

A possible explanation is that 4-(carbophenoxy-amino)salicylic acid (II) can bind weakly to some site other than the active site, then form a covalent link that makes the active site inaccessible to the substrate; such reversible binding to an extraneous site would have to have a dissociation constant larger than 10^{-2} to show the lack of a rate saturation effect within experimental error. The fact that one compound does not show a rate saturation effect actually strengthens the interpretation that those compounds showing a rate saturation effect might operate by the exo-alkylation mechanism.

Although no compound in Table II inactivated LDH selectively by the exo-alkylation mechanism, it is probable that such a compound could be found by further investigation.

Of importance to chemotherapy is the irreversible specificity noted with the phenyl esters (VII-IX), this specificity being because of the difference in the nucleophilic character of the enzymic groups being covalently bound on LDH and GDH. Groups on a reversible inhibitor that can bridge to and specifically bind other enzymic functional groups would be of use in both chemotherapy and protein structure

studies; such a study is continuing in these laboratories.

REFERENCES

- (1) Baker, B. R., *Biochem. Pharmacol.*, **12**, 293(1963).
- (2) Baker, B. R., and Patel, R. P., *Biochem. Biophys. Res. Commun.*, **9**, 199(1962).
- (3) Baker, B. R., *Cancer Chemotherapy Rep.*, No. 4, 1(1959).
- (4) Baker, B. R., Lee, W. W., Skinner, W. A., Martinez, A. P., and Tong, E., *J. Med. Pharm. Chem.*, **2**, 633(1960).
- (5) Baker, B. R., Lee, W. W., Tong, E., and Ross, L. O., *J. Am. Chem. Soc.*, **83**, 3713(1961).
- (6) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 459(1962).
- (7) Lawson, W. B., and Schramm, H. J., *J. Am. Chem. Soc.*, **84**, 2017(1962).
- (8) Wofsy, L., Metzger, H., Singer, S. J., "Abstracts of the 141st Meeting of the American Chemical Society," Washington, D. C., 1962, p. 18C; *Biochemistry*, **1**, 1031(1962).
- (9) Baker, B. R., *J. Med. Pharm. Chem.*, **5**, 654(1962).
- (10) Baker, B. R., *Biochem. Pharmacol.*, **11**, 1155(1962).
- (11) Crosby, D. G., and Niemann, C., *J. Am. Chem. Soc.*, **76**, 4458(1954).
- (12) Baker, B. R., Lee, W. W., Martinez, A. P., Ross, L. O., and Goodman, L., *J. Org. Chem.*, **27**, 3283(1962).
- (13) Zollinger, H., *Angew. Chem.*, **73**, 125(1961); cf. French pat. 1,217,337.
- (14) Albert, A., *J. Chem. Soc.*, **1941**, 121.
- (15) Goldberg, A. A., and Thomas, N. L., *Brit. pat.* 665,675.
- (16) Dixon, M., and Webb, E. C., "Enzymes," Academic Press, Inc., New York, N. Y., 1958, p. 75.
- (17) Weygand, F., and Röpsch, A., *Chem. Ber.*, **92**, 2095 (1959).

Nonclassical Antimetabolites XIII

Simulation of the 5'-Phosphoribosyl Moiety of 5'-Adenylic Acid at the Enzyme Level by ω -Carboxyalkyl and Aralkyl Groups Attached to Adenine

By B. R. BAKER and H. S. SACHDEV

5'-Adenylic acid can inhibit both lactic dehydrogenase and glutamic dehydrogenase; since both the phosphate and adenine moieties are necessary for good inhibition, the 5'-adenylic acid presumably competes with DPN. This inhibition by 5'-adenylic acid can be duplicated by 9-(4'-carboxybutyl)-adenine (XVIII) on both enzymes, indicating that the ω -valeric acid side chain can simulate the binding of the 5-phosphoribosyl group when the latter is attached to adenine. 9-(*para*-Carboxybenzyl)-adenine (XVI), a compound with a conformation-fixed side chain, can simulate the binding of 5'-adenylic acid to glutamic dehydrogenase but not lactic dehydrogenase; this difference has been attributed to the difference in conformation of 5'-adenylic acid (and DPN) when bound to the two enzymes.

MANY IMPORTANT ENZYMES have substrates bearing the 5-phosphoribosyl moiety such as the ribotides involved in nucleic acid biosynthesis. A number of purines and pyrimidines useful in cancer chemotherapy operate by en-

zymic blockade at the nucleotide level (2). For example, 5-fluorouracil is converted intracellularly to 5-fluorouracil deoxyribotide; the latter inhibits cell growth by blockade of thymidylate synthetase (3, 4). Similarly, in the cell 6-mercaptopurine (I) is converted by inosinic pyrophosphorylase to 6-mercaptopurine ribotide (II) (5, 6). The latter compound is a potent inhibitor for inosinic dehydrogenase, the enzyme converting inosinic acid (IV) to xanthylic acid (V); at a somewhat higher concentration, II also inhibits adenylosuccinate synthetase, the enzyme forming adenylosuccinate (III) from inosinic acid (IV) (7, 8).

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Unfortunately, cancer cells all too soon become resistant to the effects of 6-mercaptopurine (I) because of the mutational loss of the inosinic pyrophosphorylase that converts I and hypoxanthine to their ribonucleotides, II and IV (5, 8). Although 6-mercaptopurine ribotide (II) is still effective as an inhibitor of the enzymes utilizing inosinic acid (IV) in a cell-free extract of resistant cell lines (7), II has little effect on the intact resistant cells (7, 9, 10). This lack of activity probably is because 6-mercaptopurine ribonucleotide (II), like natural nucleotides (11), suffers enzymic cleavage back to the corresponding base (I) during entrance into the cell by active transport.

One approach to the solution of the enigma of this resistance problem has been to block completely the phosphate group of II as a triester (12); the latter type could presumably enter the cell by passive transport, then be reconverted intracellularly to the free nucleotide (II) by a nonspecific phosphoesterase. This approach has been partially successful (13).

A second approach would be to utilize a 9-substituted derivative of 6-mercaptopurine wherein this 9-substituent could simulate the binding of the ribotide moiety of II to the enzymes utilizing inosinic acid (IV) as a substrate but still have the

ability to penetrate a cell wall and not suffer enzymic cleavage to the base. Since a major portion of the binding of the ribose phosphate moiety can certainly be attributed to the binding of the ionized phosphate to an enzymic cationic site, other anionic groups such as sulfates, phosphonates, or carboxylates should be able to replace the phosphate group in binding. In addition, since the ribose part of this moiety probably contributes much less than half of the total binding of this moiety, the ribose fragment should be replaceable by any group that is smaller than β -D-ribofuranose, providing that this group can properly juxtaposition the anionic binding group and the purine moiety. This juxtapositioning will be dependent upon the conformation of the nucleotide as it binds to a given enzyme. For reasons to be presented in the *Discussion*, the *p*-carboxybenzyl and the 4-carboxybutyl groups attached to the 9-position of some purines were selected for initial investigation.

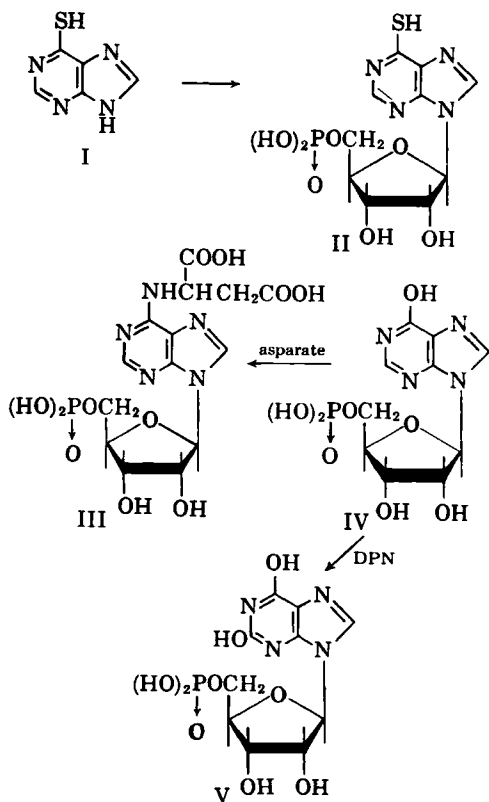
Although crude preparations of inosinic dehydrogenase (14, 15) and adenylosuccinate synthetase (16) (two enzymes utilizing inosinic acid (IV)) have been described, we considered it expedient to use commercially available crystalline enzymes to determine—as a first approximation—if the binding of a nucleotide to one of these enzymes could be simulated by replacement of the ribose phosphate moiety with a suitable group. Since LDH and GDH¹ have been used extensively in these laboratories for other inhibitor studies (1, 17, 18), and since both enzymes use the dinucleotide, DPN, as a cofactor, these two enzymes were selected for initial studies of the relative binding of AMP, 9-(*p*-carboxybenzyl)adenine (XVI), and 9-(4'-carboxybutyl)-hypoxanthine (XVIII). The synthesis and evaluation of the latter two compounds as DPN inhibitors of LDH and GDH is the subject of this paper.

EXPERIMENTAL

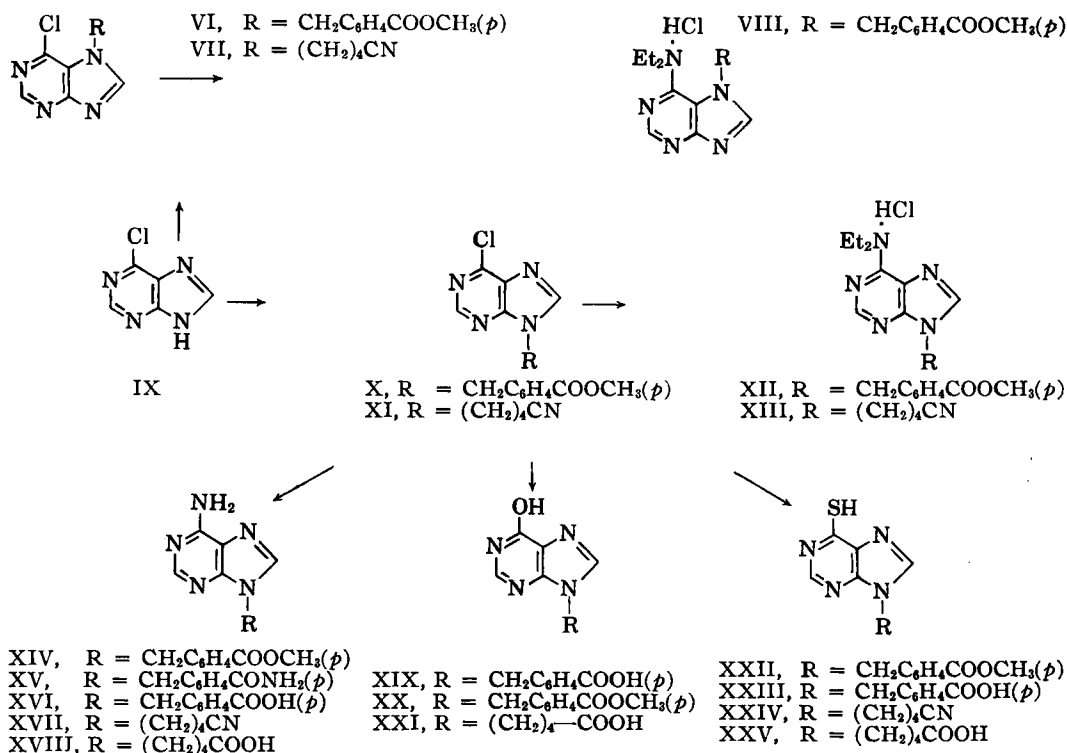
Enzyme Measurements

Reagents.—DL-Lactate, L-glutamate, DPN, and adenosine were purchased from Nutritional Biochemicals Corp. AMP, IMP, and AMP-2'(3') were purchased from Pabst Laboratories. Crystalline LDH (isolated from rabbit skeletal muscle) and crystalline GDH (isolated from mammalian liver) were purchased from the Sigma Chemical Co.

Methods of Assay.—The enzymatic rates for LDH and GDH were determined by the rate of formation of DPNH as measured by the rate of



¹ Abbreviations used: LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase; DPN, diphosphopyridine nucleotide; AMP, 5'-adenylic acid; IMP, 5'-inosinic acid; AMP-2'(3'), a 1:1 mixture of 2'- and 3'-adenylic acid; Ad, adenine; Hx, hypoxanthine; ADP, adenosine 5'-diphosphate.



appearance of DPNH at 340 μ in a Cary 11 spectrophotometer. All reactions were run in 0.05 *M* Tris buffer (pH 8.4).

For LDH, the cell contained 3.2 *mM* DPN, 8 *mM* DL-lactate, and the inhibitor when necessary in a total volume of 3.0 ml. The reaction was initiated by the addition of the appropriate amount of enzyme in 100 λ of buffer. Sufficient enzyme was used to give an initial rate of formation of DPNH of 0.4–0.6 optical density units per minute (17, 19).

The GDH assays were run similarly using 3.2 *mM* DPN, 2 *mM* L-glutamate and the inhibitor when necessary. Sufficient enzyme was used to give an initial rate of 0.9–1.2 optical density units per minute (17, 20).

Chemistry

Methods.—Although some purines can be alkylated directly to 9-alkylpurines—for example, 2-methylthio-6-dimethylaminopurine (21) and purine (22)—other purines react poorly or even fail to react properly (23). More often mixtures of 7- and 9-alkyl derivatives are obtained which must be separated (21, 24). The elegant methods developed by Montgomery and Temple (24, 25) for synthesis of 9-alkylpurines *via* 9-alkyl-6-chloropurine are particularly suitable if one wishes to have several different substituents at the 6-position. Their first route for 9-alkyl-6-chloropurines *via* 5-amino-4,6-dichloropyrimidine and an appropriate amine is favored if the necessary alkylamine is readily available, since only a 9-substituted purine can be obtained after ring closure with ethyl orthoformate-acetic anhydride (25–28). The second route involving the reaction of 6-chloropurine with an alkyl halide is useful when an alkyl halide is more available than the amine; dimethylformamide or dimethylsulfoxide as the

solvent and potassium carbonate as the acid acceptor were the keys to the success of this reaction (24, 29). In most cases a mixture of 7- and 9-alkyl-6-chloropurines were obtained with the 9-isomer predominating as is usually the case in such alkylations (21). Once the separation has been completed, the chloro group can then be displaced by a large variety of nucleophiles (24, 25, 29) in a direct manner that gives no further isomer problems.

The second route of Montgomery and Temple was chosen for our syntheses of carboxyalkyl and aralkyl purines since bromo esters give considerably less difficulty in nucleophilic reactions than amino esters; the latter cause complications by polymerization. Condensation of methyl α -bromo-*p*-toluate (30) with 6-chloropurine (IX) proceeded smoothly. The 9-substituted purine (X) was obtained in 65% yield and was readily separated from the 7-isomer (VI) formed in 4% yield; the latter is readily soluble in hot methanol and X is insoluble. That the major isomer was the 9-isomer (X) was readily shown by conversion of X and VI to the corresponding 6-diethylaminopurines (XII and VIII) by the use of diethylamine in boiling methanol. The two 6-diethylaminopurines were readily distinguished by the large difference in their ultraviolet absorption maxima; XII showed a peak at 276 μ in ethanol, whereas VIII showed a maximum at 298 μ , in agreement with other 7- and 9-alkyl-6-dimethylaminopurines (21). The use of diethylamine is simpler than the use of dimethylamine for these structure proofs (24), since dimethylamine requires a sealed vessel for reaction.

Reaction of 9-(*p*-carbomethoxybenzyl)-6-chloropurine (X) with methanolic ammonia at 105° for 75 minutes gave the corresponding adenine derivative (XIV) in 85% yield. However, longer reaction

times and higher temperatures (115°) or both led to the additional ammonolysis of the ester function with formation of appreciable quantities of the amide (XV). Saponification of XIV with hot 1 *N* sodium hydroxide afforded the desired 9-(*p*-carboxybenzyl)-adenine (XVI) in 80% yield.

When the chloropurine, X, was refluxed with concentrated hydrochloric acid for 2 hours, hydrolysis of the 6-chloro and ester groups occurred with formation of 9-(*p*-carboxybenzyl)-hypoxanthine (XIX) in 89% yield. The use of hot 0.1 *N* hydrochloric acid gave only hydrolysis of the 6-chloro group; the resultant hypoxanthine ester (XX) was isolated in 74% yield.

The chloropurine X also reacted smoothly with thiourea in boiling methanol to give the 6-mercaptopurine ester XXII in 90% yield. Further saponification of XXII with hot 1 *N* sodium hydroxide afforded 9-(*p*-carboxybenzyl)-6-mercaptopurine (XXIII) in 70% yield.

Alkylation of 6-chloropurine (IX) with 5-bromovaleronitrile in dimethylformamide proceeded smoothly. Apparently considerable amounts of 7-isomer were present which made purification somewhat difficult and the yield of usable 9-isomer (XI) was 27%. That the crystalline isomer obtained was indeed the 9-isomer was shown by conversion of XI to the 6-diethylaminopurine derivative (XIII), isolated as the crystalline hydrochloride; the latter showed an ultraviolet maximum at 276 μ in ethanol, as expected for a 9-isomer (21). Conversion of XI to 9-(4'-carboxybutyl)-hypoxanthine (XXI), 9-(4'-cyanobutyl)-6-mercaptopurine (XXIV) and 9-(4'-cyanobutyl)-adenine (XVII) proceeded smoothly as described for the *p*-carboxybenzyl series. However, conversion of XXIV and XVII to the corresponding 9-(4'-carboxybutyl)-6-mercaptopurine (XXV) and 9-(4'-carboxybutyl)-adenine (XVIII), respectively, was more expediently done with hot concentrated hydrochloric acid than with the alkaline hydrolysis used in the *p*-carboxybenzyl series.

Synthesis.²—9-(*p*-Carbomethoxybenzyl)-6-chloropurine (X).—To a solution of 6.87 Gm. (0.03 mole) of methyl α -bromo-*p*-toluate (30) in 50 ml. of dimethylformamide was added 4.4 Gm. (0.032 mole) of anhydrous potassium carbonate followed by 4.63 Gm. (0.03 mole) of 6-chloropurine. The mixture was stirred for 2 hours protected from moisture during which time a buff-colored solid separated. The mixture was poured into 150 ml. of ice water. The solid was collected on a filter and washed with 50 ml. of water. The dry filter cake weighed 8.2 Gm., m.p. 165–190°. The dry solid was heated to boiling with 125 ml. of methanol and filtered hot. The undissolved product melted at 196–201°; yield, 5.9 Gm. (65%). Recrystallization from benzene gave analytically pure material; yield 4.2 Gm. (46%), m.p. 200–201°; $\nu_{\text{max}}^{\text{KBr}}$ 1715 (ester); 1600, 1560, 1510 cm^{-1} (C=C, C=N).

Anal.—Calcd. for $\text{C}_{14}\text{H}_{11}\text{ClN}_4\text{O}_2$: C, 55.5; H, 3.70; N, 18.5. Found: C, 55.6; H, 3.83; N, 18.5.

The hot methanol mother liquor was evaporated to residue *in vacuo*. Two recrystallizations from

methanol gave 0.40 Gm. (4%) of 7-(*p*-carbomethoxybenzyl)-6-chloropurine (VI), m.p. 174°.

Anal.—Calcd. for $\text{C}_{14}\text{H}_{11}\text{ClN}_4\text{O}_2$: C, 55.5; H, 3.70; N, 18.5. Found: C, 55.6; H, 3.62; N, 18.4.

9-(4'-Cyanobutyl)-6-chloropurine (XI).—A mixture of 6.18 Gm. (0.04 mole) of 6-chloropurine (IX), 7.24 Gm. (0.044 mole) of 5-bromovaleronitrile, 5.52 Gm. (0.04 mole) of anhydrous potassium carbonate, and 60 ml. of dimethylformamide was stirred at room temperature for 8 hours. After dilution with 180 ml. of ice cold water, the mixture was extracted with chloroform (4 $\times$ 50 ml.). The combined extracts were dried with anhydrous magnesium sulfate and the solvent was spin-evaporated *in vacuo*; the last traces with dimethylformamide were removed at oil pump pressure. The oily residue solidified after standing overnight. Two recrystallizations from benzene-petroleum ether gave 2.5 Gm. (27%) of product, m.p. 87–89°, that was suitable for further transformations. Repeated recrystallization afforded the analytical sample, m.p. 90–91°; $\nu_{\text{max}}^{\text{KBr}}$ 2250 (C $\equiv$ N); 1590, 1550, 1490 cm^{-1} (C=C, C=N).

Anal.—Calcd. for $\text{C}_{10}\text{H}_{10}\text{ClN}_5$: C, 51.0; H, 4.28; N, 29.7. Found: C, 51.1; H, 4.31; N, 29.5.

9-(*p*-Carbomethoxybenzyl)-6-diethylaminopurine Hydrochloride (XII).—A 160-mg. (2.2 mmoles) quantity of diethylamine was added to a solution of 302 mg. (1 mmole) of X in 25 ml. of reagent grade methanol. After being refluxed for 2 hours, the solution was spin-evaporated to a syrup *in vacuo*. The residual gum was triturated with 15 ml. of 1:1 reagent acetone-dry ether; the diethylamine hydrochloride was removed by filtration and washed with 1:1 acetone-ether. With cooling, dry hydrogen chloride was passed into the filtrate until precipitation was complete; the product rapidly separated. It was collected on a filter and washed with ether. The solid was dissolved in reagent acetone and dry ether was added to turbidity. On standing at 3°, the pure product separated as white crystals; yield, 170 mg. (45%), m.p. 183–184°; $\lambda_{\text{max}}^{\text{EtOH}}$ 276 μ (ϵ 19,500).

Anal.—Calcd. for $\text{C}_{18}\text{H}_{22}\text{ClN}_5\text{O}_2$: C, 57.5; H, 5.91; N, 18.6. Found: C, 57.7; H, 6.05; N, 18.9.

No attempt was made to obtain a second crop.

9-(4'-Cyanobutyl)-6-diethylaminopurine Hydrochloride (XIII).—This was prepared in a similar way and recrystallized from 1:1 ethyl acetate-acetone; yield of pure material 52%, m.p. 120–123°; $\lambda_{\text{max}}^{\text{EtOH}}$ 277 μ (ϵ 20,000).

Anal.—Calcd. for $\text{C}_{14}\text{H}_{21}\text{ClN}_6$: C, 54.5; H, 6.86; N, 27.3. Found: C, 54.3; H, 6.96; N, 27.2.

7-(*p*-Carbomethoxybenzyl)-6-diethylaminopurine Hydrochloride (VIII).—This was also prepared as described for XII. Recrystallization from acetone-ether gave a 64% yield of pure product, m.p. 193–195°; $\lambda_{\text{max}}^{\text{EtOH}}$ 298 μ (ϵ 15,900).

Anal.—Calcd. for $\text{C}_{18}\text{H}_{22}\text{ClN}_5\text{O}_2$: C, 57.5; H, 5.91; N, 18.6. Found: C, 57.4; H, 6.01; N, 18.4.

9-(*p*-Carbomethoxybenzyl)-adenine (XIV).—A 1.00-Gm. (3.3 mmoles) quantity of X was added to 75 ml. of methanol saturated with ammonia gas at 0° and placed in a steel bomb. The bomb was heated at 105–106° for 75 minutes, then cooled in cold water. A white solid separated out on the walls of

² Melting points were taken on a Mel-temp. apparatus in capillary tubes and are uncorrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer recording spectrophotometer 137B. Ultraviolet spectra were determined with a Cary 11 recording spectrophotometer.

the bomb. The product was collected on a filter and washed with a little cold methanol; yield, 0.80 Gm. (85%), m.p. 228–229°, that was sufficiently pure for further transformations. An analytical sample was obtained by recrystallization from methanol as white crystals, m.p. 230–231°; $\nu_{\text{max}}^{\text{KBr}}$ 3300 (NH); 1730 (ester C=O); 1675, 1610, 1570 cm^{-1} (NH, C=C, C=N).

Anal.—Calcd. for $\text{C}_{14}\text{H}_{13}\text{N}_5\text{O}_2$: C, 59.4; H, 4.62; N, 24.7. Found: C, 59.2; H, 4.56; N, 24.6.

9-(4'-Cyanobutyl)-adenine (XVII).—This was prepared as described for XIV. Recrystallization from chloroform with the aid of decolorizing carbon gave 54% of white needles, m.p. 156–157°; $\nu_{\text{max}}^{\text{KBr}}$ 3300 (NH); 2240 (C≡N); 1670, 1600, 1575 cm^{-1} (NH, C=C, C=N).

Anal.—Calcd. for $\text{C}_{10}\text{H}_{12}\text{N}_6$: C, 55.5; H, 5.62; N, 38.9. Found: C, 55.3; H, 5.52; N, 38.9.

9-(p-Carbomethoxybenzyl)-6-mercaptapurine (XXII).—A mixture of 0.302 Gm. (1 mmole) of X, 15 ml. of methanol, and 0.076 Gm. (1 mmole) of thiourea was refluxed with stirring for 2 hours. During this time the solid nearly dissolved, then the product separated. The cooled mixture was filtered and the product washed with 20 ml. of cold water; yield, 270 mg. (90%), m.p. 289–290°, that was suitable for further transformation. Recrystallization from 2-methoxyethanol gave 200 mg. (67%) of pure product as pale-buff crystals, m.p. 290–291°; $\nu_{\text{max}}^{\text{KBr}}$ 1710 (ester C=O); 1600, 1575, 1530 (C=C, C=N); 1275 cm^{-1} (ester C—O—C).

Anal.—Calcd. for $\text{C}_{14}\text{H}_{12}\text{N}_4\text{O}_3\text{S}$: C, 56.0; H, 4.03; N, 18.7. Found: C, 56.2; H, 4.16; N, 18.5.

9-(4'-Cyanobutyl)-6-mercaptapurine (XXIV).—This was prepared as described for XXII; yield, 200 mg. (86%), m.p. 284–285°. Recrystallization from ethanol gave pale-colored needles, m.p. 286–287°; $\nu_{\text{max}}^{\text{KBr}}$ 2255 (C≡N); 1600, 1540 cm^{-1} (C=N, C=C).

Anal.—Calcd. for $\text{C}_{10}\text{H}_{11}\text{N}_5\text{S}$: C, 51.5; H, 4.77; N, 30.0. Found: C, 51.7; H, 4.90; N, 30.0.

9-(p-Carboxybenzyl)-adenine (XVI) Hydrochloride.—A mixture of 150 mg. (0.53 mmole) of XIV and 15 ml. of 1 *N* aqueous sodium hydroxide was refluxed for 45 minutes; the ester rapidly dissolved. The cooled solution was acidified to pH 2 with 6 *N* hydrochloric acid. The white solid was collected on a filter, washed with water, and dried at 100°; yield, 130 mg. (80%), m.p. 348–349°. Recrystallization from absolute ethanol gave analytically pure material, m.p. 350°; $\nu_{\text{max}}^{\text{KBr}}$ 3450 (NH); 1700 (acid C=O, C=NH⁺); 1600, 1510 cm^{-1} (NH, C=C, C=N).

Anal.—Calcd. for $\text{C}_{13}\text{H}_{12}\text{ClN}_5\text{O}_2$: C, 51.1; H, 3.97; N, 22.8. Found: C, 51.3; H, 4.15; N, 22.8.

9-(p-Carboxybenzyl)-6-mercaptapurine (XXIII).—From 150 mg. (0.50 mmole) of XXII, as described for the preparation of XVI, was obtained after recrystallization from 2-methoxyethanol 100 mg. (70%) of product, m.p. 318–320°; $\nu_{\text{max}}^{\text{KBr}}$ 1685 (carboxyl C=O); 1595, 1570, 1525 cm^{-1} (C=N, C=C).

Anal.—Calcd. for $\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_3\text{S}$: C, 54.5; H, 3.57; N, 19.6. Found: C, 54.5; H, 3.70; N, 19.7.

9-(p-Carbomethoxybenzyl)-hypoxanthine (XX).—A solution of 500 mg. (1.65 mmoles) of X in 50 ml. of methanol and 5 ml. of 1 *N* aqueous hydrochloric

acid was refluxed for 3.5 hours. Most of the solvent was spin evaporated *in vacuo*, then 25 ml. of water was added. The product was collected on a filter and recrystallized from methanol; yield, 350 mg. (75%) of white crystals, m.p. 288°; $\nu_{\text{max}}^{\text{KBr}}$ 1715 (ester C=O); 1600, 1555 (C=O, C=N, C=C); 1280 cm^{-1} (ester C—O—C).

Anal.—Calcd. for $\text{C}_{14}\text{H}_{12}\text{N}_4\text{O}_3$: C, 59.1; H, 4.27; N, 19.7. Found: C, 58.9; H, 4.06; N, 19.5.

9-(p-Carboxybenzyl)-hypoxanthine (XIX).—A solution of 151 mg. (0.50 mmole) of X in 7 ml. of concentrated hydrochloric acid was refluxed for 2.5 hours with magnetic stirring during which time the product separated. The mixture was diluted with 15 ml. of water and cooled. The product was collected on a filter and washed with water; yield, 120 mg. (89%), m.p. 324–327°. Recrystallization from 2-methoxyethanol gave white crystals, m.p. 330–331°; $\nu_{\text{max}}^{\text{KBr}}$ 1670 (carboxyl C=O); 1600, 1580 cm^{-1} (C=O, C=C, C=N).

Anal.—Calcd. for $\text{C}_{13}\text{H}_{10}\text{N}_4\text{O}_3$: C, 57.8; H, 3.77; N, 20.7. Found: C, 57.5; H, 3.91; N, 20.9.

9-(4'-Carboxybutyl)-hypoxanthine (XXI).—Hydrolysis of 235 mg. (1 mmole) of XI, as described for the preparation of XIX gave, after recrystallization from 2-methoxyethanol, 150 mg. (64%) of product, m.p. 260–261°; $\nu_{\text{max}}^{\text{KBr}}$ 1690 (carboxyl C=O); 1650, 1600 (C=C, C=N, C=O).

Anal.—Calcd. for $\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_3$: C, 50.9; H, 5.14; N, 23.8. Found: C, 50.9; H, 5.22; N, 23.9.

9-(4'-Carboxybutyl)-adenine (XVIII).—Hydrolysis of 216 mg. (1 mmole) of XVII with concentrated hydrochloric acid for 3 hours, as described for the preparation of XIX, gave 190 mg. (81%) of product, m.p. 267–270°. Recrystallization from 2-methoxyethanol gave 140 mg. (60%) of white crystals, m.p. 272–273°.

Anal.—Calcd. for $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_2$: C, 51.1; H, 5.58; N, 29.8. Found: C, 51.0; H, 5.63; N, 30.0.

9-(4'-Carboxybutyl)-6-mercaptapurine (XXV).—A mixture of 233 mg. (1 mmole) of XXIV and 10 ml. of concentrated hydrochloric acid was refluxed for 6 hours, then cooled and diluted with 25 ml. of ice water. The crude product was collected on a filter and washed with water, then dissolved in excess 5% aqueous sodium bicarbonate. Acidification gave 90 mg. (36%) of tan crystals, m.p. 257–258°. Recrystallization from 2-methoxyethanol gave an analytical sample, m.p. 258–259°; $\nu_{\text{max}}^{\text{KBr}}$ 1710 (carboxyl C=O); 1600, 1575, 1545 cm^{-1} (C=C, C=N).

Anal.—Calcd. for $\text{C}_{10}\text{H}_{12}\text{N}_4\text{O}_2\text{S}$: C, 47.7; H, 4.82; N, 22.2. Found: C, 47.7; H, 4.90; N, 22.1.

Results

By plotting V_0/V against I for four or more concentrations of I , the concentration of I necessary to give 50% inhibition ($V_0/V_1 = 2$) was readily determined, where V_0 = velocity of the enzymic reaction with no inhibitor, V_1 = velocity of reaction in the presence of inhibitor, and I = concentration of inhibitor (17). At least two of the points should be in the 30–70% inhibition range where the least error occurs. In a few cases where a saturated solution gave only 35–49% inhibition, such as with compound XX, the plot was extended to $V_0/V = 2$ to determine the concentration of I that would

TABLE I.—50% INHIBITION OF LDH AND GDH BY NUCLEOTIDE ANALOGS

Group	Compound ^a	mM Concentration for 50% Inhibition	
		LDH	GDH
A	AMP	6.7	35
	IMP	67	66
B	Carboxybenzyl-Ad (XVI)	15	27
	Carboxybenzyl-Hx (XIX)	22	64
C	Carboxybutyl-Ad (XVIII)	30	40
	Carboxybutyl-Hx (XXI)	82	D ^c
	Valerate	340	210
D	Benzoate	27	95
	<i>p</i> -Toluate	24	
	{ Adenosine }	17	B ^c
	{ Benzoate }		

^a For abbreviations see Footnote 1. ^b Equal concentrations of both compounds. ^c Compound not sufficiently soluble to determine 50% inhibition. See this group in Table II for partial inhibition data.

give 50% inhibition. These 50% inhibition values for LDH and GDH are listed in Table I.

In cases where a compound gave less than 35% inhibition with a saturated solution, a group run was made for simultaneous comparison of two to four compounds at the same concentration (Table II); if one of the compounds in the group did give a normal 50% inhibition plot, the inhibition at lower concentration was taken from V_0/V_1 versus I plot—for example compound XVIII in Table II, group C.

DISCUSSION

Both riboflavin-5'-sulfate and adenosine-5'-sulfate have been observed to be flavin-adenine dinucleotide inhibitors in the D-amino acid oxidase system (31, 32). In addition, AMP, IMP, and adenosine have been found to be inhibitors in alcohol dehydrogenase systems, whereas nicotinamide mononucleotide

was not (33, 34). ADP-ribose, an hydrolysis product of DPN is a strong competitive inhibitor of LDH (35) and alcohol dehydrogenase (36). Therefore it was not unreasonable to expect that AMP would be an inhibitor in the LDH and GDH systems.

That AMP was an inhibitor in both the LDH and GDH systems is shown in Table I. Attempts to show that AMP was a "competitive" DPN inhibitor by Lineweaver-Burk plots were not conclusive since results varied between "competitive" and "partially competitive" (20, 37). Therefore, we chose to rely on molecular changes in inhibitor structure to determine whether the synthetic compounds were binding to the enzymes in the same manner as AMP, an approach which Reiner (38) considers to be more definitive and with which we concur (17). Thus, both the LDH and GDH systems could be used to search for chemical groupings that simulate the 5'-phosphoribosyl moiety of AMP as an inhibitor.

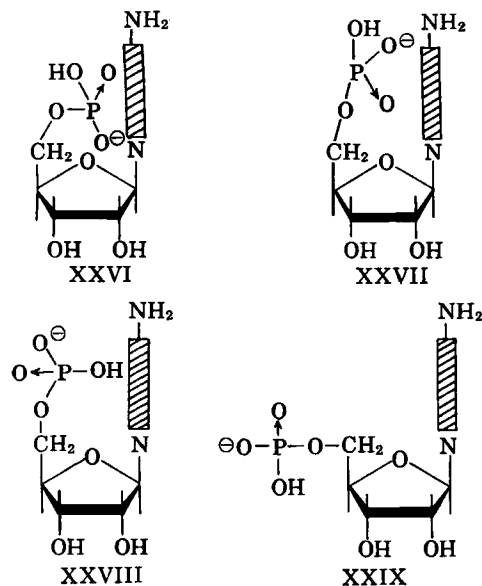
To check on the mode of binding of AMP to LDH and GDH, IMP and adenosine were measured as inhibitors. IMP inhibited both LDH and GDH, but considerably higher concentrations than AMP were needed for 50% inhibition (Table I). It can be concluded that the adenine moiety is necessary for binding to both enzymes, and the hypoxanthine moiety binds less effectively, probably because it is less basic. Adenosine was not sufficiently soluble to determine the concentration necessary for 50% inhibition. However, 7 mM adenosine gave no measurable inhibition of LDH, contrasted to AMP at 6.7 mM which gave 50% inhibition (Table I). Similarly, 15 mM adenosine gave only 7% inhibition of GDH, whereas AMP at 15 mM gave 29% inhibition (Table II, group C). Therefore it can be concluded that the phosphate group of AMP is necessary for binding to LDH and GDH when AMP acts as an inhibitor.

In the AMP structure, the purine ring has a rigid structure and the furanose ring can vary only slightly in conformation because of its slight pucker. The main possible changes in conformation of binding of AMP to an enzyme is because of the free rotation of the 1'-9 bond and all of the bonds of the phosphomethylene group. Examination of Leybold

TABLE II.—PARTIAL INHIBITION OF LDH AND GDH BY NUCLEOTIDE ANALOGS

Group	Enzyme	Compound ^a	mM Conc.	V_0/V_1	Inhibition, %
A	LDH	Valerate	18	1.15	13
		{ Adenosine }	18	1.19	16
		{ Valerate }			
		Carboxybutyl-Ad (XVIII)	18	1.60	38
B	GDH	Benzoate	30	1.12	11
		AMP-2'(3')	30	1.12	11
		{ Benzoate }	30	1.16	16
		{ AMP-2'(3') }			
		Carboxybenzyl-Ad (XVI)	30	2.30	67
C	GDH	AMP	30	1.88	47
		Valerate	15	1.00	0
		Adenosine	15	1.07	7
		{ Adenosine }	15 ^b	1.12	11
		{ Valerate }			
		Carboxybutyl-Ad (XVIII)	15	1.35	26
		AMP	15	1.40	29
D	GDH	Carboxybutyl-Ad (XVIII)	52	2.30	67
		Carboxybutyl-Hx (XXI)	52	1.23	19

^a For abbreviations see Footnote 1. ^b Equal concentrations of both compounds.



models shows that there are two conformations of AMP with extremes in the distance of the $\text{P-O}^\ominus$ from the 6-amino group of AMP³, 13.0 Å. (XXIX) and 3.0 Å. (XXVII). Another way of measuring the relative conformation is the distance between the $\text{P-O}^\ominus$ and the 9-nitrogen; in the case of XXIX, this distance³ is 9.0 Å. Another type of extreme conformation is XXVI. In this case the $\text{P-O}^\ominus$ is tucked in towards the 9-nitrogen and the two atoms can almost touch. The latter conformation for enzyme binding is highly improbable because of the crowding of these ionic binding groups and probable steric hindrance to ionic binding to the enzyme. However, if the $\text{P-O}^\ominus$ and 9-nitrogen were separated about 3 Å., this type of conformation would be possible. One of the many possible intermediate conformations is shown by structure XXVIII.

Two side chains were selected for investigation to determine whether the 5'-phosphoribose moiety of AMP could be simulated in the binding of AMP to GDH or LDH or both. 9-(*p*-Carboxybenzyl)-adenine (XVI) has a fixed relationship between the 9-nitrogen of the adenine moiety and the charge

of the $\text{O}^\ominus$ of 8.6°. This distance compares favorably to that of 9.0 Å. between the $\text{O}^\ominus$ and 9-N of AMP³ for the most extended conformation of AMP (XXIX). Slight rotation of the phosphate of XXIX group can bring this distance down to 8.6 Å. The most extended conformation of AMP and XVI both have a distance between the nitrogen of the 6-amino group³ and the anionic group of 13 Å. All of the *p*-carboxybenzyl group except one hydrogen fits within the space occupied by the 5'-phosphoribosyl group. Thus XVI has the binding potential of the most extended conformation of AMP.

The second compound chosen was (4'-carboxybutyl)-adenine (XVIII). This compound has a flexible side chain which can vary in conformation

from nearly the most extended conformation of AMP (XXIX) to nearly the least extended (XXVII) and include other conformations such as XXVI and XXVIII. In most (if not all cases) the bulk of the carboxybutyl side chain fits within the space occupied by the ribose-5'-phosphate moiety.

To determine whether 9-(*p*-carboxybenzyl)-adenine (XVI) and 9-(4'-carboxybutyl)-adenine (XVIII) simulate the inhibitor properties of AMP for LDH and GDH, three criteria must be met:

1. The compound should inhibit the same magnitude as AMP.
2. The hypoxanthine analog must inhibit less effectively than the adenine analog.
3. A given concentration of a compound such as XVI with two ionic points of attachment should show considerably greater inhibition than a solution of equal concentration of both of its moieties with one ionic point of attachment, such as benzoate and adenine. Actually adenosine and AMP-2'(3') were used in place of adenine since the latter was not sufficiently soluble and has an undesirable acidic NH.

9-(*p*-Carboxybenzyl)-adenine (XVI) was an inhibitor of both LDH and GDH (Table I, group B) but inhibited in different ways. In the case of GDH, all three criteria for simulating AMP were met. First, XVI inhibited GDH slightly better than AMP. Second, 9-(*p*-carboxybenzyl)hypoxanthine (XIX) was required at 2.4 times the concentration of XVI to give 50% inhibition (Table I, group B); IMP was required in 1.9 times the concentration of AMP to give 50% inhibition, a favorable comparison. Third, 30 mM each of benzoate and AMP-2'(3') in the same solution gave only 16% inhibition, whereas 30 mM of XVI gave 67% inhibition. The simulation of the inhibition of AMP by XVI is evidence that AMP has the fully extended conformation of the 5'-phosphoribosyl moiety when AMP binds to GDH, as discussed earlier.

With LDH, the criteria for simulating AMP were not met by 9-(*p*-carboxybenzyl)-adenine (XVI). First, the compound inhibited LDH half as well as did AMP (Table I). However, at only 1.5 times the concentration of XVI, 9-(*p*-carboxybenzyl)-hypoxanthine (XIX) gave 50% inhibition; this was far too inhibitory since there is a tenfold difference in concentration between AMP and IMP necessary for 50% inhibition. The reason for the high inhibition of XIX is apparent when one notes that 24 mM *p*-toluate gives 50% inhibition and benzoate at 27 mM (Table I); apparently XIX binds primarily as a derivative of benzoate and XIX has no second ionic binding point as required in the simulation of AMP. In addition, the third criterion was not met, since 17 mM concentrations of benzoate and adenosine in the same solution gave the same inhibition as 15 mM of XVI (Table I, group D). Apparently, XVI does not bind in a two ionic point attachment to LDH, but the adenine moiety of one molecule and the carboxybenzyl moiety of another molecule bind to one enzymic active site. Since the binding of AMP to LDH cannot be simulated by XVI, it can be concluded that AMP most probably does not have the most extended conformation of its ribose phosphate moiety when bound to LDH.

9-(4'-Carboxybutyl)-adenine (XVIII) was an

³ Distances measured from the center of the $\text{O}^\ominus$ and N atoms.

inhibitor for both enzymes (Table I), and in both cases XVIII met the three criteria for simulating AMP. With GDH, XVIII gave 50% inhibition at only a slightly higher concentration than that required for AMP. The hypoxanthine analog (XXI) was not sufficiently soluble to determine the 50% inhibition point; therefore the equal concentration comparison listed in Table II, group D was made. At the highest obtainable concentration (52 mM), the hypoxanthine analog (XXI) gave 19% inhibition, whereas 9-(4'-carboxybutyl)-adenine (XVIII) gave 67% inhibition at 52 mM, thus meeting the second criterion. Third, in Table II, group C, 15 mM each of a mixture of valerate and adenosine gave only 11% inhibition, whereas 15 mM 9-(4'-carboxybutyl)-adenine (XVIII) gave 26% inhibition and 15 mM AMP gave 29% inhibition.

With LDH, a 30 mM concentration of the adenine analog (XVIII) was needed to give 50% inhibition, compared to 6.7 mM AMP. The other two criteria for simulation of AMP by XVIII were also met. As shown in Table I, group C, the AMP analog (XVIII) showed 50% inhibition at about one-third the concentration of the IMP analog (XXI). XVIII and XXI do not show the tenfold spread in concentration shown by AMP and IMP, for 50% inhibition of LDH is apparently due to appreciable one ionic-point attachment of the valerate side chain at the high concentration (82 mM) necessary for 50% inhibition. As shown in Table II, group A, 9-(4'-carboxybutyl)-adenine (XVIII) shows considerably higher inhibition than a solution of equal concentrations of valerate and adenosine.

In view of the reference material at the beginning of the *Discussion*, the data obtained in this paper, and the similarity in structure between AMP and half of the DPN molecule, it is probable that AMP binds to LDH and GDH in the same part of the active site as the AMP half of DPN. It follows that DPN probably has two different conformations when bound to LDH and GDH. The evidence is strong that 9-(4'-carboxybutyl)-adenine (XVIII) can simulate either conformation that AMP assumes when bound to these two different enzymes; it is probable that most of the conformations that AMP can assume when bound to other enzymes can be simulated by XVIII.

Side chains in addition to the *p*-carboxybenzyl group with a fixed conformation—or a limited locus of conformations—should be more specific as inhibitors when they simulate AMP. Of course these arguments can also apply to nucleotide substrates of purine and pyrimidine bases. One could even speculate that substrate-identical enzymes from different tissues might be expected to have different conformational requirements for the substrate.

In our opinion, this pilot study on simulation of the inhibitor properties of AMP against LDH and GDH by adenine derivatives substituted with an

anionic side chain has been sufficiently definitive to warrant further study of these compounds including the mercaptopurines (XXIII, XXV) as inhibitors of enzymes interconverting nucleotides. By blocking the carboxylate group, a variety of possibilities for laticiation are possible to aid transport and membrane permeability *in vivo*, if necessary.

The search for other anionic side chains, particularly with a limited locus of conformational possibilities, attached to purines and pyrimidines that may inhibit biosynthesis of nucleic acid precursors is also warranted.

REFERENCES

- (1) Baker, B. R., and Patel, R. P., *THIS JOURNAL*, **52**, 927(1963).
- (2) Welch, A. D., *Cancer Res.*, **11**, 1475(1961).
- (3) Hartmann, K.-U., and Heidelberger, C., *J. Biol. Chem.*, **236**, 3006(1961).
- (4) Flaks, J. G., and Cohen, S. S., *ibid.*, **234**, 2981(1961).
- (5) Brockman, R. W., *Cancer Res.*, **20**, 643(1960).
- (6) Carter, C. E., *Biochem. Pharmacol.*, **2**, 105(1959).
- (7) Salser, J. S., Hutchison, D. J., and Balis, M. E., *J. Biol. Chem.*, **235**, 429(1960).
- (8) Davidson, J. D., *Cancer Res.*, **20**, 225(1960).
- (9) Brockman, R. W., *Clin. Pharmacol. Therap.*, **2**, 237(1961).
- (10) Tomizawa, S., and Aronow, L., *J. Pharmacol. Exptl. Therap.*, **128**, 107(1960).
- (11) Roll, P. M., Weinfeld, H., Carroll, E., and Brown, G. B., *J. Biol. Chem.*, **220**, 439(1956).
- (12) Montgomery, J. A., Thomas, H. J., and Schaeffer, H. J., *J. Org. Chem.*, **26**, 1929(1961).
- (13) Montgomery, J. A., private communication.
- (14) Magasanik, B., Moyed, H. S., and Gehring, L. B., *J. Biol. Chem.*, **226**, 339(1957).
- (15) Lagerkvist, U., *ibid.*, **233**, 138(1958).
- (16) Lieberman, A., *ibid.*, **223**, 327(1956).
- (17) Baker, B. R., Lee, W. W., Skinner, W. A., Martinez, A. P., and Tong, E., *J. Med. Pharm. Chem.*, **2**, 633(1960).
- (18) Baker, B. R., Lee, W. W., and Tong, E., *J. Theoret. Biol.*, **3**, 458(1962).
- (19) Neilands, J. B., "Methods in Enzymology," Vol. I, edited by Colowick, S. P., and Kaplan, N. O., Academic Press, Inc., New York, N. Y., 1955, p. 449.
- (20) Caughey, W. S., Smiley, J. D., and Hellerman, L., *J. Biol. Chem.*, **224**, 591(1957).
- (21) Baker, B. R., Schaub, R. E., and Joseph, J. P., *J. Org. Chem.*, **19**, 638(1954).
- (22) Brederick, H., Ulmar, H., and Waldmann, H., *Chem. Ber.*, **89**, 12(1956).
- (23) Albert, A., and Brown, D. J., *J. Chem. Soc.*, **1954**, 2060.
- (24) Montgomery, J. A., and Temple, C., Jr., *J. Am. Chem. Soc.*, **83**, 630(1961).
- (25) *Ibid.*, **79**, 5239(1957).
- (26) Montgomery, J. A., *ibid.*, **78**, 1928(1956).
- (27) Goldman, L., Marsico, J. W., and Gazzola, A. L., *J. Org. Chem.*, **21**, 599(1956).
- (28) Baker, B. R., Querry, M. V., Kadish, A. F., and Williams, J. H., *ibid.*, **17**, 35(1952).
- (29) Schaeffer, H. J., and Weimar, R. D., Jr., *J. Am. Chem. Soc.*, **81**, 197(1959).
- (30) Fuson, R. C., and Cooke, H. C., Jr., *ibid.*, **62**, 1180(1940).
- (31) Yagi, K., Ozawa, T., and Okada, K., *Biochim. Biophys. Acta*, **35**, 102(1959).
- (32) Yagi, K., Ozawa, T., and Nagatsu, T., *ibid.*, **43**, 310(1960).
- (33) Hoch, F. L., Martin, R. G., Wacker, W. E. C., and Vallee, B. L., *Arch. Biochem. Biophys.*, **91**, 166(1960).
- (34) Li, T.-K., and Vallee, B. L., *Abst. 142nd Meeting American Chemical Society*, 1962, p. 67C.
- (35) Winer, A. D., *ibid.*, p. 23C.
- (36) Dalziel, K., *Biochem. J.*, June, 1962, 29P.
- (37) Friedenwald, J. S., and Maengwyn-Davies, G. D., in McElroy, W. D., and Glass, B., "The Mechanism of Enzyme Action," Johns-Hopkins Press, Baltimore, Md., 1954, p. 154.
- (38) Reiner, J. M., "Behavior of Enzyme Systems," Burgess Publishing Co., Minneapolis, Minn., 1959, pp. 151-152.