ready permeation of the central nervous system by certain compounds with low lipid solubility. It would seem important to determine in which cellular fraction, if any, the drug is fixed and the degree of stability of any drug-cellular substituent bonds.

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Aspects of the Metabolism of Isoniazid and Acetylisoniazid in the Human and the Dog[1,2]

ALLEN S. YARD AND HERBERT MCKENNIS, JR.

Department of Pharmacology, Medical College of Virginia, Richmond, Va.

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After administration of 1-acetyl-2-isonicotinylhydrazine, a known metabolite of isonicotinylhydrazine, an adult male excreted 1,2-diacetylhydrazine. In contrast, the dog, following administration of 1-acetyl-2-isonicotinylhydrazine, excreted acetylhydrazine. In view of the foregoing and previous data indicating the inability of the dog to form 1,2-diacetylhydrazine from hydrazine or acetylhydrazine, it is concluded that the metabolism of isoniazid in man and other species such as the rabbit involves the route: isonicotinylhydrazine → 1-acetyl-2-isonicotinylhydrazine → acetylhydrazine → 1,2-diacetylhydrazine.

The metabolism of isonicotinylhydrazine (isoniazid) to yield 1-acetyl-2-isonicotinylhydrazine (acetylisoniazid) has been recorded for

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various animal species[3,4] including man.[3,5] Hughes[3] reported that in one patient under treatment with isoniazid as much as 91% of the total urinary isoniazid-like components were present as acetylisoniazid. It was suggested that variations in the degree to which acetylation of isoniazid was accomplished might account for observed differences in the toxicity of isoniazid to humans.

Hughes, *et al.*,[6] in a later report in which the excretion pattern of seventeen patients who were receiving isoniazid was examined, concluded that there existed three different mechanisms for the "degradation" of isoniazid: acetylation of acetylisoniazid, cleavage to form isonicotinic acid or a closely related derivative, and unidentified processes leading to one or more metabolites.

Studies in this laboratory in the rabbit, which in many instances has acetylation processes similar to that of the human, have shown[7] that administered acetylhydrazine or its isopropylidene derivative is excreted in the urine partially as 1,2-diacetylhydrazine. In general it was observed that the excretion of 1,2-diacetylhydrazine was more effectively accomplished following administration of acetylhydrazine than after the administration of hydrazine. Wenzel[8] reported that acetylisoniazid is enzymatically cleaved by serum with the production of isonicotinic acid but not of isoniazid. After intraperitoneal administration of acetylisoniazid, the urine of rabbits was found by chromatography to contain acetylisoniazid and isonicotinic acid but no detectable isoniazid.[9] These observations and related findings led to the suggestion[7] that acetylisoniazid (I) which arises from the metabolism of isoniazid could be in turn degraded by a metabolic route with eventual formation of 1,2-diacetylhydrazine (II).

$$\underset{\text{I}}{\text{4-PyCONHNHAc}} \longrightarrow \text{4-PyCO}_2\text{H} + \underset{\text{III}}{\text{AcNHNH}_2} \longrightarrow \underset{\text{II}}{\text{AcNHNHAc}}$$

In the present study, the urine of both the dogs and the human has been examined after administration of acetylisoniazid. This has led to the isolation of the metabolite 1,2-diacetylhydrazine from the

(3) H. B. Hughes, *J. Pharmacol. Exptl. Therap.*, **109**, 444 (1953).
(4) A. DeFranceschi and V. Zamboni, *Biochim. et Biophys. Acta*, **13**, 304 (1954).
(5) V. Zamboni and A. DeFranceschi, *Arch. Fisiol.* (Naples), **10**, 184 (1955).
(6) H. B. Hughes, J. P. Biel, A. P. Jones, and L. H. Schmidt, *Am. Rev. Tuberc.*, **70**, 266 (1954).
(7) H. McKennis, Jr., A. S. Yard, J. H. Weatherby, and J. A. Hagy, *J. Pharmacol. Exptl. Therap.*, **126**, 109 (1959).
(8) M. Wenzel, *Naturwissenschaften*, **42**, 370 (1955).
(9) A. S. Yard and H. McKennis, Jr., *Federation Proc.*, **17**, 421 (1958).

urine of a male human subject and the isolation of the metabolite acetylhydrazine (III) from dog urine.

Examination of the urine of the human subject for hydrazino nitrogen (iodate method) following administration of acetylisoniazid revealed that in a 48-hour period subsequent to ingestion (Table I)

TABLE I

HYDRAZINO NITROGEN IN HUMAN URINE AFTER ORAL ADMINISTRATION OF ACETYLISONIAZID, 500 mg.

Urine collection	24 hr. (1760 ml.)	48 hr. (900 ml.)
Type II hydrazino nitrogen μl./ml.	22.0	9.5
Molar % of dose present as 1,2-diacylhydrazine	67.23	21.13
Diacetylhydrazine isolated, mg.	54.6	19.4
Molar % of dose isolated as 1,2-diacetylhydrazine	16.8	6.0

almost 90% of the dose was excreted as 1,2-diacylhydrazine. The 24-hour urine sample of the third day subsequent to ingestion showed also some evidence of 1,2-diacetylhydrazine. This urine was treated with decolorizing carbon and then placed upon Dowex 50 (H^+). The paper chromatograms of the eluates upon careful examination revealed a small amount of material giving a yellow-orange color with acidic *p*-dimethylaminobenzaldehyde at R_f 0.54. This chromatogen, which was not encountered in normal urine, co-chromatographed with authentic 1,2-diacetylhydrazine. The low intensity of color and the slowness of color development indicated that excretion on the third day was in the range of 3–4 μg. In consequence, only the urine of the first two days was employed for isolation of the metabolite 1,2-diacetylhydrazine.

Approximately 45% of the 1,2-diacylhydrazine (Type II hydrazino nitrogen) excreted in the first 48-hour period was retained by Dowex 50 (H^+). Authentic samples of acetylisoniazid added to control samples of urine were similarly retained by the resin. Subsequent treatment with dilute alkali served effectively to remove this compound. The fraction retained by the resin in the metabolic experiment was not, however, subjected to investigation, and material other than acetylisoniazid may be in this fraction. The effluents and water washes from the column of the metabolic experiments were examined by paper chromatography after passage through Dowex 3

(OH^-). The initial chromatograms, in some cases, showed upon treatment with acidic *p*-dimethylaminobenzaldehyde, an orange-yellow area covering approximately the first two-thirds of the paper. Extracts of this area yielded upon rechromatography a relatively homogeneous zone at R_f 0.54, corresponding to authentic 1,2-diacetylhydrazine. Extracts of the latter zones readily afforded crystalline 1,2-diacetylhydrazine which was identified by melting point, mixed melting point and analysis in the yields indicated in Table I. The total yield (74 mg.) represents on a molar basis approximately 25% of the ingested compound (approximately 50% when corrected for losses inherent in the procedures[7]).

After intravenous administration of isoniazid (100–150 mg./kg.) to the dog under pentobarbital anesthesia, the urine of the subsequent 4- to 5-hour period was analyzed for Type I and Type II hydrazino nitrogen by the iodate method. Only Type I hydrazino nitrogen ranging from approximately 10–35% of the administered dose was found in the series which included 11 male mongrel dogs. The urine of one animal was selected for study and following treatment with benzaldehyde yielded benzaldehyde isonicotinylhydrazone equivalent to 92.6% of the excreted hydrazino nitrogen. Identity of the hydrazone was confirmed by melting point, mixed melting point and analyses.

Examination of the urine of the anesthetized dog 3.5 hours after administration of acetylisoniazid gave clear evidence of Type I hydrazino nitrogen. Upon treatment with benzaldehyde this urine yielded the benzylidene derivative of acetylhydrazine which was extracted with chloroform. Identity of the compound (accounting for 48.2% of the excreted Type I hydrazino nitrogen) was confirmed by melting point, mixed melting point, and analyses. Excretion of hydrazino nitrogen in the urine was followed over a 6-hour period. The urine of this period contained a total of 8.3% of the administered dose as Type I hydrazino nitrogen. The single dose of acetylisoniazid (265 mg./kg.) employed appeared to be well tolerated under the experimental conditions. In contrast equimolar doses of isoniazid produced convulsions of such severity that the experiments at this dose level were voluntarily or involuntarily terminated. The urine of two dogs receiving acetylisoniazid (265 mg./kg.) showed during a 5-hour period Type I hydrazino nitrogen equivalent to 5.3 and 5.6% of the administered dose, while the values for Type II hydrazino ni-

trogen were 19.3 and 30.3%, respectively. Since previous studies have indicated the inability of the dog to excrete hydrazine or acetylhydrazine in the urine in a 1,2-diacylated form, it is presumed that the Type I hydrazino nitrogen in the urine represents acetylhydrazine (and possibly some hydrazine) while the 1,2-diacylhydrazine (Type II hydrazino nitrogen) represents 1-acetyl-2-isonicotinylhydrazine or a closely related derivative.

In numerous clinical studies it has been concluded that wide variations exist in the ability of the human to acetylate isoniazid. In addition great variations in urinary excretion of isoniazid have been reported.[10,11] The present studies in which acetylisoniazid was administered to man clearly implicate 1,2-diacetylhydrazine in the metabolism of isoniazid. As previous discussions[7] indicate, 1,2-diacetylhydrazine could arise from isoniazid by a variety of processes. These include the sequences in which either isoniazid or acetylhydrazine are hydrolyzed to hydrazine which in turn is acetylated to give 1,2-diacetylhydrazine by way of the reformation or neoformation of acetylhydrazine. Porcellati and Preziosi[12] have reported the metabolic formation of hydrazine from isoniazid. This would suggest that some species, such as the rabbit, would excrete as 1,2-diacetylhydrazine at least some of the hydrazine formed in this manner.

In the present study acetylisoniazid was metabolized by the dog largely to acetylhydrazine (isolated as the benzylidene derivative). The previously noted studies have indicated the inability of the dog, in contrast to the rabbit, to acetylate acetylhydrazine and excrete it as 1,2-diacetylhydrazine. The current finding together with the observation[13] that following administration of acetylisoniazid the dog excretes isonicotinic acid but no isoniazid support fully the suggestion that acetylhydrazine is an intermediate in the metabolism of the hydrazino moiety of isoniazid in man and other species, such as the rabbit and the rat. Consistent also with this metabolic scheme are the experiments by Wenzel[8] with human serum.

In the development of this interpretation advantage has been taken of the distinctive inability of the dog to acetylate isoniazid, and

(10) D. F. Elmendorf, D. W. Cawthon, C. Muschenheim, and W. McDermott, *Am. Rev. Tuberc.*, **70**, 420 (1952).

(11) W. F. J. Cuthbertson, D. M. Ireland, and W. Wolff, *Biochem. J.*, **55**, 669 (1953).

(12) G. Porcellati and P. Preziosi, *Boll. soc. ital. Biol. Sper.*, **29**, 209 (1953).

(13) Personal communication, Dr. L. H. Schmidt, Institute of Medical Research, Christ Hospital, Cincinnati 19, Ohio.

all of the studies in the present series are consistent with the observations previously reported by Hughes, Schmidt, and Peters.[14]

Enzymatic experiments by Johnson[15,16] and subsequent studies show the inhibition of the acetylation of isoniazid by sulfanilamide and various aromatic amines. As a result of these findings, it has become tempting to conclude that the general inability of the dog to excrete or form acetylisoniazid following administration of isoniazid is related to the same coenzymatic defect which is involved in the failure of this species to acetylate the amino group of sulfanilamide,[17] related compounds, and various aromatic amines. In this connection it is interesting to note the report by Krebs *et al.*,[18] on the acetylation of the amino group of sulfamezathine, a sulfonamide. Williams,[19] who has reviewed this matter, summarizes that the dog "does not acetylate sulphanilamide or any of its derivatives" and, in another connection, "whether the dog actually acetylates aromatic amines which are then deacetylated before excretion or whether it is completely unable to acetylate aromatic amines has not been satisfactorily decided."

In our first survey of the acylation and deacylation of hydrazine compounds, in collaboration with Dr. J. H. Weatherby, it was found that after administration of γ-glutamylhydrazine and hydrazine, the dog, under conditions comparable to those of the present study, excreted in the urine no or negligible amounts of 1,2-diacylhydrazino compounds (as adjudged by the iodate method). 1,2-Diacetylhydrazine was not excreted in deacylated form as opposed to acetylisoniazid[20] which underwent metabolic hydrolysis to a degree comparable to that observed in the present study. Significantly it was observed in the urine of three animals, again under comparable conditions, that isoniazid was excreted (approximately 7–16% of the dose) in a form behaving as 1,2-diacylhydrazine in the iodate reaction. During moving of our laboratory facilities, the loss and destruction of the collections of urine from these animals eliminated the possibility

(14) J. H. Peters, *Am. Rev. Tuberc. Pulmonary Diseases*, **81**, 485 (1960).
(15) W. J. Johnson, *Nature* (London), **174**, 744 (1954).
(16) W. J. Johnson, *Can. J. Biochem. and Physiol.*, **33**, 107, 523 (1955).
(17) E. K. Marshall, Jr., *J. Biol. Chem.*, **211**, 499 (1954).
(18) H. A. Krebs, W. O. Sykes, and W. C. Bartley, *Biochem. J.*, **41**, 622 (1947).
(19) R. T. Williams, "Detoxication Mechanisms," 2nd Edition, John Wiley & Sons, Inc., New York, N. Y., 1959, pp. 439, 504.
(20) A. S. Yard, H. McKennis, Jr., and J. H. Weatherby, *J. Pharmacol. Exptl. Therap.*, **119**, 195 (1957).

for further exploration of the nature of the metabolites. Although our present series is in accord with the data of Peters[14] and others and could support a contention that acetylisoniazid is not involved to a measurable degree in the metabolism of isoniazid in the dog, it would appear from the early studies that the role of acetylation in this species may not be fully settled. There may indeed be instances where acetylation is an important factor.

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Experimental[21]

Metabolic Experiments.—Acetylisoniazid[22,23] (m.p. 158–159°) (500 mg.) was given as a single oral dose in 75 ml. of water to a normal adult male (86 kg.). The subject was allowed food and water *ad libitum* but no medication. The voluntarily voided urine was collected over sodium fluoride. Male mongrel dogs under pentobarbital anesthesia received acetylisoniazid and isoniazid via the femoral vein. Urine was collected by means of an indwelling bladder catheter. All urine samples were used immediately at the end of collection periods or stored in the frozen state prior to analyses.

Analytical Methods.—Hydrazine nitrogen was assayed as described previously.[7,24] Paper chromatograms were performed as described earlier.[7]

Isolation of 1,2-Diacetylhydrazine from Human Urine.—The 24-hr. urine samples were treated with decolorizing carbon and then passed through Dowex 50 (H^+) and Dowex 3 (OH^-) in accordance with the procedure previously employed on rabbits.[7] The effluent and water washes from the resin columns were concentrated to dryness under diminished pressure. The residue was extracted with ethanol. The ethanol solutions were chromatographed on paper, and the zone corresponding to that of authentic 1,2-diacetylhydrazine was extracted with ethanol. The ethanol solutions were rechromatographed, and the zone was extracted as before. The filtered solution was evaporated to dryness. The residue was dissolved in a minimum amount of butanone. 1,2-Diacetylhydrazine was precipitated by addition of hexane and was recrystallized from acetone-hexane, m.p. 138–139°.

Anal. Calcd. for $C_4H_8N_2O_2$: C, 41.37; H, 6.95; N, 24.13. Found: C, 41.28; H, 6.93; N, 24.25.

Metabolism of Acetylisoniazid to Acetylhydrazine by the Dog.—A male mongrel

(21) Microanalyses by Weiler and Strauss, Oxford, and Spang Microanalytical Laboratory, Ann Arbor, Michigan.

(22) J. Bernstein, W. A. Lott, B. A. Steinberg, and H. L. Yale, *Am. Rev. Tuberc.*, **65**, 357 (1952).

(23) H. McKennis, Jr., A. S. Yard, and E. V. Pahnelas, *Am. Rev. Tuberc. Pulmonary Diseases*, **73**, 956 (1956).

(24) H. McKennis, Jr., J. H. Weatherby and E. P. Dellis, *Anal. Chem.*, **30**, 499 (1958).

dog (8.0 kg.) under pentobarbital anesthesia received acetylisoniazid (265 mg./kg.) intravenously. An aliquot of urine (30 ml.) from the collection obtained during a period of 3.5 hr. following the administration was filtered and then shaken vigorously with 0.1 ml. of freshly distilled benzaldehyde. After standing for 4 hr., the mixture then was extracted continuously with chloroform. The residue from evaporation of the chloroform under diminished pressure was dissolved in a small volume of warm water. Upon chilling, the solution deposited almost colorless crystals of 1-acetyl-2-benzylidenehydrazine which was recrystallized (30.8 mg.), micro m.p. 139–140° (uncor.), undepressed by admixture with an authentic sample, micro m.p. 139–140°. For analysis the material was dried *in vacuo* over potassium hydroxide, λ_{max} 282.5 mμ, ϵ 20,630 ($c = 9.88 \times 10^{-5}M$ in ethanol).

Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.21; N, 17.28. Found: C, 66.65; H, 6.18; N, 17.04.

Excretion of Isoniazid by the Dog.—A male mongrel dog (11.5 kg.), under pentobarbital anesthesia, received isoniazid (100 mg./kg.) intravenously. An aliquot (35 ml.) of the urine (72 ml.) obtained during the 5-hr. period following administration of the drug was stirred vigorously with 0.1 ml. of benzaldehyde. 1-Benzylidene-2-isonicotinylhydrazine (150 mg.) formed slowly and was separated by filtration, micro m.p. 200° (uncor.). In comparison an authentic sample[25] of the hydrazone, cap. m.p. 197–198°, melted at 200° on the hot stage. There was no depression of melting point upon admixture of the two samples. A further quantity of the hydrazone was obtained by extracting the aqueous mother liquor (above) continuously with chloroform. The chloroform solution was evaporated to a glassy residue which gave crystals of the benzalhydrazone upon the addition of a drop of water. The product was dissolved in 15 ml. of boiling water. Upon cooling, the solution yielded 30 mg. to give a total yield of 180 mg.

Anal. Calcd. for $C_{13}H_{11}N_3O$: C, 69.33; H, 4.92. Found: C, 69.59; H, 5.20.

(25) P. P. T. Sah and S. A. Peoples, *J. Am. Pharm. Assoc. Sci. Ed.*, **43**, 513 (1954).