and 1-acetyl-2-benzoylhydrazine, respectively.

TABLE II

METABOLISM OF 1,2-DIACETYLHYDRAZINE-C^{14} IN THE MALE ALBINO RAT

	% Radioactivity recovered											
	0–24 hr.			25–48 hr.			49–72 hr.			73–96 hr.		
Animal[a] and weight	Urine	Feces	CO_2	Urine	Feces	CO_2	Urine	Feces	CO_2	Urine	Feces	CO_2	Total
Rat No. 4[b] (117 g.)	83.7	...	0.6	7.1	...	...	0.3	...	...	0.3	3.9	...	95.9
Rat No. 5 (180 g.)	88.4	...	0.7	1.4	...	0.6	0.1	6.4	...	...	...	...	97.6
Rat No. 6 (160 g.)	83.3	...	0.7	2.0	...	...	0.3	5.3	...	...	...	...	91.6

[a] All animals received a single oral dose of 1,2-diacetylhydrazine-C^{14} (8.73 mg., 6.93×10^5 c.p.m.). [b] The 24-hr. urine of this animal was filtered and concentrated to dryness under diminished pressure. The residue was triturated with hot alcohol. An aliquot of the filtrate showed upon paper chromatography only one radioactive zone, corresponding in R_f value to authentic 1,2-diacetylhydrazine. [c] The determinations on the feces in all cases represent cumulative values.

iodate reaction. The high incidence of death at this dose level[12] prompted the employment of the lower, more satisfactory, and non-convulsant dose of 80 mg./kg. for most of our chemical studies. From the pooled urine of 8 rabbits which had received a total of 1.09 g. of benzhydrazide, 1,2-diacetylhydrazine (55 mg.), m.p. 139°, was obtained by chromatography and fractional crystallization. This yield, corrected for losses in the procedure, accounted for 12% of the administered dose. Under conditions similar to the foregoing the urine of 4 rabbits that had received 1-acetyl-2-benzoylhydrazine yielded 17.8 molar per cent of the administered dose in the form of crystalline 1,2-diacetylhydrazine. This demonstration that administration of either 1-acetyl-2-benzoylhydrazine or benzoylhydrazine leads to the formation of 1,2-diacetylhydrazine is analogous to that of our previous studies which showed that both isonicotinyl hydrazide and 1-acetyl-2-isonicotinylhydrazine are metabolized to 1,2-diacetylhydrazine.

In order to obtain further confirmation for the general metabolic scheme in which acetylhydrazine and 1-acetyl-2-aroylhydrazines are intermediates in the metabolism of aroylhydrazines, 1-acetyl-C^{14}-benzoylhydrazine was prepared by reaction of benzoylhydrazine and acetic anhydride-1-C^{14} in pyridine. The radioactive 1-acetyl-2-benzoylhydrazine (total dose of 135 mg.) was given to three male albino rats. The pooled urine from these animals at 24 hours was processed by methods similar to those previously employed,[5] and 1,2-diacetylhydrazine-C^{14} with a specific activity corresponding to 81% of the parent compound was obtained.

In the present studies, it was also shown (Table I) that C^{14} of the acetyl group in labeled 1-acetyl-2-benzoylhydrazine is metabolized to $C^{14}O_2$. From this and previous studies it appears that the major metabolism of the labeled carbon of 1-acetyl-C^{14}-2-benzoylhydrazine to $C^{14}O_2$ occurs prior to the formation of 1,2-diacetylhydrazine. The studies in which acetylhydrazine, but not 1,2-diacetylhydrazine, was shown[5] to produce fatty livers in rabbits in a manner comparable to hydrazine serve also to suggest a relative stability of 1,2-diacetylhydrazine to hydrolysis *in vivo*. Such stability was again indicated in the present investigation by studies on the metabolism of 1,2-diacetylhydrazine-C^{14} to $C^{14}O_2$ in the rat.

1,2-Diacetylhydrazine-C^{14} was conveniently synthesized by treating hydrazine with acetic anhydride-1-C^{14} in pyridine. The radioactivity of 1,2-diacetylhydrazine-C^{14} following oral administration to the rat was excreted in the urine to the extent of 85–90% during a

(12) A. S. Yard and H. McKennis, Jr., *J. Pharmacol. Exptl. Therap.*, **114**, 391 (1955).

48-hour period (Table II). The combined recovery of radioactivity from urine, feces, and expired air averaged 95.0% of the dose. Only 0.6-1.3% of the administered radioactivity was found in the form of respiratory carbon dioxide. The excretion of radioactivity as carbon dioxide indicated that in the rat hydrolysis and resynthesis of 1,2-diacetylhydrazine probably would not be sufficient to account for the observed metabolic dilution of the C^{14} label in the sequence going from 1-acetyl-2-benzoylhydrazine to 1,2-diacetylhydrazine.

Of the two remaining metabolic steps which would make acetate available for oxidation to carbon dioxide, hydrolysis of acetylbenzhydrazide to benzhydrazide and acetate and hydrolysis of acetylhydrazine to hydrazine and acetate, only the latter was investigated in the dog, taking advantage of its inability to acetylate acetylhydrazine and excrete 1,2-diacetylhydrazine therefrom.[5] A male mongrel dog under pentobarbital anesthesia received an intravenous injection of acetylhydrazine fumarate. The combined urine at 2 hours after administration of the compound contained hydrazine which was isolated as benzalazine upon addition of benzaldehyde.

All of the foregoing data are consistent with the concept that aroylhydrazines can be metabolized in certain species, *viz.*, man, rat, and rabbit, by way of a metabolic route involving the intermediates 1-aroyl-2-acetylhydrazine, acetylhydrazine, and finally the relatively stable 1,2-diacetylhydrazine. The hydrolysis of acetylhydrazine, as indicated in experiments in the dog, suggests acetylhydrazine may be in part the immediate precursor of the hydrazine which has been observed by some investigators in studies on the metabolism of aroylhydrazines.

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Experimental

Paper Chromatograms.—All paper chromatograms were prepared by the descending method at ambient temperature using Whatman No. 1 paper. The solvent system was ammonia–ethanol–butanol[5] and acidic *p*-dimethylaminobenzaldehyde was used[5] to disclose the hydrazine compounds.

Metabolism of 1-Acetyl-2-benzoylhydrazine by the Rabbit.—The urine of 4 New Zealand White female rabbits (1.25–1.70 kg.) that had received a total of 774 mg. of 1-acetyl-2-benzoylhydrazine (133.6 mg./kg.) intraperitoneally was collected up to 30 hr. after administration of the compound. After filtration, the pooled urine was passed through Dowex 50 (H^+) and Dowex 3 (OH^-) as previously described.[5] The combined effluent and water wash was treated with decolorizing carbon and then concentrated almost to dryness under diminished pressure. A solution of the residue in 20 ml. of ethanol was placed on six sheets of Whatman

No. 1 paper (18 1/4″ × 22 1/2″) and chromatographed. The areas corresponding[5] in R_f value to 1,2-diacetylhydrazine were extracted with ethanol. The residue from evaporation of the solvent was recrystallized from methyl ethyl ketone-hexane to give 90 mg. (17.8%) of 1,2-diacetylhydrazine, m.p. 139°, corresponding in R_f value and melting point to an authentic sample. Admixture of the authentic and metabolic compounds caused no depression in melting point.

Metabolism of Benzhydrazide to 1,2-Diacetylhydrazine by the Rabbit.—The urine from 8 New Zealand White female rabbits (1.20–1.75 kg.) that had received a total of 1.09 g. of benzhydrazide intraperitoneally was collected up to 30 hr. after administration of the compound. By the procedure employed for the isolation of 1,2-diacetylhydrazine after administration of 1-acetyl-2-benzoylhydrazine (above) 21.4 mg. (5.9%) of 1,2-diacetylhydrazine, m.p. 139°, was obtained. The identity of the compound was confirmed as described above.

Hydrazine from Acetylhydrazine in the Dog.—A male mongrel dog (10.5 kg.) under pentobarbital anesthesia was given 2.96 g. of acetylhydrazine fumarate[5] *via* the femoral vein. The urine (83 ml.) was collected during the subsequent 2-hr. period by means of an indwelling bladder catheter. An aliquot of the urine (50 ml.) was shaken with 0.15 ml. of freshly distilled benzaldehyde. Benzalazine precipitated slowly and was collected by filtration. The product (10.2 mg.) was washed with hot water and dried *in vacuo*, micro m.p. 93°. Upon admixture with an authentic sample of m.p. 93°, there was no depression of the melting point.

The water wash from the foregoing benzalazine yielded 5.4 mg. of the acetylhydrazone of benzaldehyde upon cooling in the ice-bath, micro m.p. 139–139.5°. A further quantity of the acetylhydrazone of benzaldehyde (73.8 mg.) was obtained by continuously extracting the mother liquors from the azine (above) with chloroform. The residue from evaporation of the chloroform was extracted with hot water. The aqueous solution was filtered and cooled to give the crystalline product, micro m.p. 139–139.5°.

1-Acetyl-C^{14}-2-benzoylhydrazine.—A solution containing 30 mg. of benzhydrazide (Aldrich Chemical Co.) and 22 mg. of acetic-1-C^{14} anhydride (New England Nuclear Corp.) in 1 ml. of dry pyridine was allowed to stand at room temperature for 1 hr. The solution then was concentrated to dryness under diminished pressure. Non-isotopic 1-acetyl-2-benzoylhydrazine (30 mg.), prepared by a similar procedure, then was added to the residue, and the mixture dissolved in acetone–benzene. The product (58.5 mg.), m.p. 174.5–175.5°, was obtained in pure condition by several recrystallizations from acetone–benzene.

The melting point of 1-acetyl-2-benzoylhydrazine is usually reported[13,14] in the range of 170–171°. The standard non-isotopic material employed in our investigations recrystallized from alcohol–water or methanol–acetone to melt at 174.5–175.5° (unchanged by drying at 25° and 1 mm.).

Anal. Calcd. for $C_9H_{10}N_2O_2$: C, 60.66; H, 5.66; N, 15.72. Found: C, 60.68; H, 5.72; N, 15.92.

1,2-Diacetyl-C^{14}-hydrazine.—Acetic-1-C^{14} anhydride (53.7 mg., 1 mc.) was diluted with 133 mg. of non-isotopic acetic anhydride and then treated with 1 ml. of dry pyridine containing 29.4 mg. of 95% hydrazine hydrate. After standing overnight at room temperature, the mixture was concentrated to dryness under diminished pressure. The residue was recrystallized from acetone. The

(13) J. P. Horwitz and V. A. Grakauskas, *J. Org. Chem.*, **19**, 194 (1954).
(14) S. Takagi and A. Sugii, *Yakugaku Zasshi*, **79**, 103 (1959); *C. A.* **53**, 10086 (1959).

colorless crystals, m.p. 137.5–139°, were collected and washed with cold acetone. The yield of dried product was 58.3 mg. (70%).

Metabolism of C^{14}-Hydrazides.—Male albino Wistar-strain rats were housed in glass metabolism cages. All animals were allowed food and water *ad libitum*. Respiratory carbon dioxide was collected in bubble towers containing aqueous potassium hydroxide (10% w./w.). Radioactivity of aliquots was determined on infinitely thick samples of barium carbonate following addition of barium chloride. Feces were separated from urine by collection on a screen. Urine and feces were removed from the cages daily. Feces were dried overnight at 100° and pulverized to a uniform powder for counting at infinite thickness against standards prepared by adding 1-acetyl-C^{14}-2-benzoylhydrazine to control feces. Urine samples were dried on talc at 100° and similarly counted at infinite thickness. Tables I and II summarize the data which were obtained.

Isolation of 1,2-Diacetylhydrazine and 1-Acetyl-2-benzoylhydrazine from Urine.—Urine samples were filtered through Celite and then concentrated to dryness under diminished pressure. The residue was triturated with absolute ethanol. The ethanolic solution was filtered and concentrated to dryness under diminished pressure. The residue was dissolved in water and then placed upon a column of Dowex 50 (H^+). The effluent and water wash from the column were combined and placed upon a column of Dowex 21K (OH^-). The latter was eluted with 1 *M* acetic acid. The effluent and the acetic acid eluate were then combined and processed for metabolites.

The combined urines from rats (no. 2 and no. 3) obtained during the first 24-hr. period after administration of radioactive acetylbenzhydrazide were concentrated to dryness. The ethanol-soluble components of the residue were dissolved in water and placed upon Dowex 21K (OH^-). The column was eluted with 1 *M* acetic acid. The residue from evaporation of the solvent (99% of the radioactivity which had been placed on the column) was triturated with methanol. The methanol-soluble components were chromatographed on large sheets of Whatman No. 1 paper. The radioactive area, corresponding in R_f value to 1,2-diacetylhydrazine, which was located by radioautographs, was cut from the sheets and extracted with methanol in a Soxhlet extractor. The residue (18 mg.) from evaporation of the solvent was diluted with 62 mg. of non-isotopic 1,2-diacetylhydrazine and dissolved in acetone–benzene. The solution upon cooling and evaporation deposited 38.8 mg. of 1,2-diacetylhydrazine (3.47×10^4 c.p.m./mg. at infinite thinness). The melting point was unchanged on further recrystallization and the specific activity (4.10 and 3.84×10^4 c.p.m./mg.) was constant within experimental error. The final product weighed 20.6 mg.

The radioactive zone[5] in the paper chromatograms which corresponded in R_f value to 1-acetyl-2-benzoylhydrazine was located and then extracted with methanol as described above. An aqueous solution of the solids obtained from evaporation of the methanol was processed on Dowex 21K (OH^-) as described above. The acetic acid eluate was concentrated to dryness. Non-isotopic carrier (62 mg.) was added. The mixture yielded upon repeated recrystallization from acetone 1-acetyl-2-benzoylhydrazine, m.p. 172–173°, 36.8 mg., 3.06×10^4 c.p.m./mg. at infinite thinness. Further recrystallization to give 25.1 mg., 2.86×10^4 c.p.m. and finally 16.0 mg., 2.94×10^4 c.p.m. at infinite thinness, did not appreciably affect the specific activity.

Specific Activity of Urinary 1,2-Diacetylhydrazine Arising from an Oral Dose of 1-Acetyl-C^{14}-2-benzoylhydrazine.—Three male albino rats (393, 395, and 462 g.)

were given oral doses of acetylbenzhydrazide (45 mg., 5.43×10^7 c.p.m./mmole at infinite thinness). The pooled urine at 24 hr. was neutralized with sodium hydroxide and, after filtration through Celite, placed upon a column of Dowex 50 (H^+). The effluent and water wash which contained 14.2% of the administered radioactivity were concentrated to dryness under diminished pressure. The residue was triturated with hot absolute ethanol. An aqueous solution of the solids obtained from evaporation of the ethanol was placed on Dowex 21K (OH^-). The acetic acid eluate was evaporated to dryness and chromatographed on paper as in preceding experiments to give radioactive zones corresponding in R_f value to diacetylhydrazine and acetylbenzhydrazide. The diacetylhydrazine zone was extracted with methanol. An aqueous solution of the solids from evaporation of the methanol was reprocessed on Dowex 21K (OH^-). The residue from the acetic acid eluate of the column contained 12 mg. of impure diacetylhydrazine. Trituration with acetone served to remove a small amount of non-radioactive solids. By concentration of the solvent crystals of diacetylhydrazine were obtained. After two recrystallizations the product, m.p. 138–139°, weighed 2.0 mg. Aliquots in ethanol were plated at infinite thinness on aluminum planchets. The average specific activity for three samples was 4.42×10^7 c.p.m./mmole. The specific activity was virtually unchanged by two additional recrystallizations to give a final product (0.595 mg.), m.p. 138–139°, specific activity 4.23×10^7 c.p.m./mmole.

Determination of Metabolic 1,2-Diacetylhydrazine by Isotopic Dilution with 1,2-Diacetyl-C^{14}-hydrazine.—To the pooled 24-hr. urine from 3 male albino rats (385, 400, and 500 g.) which had each received orally 50 mg. of acetylbenzhydrazide in 3 ml. of water was added 7 mg. of diacetylhydrazine (4.57×10^6 c.p.m./mg.). A pure sample of diacetylhydrazine was isolated by the procedures described above. The specific activity of the sample, m.p. 138–139°, after recrystallization from acetone was 1.84×10^6 c.p.m./mg., representing an excretion of 17.2 mg. of diacetylhydrazine equivalent on a molar basis to 13.2% of the administered acetylbenzhydrazide.