used for the standard procaine solution, by noting the response to a measured electrical stimulus applied by electrodes. Threshold determinations were made at 5-min. intervals following 1-min. applications of the compounds to the corneal surface. The strength of threshold stimulus (setting in centimeters of the secondary coil) was plotted *vs.* time, and relative potencies were thus obtained. Results are expressed in Table II in terms of averaged activity (in three or more animals) relative to procaine, both in regard to threshold stimulus and duration of action at the same setting (10 cm.). Due to variations in response in different animals, use of figures to express these potencies indicates a greater degree of quantitative accuracy than the method is capable of giving.

Acknowledgments.—The authors are indebted to Dr. Howard J. Jenkins for the determination of local anesthetic potency and to Dr. Winthrop E. Lange for the infrared spectra. They wish to express appreciation to Hoyt Pharmaceutical Corp. for financial assistance.

2-Hydroxyacetophenetidine, a New Metabolite of Acetophenetidine

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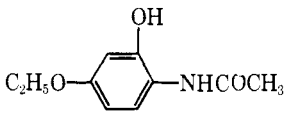
2-Hydroxyacetophenetidine (2-hydroxy-4-ethoxyacetanilide) conjugated with glucuronic acid has been shown to be a metabolite found in the urine of dogs, cats, and human subjects treated with acetophenetidine.

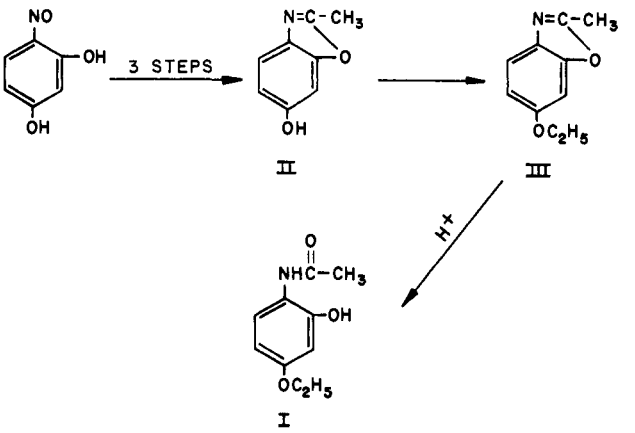
Brodie and Axelrod[1] have shown that N-acetyl-*p*-aminophenol is the major metabolite of acetophenetidine. In this communication we report the identification of a new metabolite of acetophenetidine, 2-hydroxyacetophenetidine (2-hydroxy-4-ethoxyacetanilide, I) in the urine of cats, dogs, and humans. Qualitative paper chromatography was used to detect the presence of this hydroxylated derivative which was then isolated and positively identified by comparison with a specimen of I obtained by unequivocal synthesis.



I

SCHEME I

SYNTHESIS OF 2-HYDROXYACETOPHENETIDINE (2-HYDROXY-4-ETHOXYACETANILIDE, I)



Results and Discussion

A. Preliminary Chromatographic Studies on Urinary Metabolites.—Three dogs received oral doses of acetophenetidine, and urine was collected and pooled. The $CHCl_3$ extract of urine, hydrolyzed with β-glucuronidase, was examined by paper chromatography. When the paper chromatogram was sprayed with diazotized sulfanilic acid (Pauly reagent), an orange spot was detected. Although this unknown substance could be detected in $CHCl_3$ extracts from unhydrolyzed urine, the concentration determined chromatographically was about 10 times as great in $CHCl_3$ extracts of urine treated with β-glucuronidase. Since the metabolite coupled with diazotized sulfanilic acid, it appeared that it was a ring-hydroxylated derivative of acetophenetidine, possibly I.

B. Synthesis of 2-Hydroxyacetophenetidine from Resorcinol.—2-Hydroxyacetophenetidine of unequivocal structure was synthesized as shown in Scheme I. The oxazole ring of II was used both to confirm the position of the potential 2-hydroxyl with respect to the future acetamido group, and to protect this hydroxyl during the ethylation of the 4-hydroxyl to the eventual 4-ethoxy group. The synthesis of I started with resorcinol, ensuring *meta* orientation of the final hydroxyl and ethoxyl groups. Nitrosation[2a] gave the 4-nitroso compound shown. Reduction of the nitroso group (Fe and HCl in aqueous ethanol) was followed by acetic anhydride treatment, thermal cyclization, and gentle saponification, to give 2-methyl-6-hydroxybenzoxazole (II).[2b] The phenolic impurity reported by Haginiwa was found to be II which was purified and combined with that produced by saponification of the acetoxy compound, to give an over-all yield of 20%.

Ethylation of the potassium salt of II with ethyl iodide to III, followed by cautious hydrolysis of III, led to 2-hydroxyacetophenetidine (I), m.p. 169–171° (varies with speed of heating). It formed a monoacetate (addition of acetic anhydride to the sodium salt in water) with a melting point more reproducible than that of I.

C. Isolation and Identification of the Metabolite as 2-Hydroxyacetophenetidine.—The β-glucuronidase-

(1) B. B. Brodie and J. Axelrod, *J. Pharmacol. Exptl. Therap.*, **97**, 58 (1949).

(2) (a) J. Haginiwa, *J. Pharm. Soc. Japan*, **73**, 1316 (1953). Two commercially available samples of 4-nitrosoresorcinol could not be reduced successfully. (b) The procedures described in the Experimental Section are modifications of Haginiwa's methods.

treated $CHCl_3$ extract of urine, obtained from dogs that received acetophenetidine, was examined by paper chromatography (solvent system 1, Table II). Both synthetic I and the unknown metabolite of acetophenetidine had an R_f of 0.50, and both substances gave an orange color with the Pauly reagent. In order to identify this metabolite conclusively, it was isolated and proved identical with synthetic I as described in the Experimental Section.

The amount of 2-hydroxyacetophenetidine (I) excreted in the urine of dogs, cats, and human subjects that received daily doses of 200, 200, and 15 mg./kg. of acetophenetidine, respectively, was estimated by paper chromatography of $CHCl_3$ extracts of urine previously treated with β-glucuronidase. The intensity of the color obtained after spraying the paper with diazotized sulfanilic acid reagent was compared with that obtained when an authentic sample of I was extracted from control urine in the same manner as the metabolite. These results are shown in Table I.

TABLE I

ESTIMATION OF 2-HYDROXYACETOPHENETIDINE IN THE URINE OF VARIOUS ANIMAL SPECIES TREATED WITH ACETOPHENETIDINE[a]

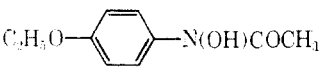
Species	Dose of acetophenetidine, mg./kg.	% of dose
Human subject I	15	0.2
Human subject II	15	0.4
Cat	200	1.0
Dog	200	1.9

[a] Hydrolysis of urine with β-glucuronidase (Ketodase) was carried out as described in the Experimental Section except that 2 ml. of β-glucuronidase was required for the complete hydrolysis of 2-hydroxyacetophenetidine glucuronide present in dog urine.

D. Metabolism of 2-Hydroxyacetophenetidine.—2-Hydroxyacetophenetidine (I) was given intravenously to two dogs at a level of 20 mg./kg. for 1 day and to eight rats orally at 50 mg./kg./day for 2 days. The urine was extracted with CH_2Cl_2 or treated with β-glucuronidase and then extracted with CH_2Cl_2. Paper chromatography of the evaporated organic solvent extracts revealed I and no metabolites. More polar solvents for the extraction of possible highly polar metabolites of I, such as 2-hydroxy-N-acetyl-*p*-aminophenol, were not used, since this reference compound was not available.

Unconjugated I in dog urine represented about 1% of the dose, whereas the I glucuronide present represented 14% of the dose. In rat urine the percentages were 14 and 21%, respectively.

E. N-Hydroxyacetophenetidine.—Because the *in vivo*[3] and *in vitro*[4] N-hydroxylation of several acetylated aromatic amines has been demonstrated, this possibility for acetophenetidine was considered. Therefore, the synthesis of N-hydroxyacetophenetidine (IV) was attempted. The synthesis was based on one carried out by Rising[5] and involved the reduction of *p*-nitrophenetole to the unstable *p*-ethoxyphenylhydroxylamine, using zinc and NH_4Cl. This was followed by acetylation with acetic anhydride, and cautious saponification to the final product. This procedure gave,



IV

among other products, a compound with an elemental analysis in agreement with that for a hydroxylated acetophenetidine, which proved to be different from a known sample of IV.[6] Analysis of the proton magnetic resonance and mass spectra[6] of our compound indicated that our synthetic hydroxyacetophenetidine was ring hydroxylated. Ultimately, it was found to be identical with the 2-hydroxyacetophenetidine (I) prepared by the unequivocal isoxazole method described above. Evidently, IV had rearranged to I[7] during the synthesis.

The $CHCl_3$ extracts from β-glucuronidase-treated urine of dogs and cats given acetophenetidine possessed a small amount of a metabolite of acetophenetidine which had the same mobility (system 3, Table II) as IV. The maximum amount of acetophenetidine that could be accounted for as the N-hydroxylated metabolite was about 0.3% of the dose in dogs, and about 0.1% of the dose in cats.

Since the N-hydroxylated derivative of acetophenetidine had apparently rearranged to the 2-hydroxylated derivative during the chemical synthetic procedure, and because it is known that migration of the N-hydroxy group to the aromatic ring occurs, *e.g.*, when N-hydroxyarylamines are heated in acid,[8] the possibility of this rearrangement occurring during the urinary hydrolysis and extraction of acetophenetidine metabolites was investigated. N-Hydroxyacetophenetidine (200 μg.) was added to 1 ml. of urine, and the urine was treated with β-glucuronidase and extracted with $CHCl_3$. Paper chromatography of the chloroform extract failed to reveal any detectable 2-hydroxyacetophenetidine, indicating that no rearrangement had occurred during the isolation procedure. A 1% conversion of the N-hydroxy derivative to 2-hydroxyacetophenetidine would have been detected.

Experimental Section

2-Methyl-6-acetoxybenzoxazole and 2-Methyl-6-hydroxybenzoxazole (II) from 4-Nitrosoresorcinol.—Freshly prepared 4-nitrosoresorcinol[2] (50 g.) was stirred with 600 ml. of 95% ethanol, and 80 ml. of concentrated HCl was added. The resulting dark brown solution was stirred under nitrogen, while 166 g. of electrolytic Fe powder was added during 8 min. with water cooling to moderate the initially vigorous reaction. The reaction was stirred under nitrogen an additional 0.5 hr. at ambient temperature and 20 min. under reflux. After cooling, the reaction mixture was treated with 120 g. of sodium acetate in 200 ml. of water at 60°, stirred briefly while still under nitrogen, and then treated with 100 ml. of acetic anhydride added over 3 min.

(3) J. W. Cramer, J. A. Miller, and E. C. Miller, *J. Biol. Chem.*, **235**, 885 (1960); J. A. Miller, J. W. Cramer, and E. C. Miller, *Cancer Res.*, **20**, 950 (1960); E. C. Miller and J. A. Miller, *Biochim. Biophys. Acta*, **40**, 380 (1960); J. A. Miller, C. S. Wyatt, E. C. Miller, and H. A. Hartman, *Cancer Res.*, **21**, 1465 (1961).

(4) M. Kiese and G. Renner, *Arch. Exptl. Pathol. Pharmakol.*, **246**, 163 (1963); M. Kiese and E. Rauscher, *Biochem. Z.*, **338**, 1 (1963); H. Uehleke *Biochem. Pharmacol.*, **12**, 219 (1963).

(5) A. Rising, *Ber.*, **37**, 43 (1904).

(6) Prepared independently by Dr. H. C. White of the Edgar C. Britton Research Laboratories, Dow Chemical Co. We are indebted to Dr. White for a supply of N-hydroxyacetophenetidine and for obtaining the p.m.r. and mass spectra and their interpretation, and allowing us to report these data prior to his publication.

(7) This rearrangement is being studied by Dr. White (personal communication).

(8) H. E. Heller, E. D. Hughes, and C. K. Ingold, *Nature*, **168**, 909 (1951).

After 10 min., the Fe and precipitated inorganic salts were removed by filtration and washed twice with ethanol. Evaporation of the combined ethanol filtrates and washings on a steam bath at *ca.* 20 mm. left a thick grease which was triturated three times in hot acetone. The solid residue of this treatment was partitioned between ether and 1 *N* HCl, and the resulting emulsion was broken by filtration. The ethereal layer was washed with aqueous $NaHCO_3$ and combined with the previously obtained acetone triturates, dried briefly, and evaporated on the steam bath at *ca.* 25 mm., leaving 41 g. of oil. This was refluxed with 360 g. of acetic anhydride for 1 hr. and distilled at atmospheric pressure; a fraction boiling at *ca.* 270° (bath temperature *ca.* 340–350°) was collected. The forerun was redistilled and the appropriate fraction was combined with the above 270° fraction and redistilled at 140–150° (5 mm.). This material (10.5 g.) solidified on scratching and seeding. Recrystallization from ethyl acetate–hexane gave 7.8 g. of 2-methyl-6-acetoxybenzoxazole (m.p. 52.5–53.5°).

Upon distillation at *ca.* 15 mm. (bath temperature *ca.* 370°), the residue of the atmospheric-pressure distillation gave 3.5 g. of a white solid which, on partitioning between 0.02 *N* aqueous NaOH and ether, followed by acidification of the aqueous alkaline solution with CO_2, gave 3.1 g. of phenol, m.p. 195.5–197.5°, undepressed on mixture with known 2-methyl-6-hydroxybenzoxazole (II) (*vide infra*).

Saponification of 8.4 g. of 2-methyl-6-acetoxybenzoxazole with 2 equiv. of KOH in 210 ml. of *ca.* 90% ethanol was complete after 5 hr. at room temperature. Distillation of the ethanol *in vacuo* and precipitation with CO_2 from a concentrated aqueous solution of the sodium salt, gave 5.6 g. of II, m.p. 198.7–200.7°.

2-Methyl-6-ethoxybenzoxazole (III).—To 5.2 g. (35 mmoles) of II in 50 ml. of commercial absolute ethanol were added 4.5 g. (40 mmoles) of potassium *t*-butoxide and after several minutes of shaking, 6.9 g. (44 mmoles) of ethyl iodide. The sealed solution was kept at an average temperature of 80° for 2.5 days. It was diluted with water and partitioned between ether and dilute, aqueous NaOH. The 3.7 g. of oil remaining after the removal of ether was distilled at 63–69° (0.13 mm.). The distillate melted at 20.5°.

Anal. Calcd. for $C_{10}H_{11}NO_2$: C, 67.78; H, 6.26; N, 7.9. Found: C, 67.46; H, 6.05; N (Kjeldahl), 7.61.

2-Hydroxyacetophenetidine (I).—A solution of 3.5 g. (19.7 mmoles) of III in 70 ml. of 95% ethanol was treated with 53 ml. of 2 *N* aqueous HCl at 30–40°. After 3 hr. the mixture was cooled and shaken to induce crystallization. Filtration gave 2.06 g. of crystals, m.p. 169.3–171.7. Storage of the mother liquor at room temperature for 4 hr. and at 4° for 16 hr. gave an additional 1.33 g. of product. It gave a reaction with ammoniacal $AgNO_3$ only after brief warming with aqueous NaOH. The product was recrystallized from water and then sublimed (air-bath temperature 133°, 0.02 mm.) for analysis.

Anal. Calcd. for $C_{10}H_{13}NO_3$: C, 61.52; H, 6.71. Found: C, 61.36; H, 6.56.

It gave an acetate, 2-acetoxy-4-ethoxyacetanilide, m.p. 131.7–132.3°.

Anal. Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37. Found: C, 60.67; H, 6.30.

Animal Experiments.—Acetophenetidine was administered orally to three adult dogs (200 mg./kg./day) for 3 days, and to two adult cats (200 mg./kg./day) for 1 day. Three human subjects each received a 1.0-g. dose of drug for 1 day and urine was collected for that day. Urine was collected before and during drug administration.

2-Hydroxyacetophenetidine in Carbowax 200 solution was administered to two dogs intravenously (200 mg./kg./day) for 1 day or as a suspension in Tween 80 solution orally (50 mg./kg./day) in divided doses to eight male Sprague–Dawley rats (140–190 g.) for 2 days. Urine was collected before and during drug treatment.

Paper Chromatography.—Paper chromatograms were equilibrated overnight with the lower phase of a toluene–benzene–water–acetic acid, 1:1:1:2 (v./v.), solvent system which is a modification of a system of Bray, *et al.*[9] Following development of the chromatogram with the upper phase (ascending) and drying, the Pauly reagent[10] was used to visualize the presence of phenolic derivatives of acetophenetidine. Acetophenetidine and N-hydroxyacetophenetidine, which do not react with Pauly reagent, were detected by their ultraviolet absorption. *p*-Phenetidine was detected by the Ehrlich reagent or by a modified Ehrlich reagent.[11] The mobilities of all reference compounds are shown in Table II.

TABLE II

MOBILITIES OF REFERENCE COMPOUNDS

Compd.	Average R_f System 1[a]	System 2[b]	System 3[c]	Detection
Acetophenetidine	0.80	0.94	0.88	*d*
N-Acetyl-*p*-aminophenol	0.08	0.13	0.80	*d, e, f*
2-Hydroxyacetophenetidine	0.50	0.76	0.86	*d, e, f*
N-Hydroxyacetophenetidine	0.84	0.42	0.17	*d, f*
p-Phenetidine	0	0.60	0.90	*d, f*
p-Aminophenol[g]	0	0	0.76, 0.36	*d, e, f*

[a] The upper phase of a toluene–benzene–water–acetic acid (1:1:1:2) mixture for paper chromatography on Whatman No. 1 paper. [b] The upper phase of benzene–water–acetic acid (2:1:2) on alumina-coated plates for thin layer chromatography. [c] The upper phase of cyclohexane–propanol–water–acetic acid (20:15:20:1) on alumina-coated plates for thin layer chromatography. [d] Ultraviolet absorption. [e] Pauly reagent. [f] Alkaline $AgNO_3$. [g] *p*-Aminophenol splits into two spots in system 3.

Thin Layer Chromatography.—Thin layer plates (200 × 200 mm.) were prepared from aluminum oxide G according to Stahl (Brinkman Instrument Co., Westbury, N. Y.) by the technique of Lees and DeMuria.[12] After air drying, the plates were activated in an oven for 30 min. at 80°. After developing the chromatograms and drying in air, the thin layer plates were heated in an oven for 5 min. to remove residual acetic acid. Visualization of reference compounds and metabolites was achieved with ultraviolet light or by spraying the plates with the alkaline $AgNO_3$ reagent of Burton, *et al.*[13] The mobilities of all reference compounds are shown in Table II.

Urine Hydrolysis.—A mixture of 1.0 ml. of urine, 1.0 ml. of 0.2 *N* acetate buffer at pH 5,[14] and 1.0 ml. of β-glucuronidase (Ketodase, Warner-Chilcott, Morris Plains, N. J.) was incubated for 16 hr. at 37.5°. The glucoronidase-treated urine was then buffered with 0.24 ml. of 0.5 *N* NaOH and 2 ml. of 1.0 *M* phosphate buffer[15] (pH 7.4) and extracted with 30 ml. of $CHCl_3$. A 25-ml. aliquot of the $CHCl_3$ extract was evaporated and the residue was dissolved in a few drops of methanol prior to chromatography. The β-glucuronidase-treated urines from animals treated with 2-hydroxyacetophenetidine were extracted with 40 ml. of CH_2Cl_2. A 30-ml. aliquot of the CH_2Cl_2 extract was evaporated, and the residue was dissolved in a small volume of methanol and subjected to chromatographic analysis.

Isolation of 2-Hydroxyacetophenetidine (I).—Urine (900 ml.) from three acetophenetidine-treated dogs was incubated at 37.5° for 18 hr. in the presence of 900 ml. of 0.2 *M* acetate buffer (pH 5) and 900 ml. of β-glucuronidase. The urine was then mixed with 108 ml. of 1 *N* NaOH and 900 ml. of 1 *M* phosphate buffer (pH 7.4) and was extracted twice with 3 vol. of $CHCl_3$. The $CHCl_3$ extracts were combined and evaporated at room temperature under vacuum. The residue (690 mg.) was dissolved in 80 ml. of $CHCl_3$ and chromatographed on an aluminum oxide column.[16] Following the addition of acetophenetidine metabolites to the column, a 90-ml. fraction of $CHCl_3$ (fraction 1) and 300 ml. of $CHCl_3$ (fraction 2) were collected. The two $CHCl_3$ fractions

(9) H. G. Bray, R. C. Clowes, and W. V. Thorpe, *Biochem. J.*, **51**, 70 (1952).

(10) I. Smith, "Chromatographic and Electrophoretic Techniques," Vol. I, Interscience Publishers, Inc., New York, N. Y., 1960, pp. 296, 297.

(11) Reference 10, pp. 193–195.

(12) T. M. Lees and P. J. DeMuria, *J. Chromatog.*, **8**, 108 (1962).

(13) R. B. Burton, A. Zaffaroni, and E. H. Keutman, *J. Biol. Chem.*, **188**, 763 (1951).

(14) "Biochemists Handbook," Van Nostrand Co., Inc., Princeton, N. J., 1961, p. 31.

(15) KH_2PO_4 (272 g.) and 5 *N* KOH (320 ml.) made up to 2 l. with water.

(16) Acid alumina, AG 4 grade, 100–200 mesh, was supplied by the BioRad Co., Los Angeles, Calif. The adsorbent (3.5 g.) was mixed with $CHCl_3$ and a 0.8 × 13.5 cm. column was prepared.

were evaporated and a sample of each residue was chromatographed on paper using solvent system 1 (Table II). A compound which absorbed ultraviolet light and had the same mobility as acetophenetidine (R_f 0.80) was obtained from fraction 1. After spraying the chromatogram with the Pauly reagent, a spot with the same mobility (R_f 0.50) and orange color as I was obtained from both fractions, and a second spot (R_f 0.33) with a red color was also obtained from fraction 2. Fraction 2 was evaporated and recrystallized twice from aqueous ethanol and once from ethyl acetate–cyclohexane. Crystals were obtained, m.p. 169–171°, alone or in mixture with an authentic sample of I. The infrared spectra of the metabolite and of I were identical. The ultraviolet spectra of the metabolite and the reference compound were similar in 0.5 *N* HCl (λ_{max} 284 mμ) and in 0.5 *N* NaOH (λ_{max} 297 mμ).

Acknowledgments. The authors wish to acknowledge the excellent assistance of Miss L. Beauchamp and Miss M. Bordun.

Nonclassical Antimetabolites. XXV.[1] Inhibitors of Thymidine Kinase. I. A New Spectrophotometric Assay. Inhibition by Nucleoside Derivatives

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A spectrophotometric assay for thymidine kinase has been devised that employs 2′-deoxy-5-fluorouridine (FUDR) as the substrate; the concentration of the resultant 2′-deoxy-5-fluorouridylate (FUDRP) was assayed by inhibition of thymidylate synthetase. The assay has a magnification of 1000-fold since FUDRP is known to bind to thymidylate synthetase 1000-fold better than the substrate, 2′-deoxyuridylate; the assay has then the same order of sensitivity as the usual type of radioactive assay. The mode of binding of the 2′-deoxyribosyl moiety of thymidine to thymidine kinase was investigated with deoxy derivatives of thymidine. Both the 5′- and 3′-hydroxyls contribute to binding, the latter more strongly. Whether or not the furanose oxygen contributed to binding could not be ascertained with any certainty. Bulk-tolerance studies indicated that larger groups, placed on the 5′-hydroxyl or 5-methyl of thymidine, caused a greater than 600-fold loss in binding. In contrast, an *n*-amyl group could be introduced on the N^3-position of thymidine with retention of enzyme binding, but the binding was decreased about 50-fold compared to thymidine.

One of the major endeavors in this laboratory is the design of suitable, active-site-directed, irreversible inhibitors[2] of dihydrofolic reductase and thymidylate synthetase.[3] An efficient blockade of either enzyme would result in "thymine-less death" of cells[4] from lack of the thymidylate necessary for DNA synthesis. Even if one did achieve the hoped-for species or tissue specificity predicted from the bridge principle of specificity with irreversible inhibitors,[2] the target cells still may be able to obtain sufficient thymidylate by converting thymidine, scavenged from the host blood stream, to thymidylate with the enzyme, thymidine kinase. Therefore, a nontissue-specific blockage of thymidine kinase might be a necessary adjunct to achieve "thymine-less death," the tissue specificity being achieved during blockage of dihydrofolic reductase or thymidylate synthetase. For this reason, a study on the mode of binding of inhibitors to thymidine kinase has been initiated.

Enzyme Studies.—The isolation of thymidine kinase from *E. coli* B and its assay with radioactive thymidine has been described by Okazaki and Kornberg.[5] These workers found that 2′-deoxyuridine and its 5-halogen derivatives were as effective as thymidine as substrates. Although their radioactive assay is quite satisfactory for evaluation of possible substrates, this assay is more laborious for quantitative evaluation of potential inhibitors; therefore, three possible methods for spectrophotometric assay of the enzyme were investigated, the third being considered the best because of the large magnification built into the assay.

(a) The measurement of ATP conversion to ADP as thymidine is converted to thymidylate (dTMP)[6] by measuring the generated ADP in a coupled system with pyruvate kinase and lactic dehydrogenase was investigated.[7] Although this coupled system worked reasonably well with the measurement of the ADP generated by the hexokinase-catalyzed phosphorylation of D-glucose, our preparation of thymidine kinase gave erratic results.

(b) The measurement of 2′-deoxyuridine conversion to 2′-deoxyuridylate (dUMP)[6] by coupling the latter as a substrate to thymidylate synthetase was investigated. This method was the simplest for detection of the presence of thymidine kinase in an extract, but the levels of thymidylate synthetase needed to make the rate of the coupled system dependent upon the thymidine kinase were uneconomically high.

(1) (a) This work was supported by Grant No. CA-05845 and CA-05867 from the National Cancer Institute, U. S. Public Health Service. (b) For the previous paper of this series see B. R. Baker and G. D. F. Jackson, *J. Pharm. Sci.*, in press. (c) Address inquiries to Department of Chemistry, University of California, Santa Barbara, Calif. 93106.

(2) For a review on the factors in the design of active-site-directed irreversible inhibitors see B. R. Baker, *ibid.*, **53**, 347 (1964).

(3) (a) B. R. Baker and J. H. Jordaan, *J. Heterocyclic Chem.*, **2**, 21 (1965); (b) B. R. Baker and J. H. Jordaan, *ibid.*, **2**, 162 (1965); (c) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965); (d) B. R. Baker and J. K. Coward, *ibid.*, **54**, 714 (1965); (e) B. R. Baker and J. H. Jordaan, *J. Med. Chem.*, **8**, 35 (1965); (f) B. R. Baker, B.-T. Ho, and T. Neilson, *J. Heterocyclic Chem.*, **1**, 79 (1964); (g) B. R. Baker and H. S. Shapiro, *J. Med. Chem.*, **6**, 664 (1963).

(4) S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, *Proc. Natl. Acad. Sci. U. S.*, **44**, 1004 (1958).

(5) R. Okazaki and A. Kornberg, *J. Biol. Chem.*, **239**, 269, 275 (1964).

(6) The following abbreviations are used: FUDR, 5-fluoro-2′-deoxyuridine; FUDRP, 5-fluoro-2′-deoxyuridylate; dUMP, 2′-deoxyuridylate; dTMP, thymidylate; M-FAH_4, 5,10-methylene-*dl*-tetrahydrofolate; FAH_2, dihydrofolate; dCTP, 2′-deoxycytidine triphosphate.

(7) This type of system has been used for the spectrophotometric assay of uridine kinase; see P. Reichard and O. Skold, *Methods Enzymol.*, **6**, 194 (1963).