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INHIBITORY EFFECTS OF 2-THIOURACIL AND 2-THIOURACIL METABOLITES ON ENZYMES INVOLVED IN PYRIMIDINE NUCLEOTIDE BIOSYNTHESIS

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Abstract—The inhibitory effects of thiouracil, thiouridine and thio-UMP on enzymes in the pyrimidine pathways leading to nucleic acid synthesis were studied. Thiouracil, but not thiouridine, was a potent competitive inhibitor of rat liver uridine phosphorylase. However, thiouridine was a potent competitive inhibitor of rat liver uridine kinase, whereas thiouracil and other antithyroid drugs produced no effect. Thio-UMP was a weak inhibitor of this enzyme but was less active than UMP. Thiouracil and thio-UMP produced little inhibition of bacterial UMP pyrophosphorylase, although thiouracil is a good substrate. Thiouracil and its analogs had no measurable effect on calf thymus or baker's yeast OMP pyrophosphorylase, but thio-UMP inhibition of rat liver OMP decarboxylase was comparable to that obtained with UMP. The results presented demonstrate that there are three sites in the pyrimidine pathways where thiouracil or its metabolites may exert substantial inhibition. The enzymes primarily affected are uridine phosphorylase and uridine kinase which are inhibited by thiouracil and thiouridine, respectively, and OMP decarboxylase which is inhibited by thio-UMP. These results support the hypothesis that some of the biological effects of thiouracil may be due to an alteration in pyrimidine nucleotide synthesis.

THIOURACIL is a classical antithyroid drug which depresses the ability of the thyroid gland to produce and secrete thyroid hormones.[1] In addition, this compound alters the extrathyroidal metabolism and potency of thyroxine[2] and produces a high incidence of toxic effects[1] including leucopenia, agranulocytosis and drug fever. Paik and Cohen[3] also observed that thiouracil inhibited [^{14}C]leucine incorporation into carbamyl phosphate synthetase in the metamorphosing tadpole, and Paschkis *et al.*[4] found that 2-thiouracil decreased the incidence of hepatoma induced in rats by 2-acetaminofluorene. Numerous studies[5] have also demonstrated that thiouracil inhibits growth in a number of bacterial, viral and plant systems and is incorporated into viral and bacterial RNA replacing uracil. Due to the close structural similarities between uracil and thiouracil, there has been speculation that each of the various actions of thiouracil may involve an effect on some aspect of nucleic acid synthesis or function. Further speculation has suggested that thiouracil may be incorporated into RNA to form a faulty RNA or that thiouracil or its metabolites may block the synthesis of uridine nucleotides depressing RNA synthesis.[5]

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Recent studies of 2-thiouracil metabolism in our laboratory[6–8] demonstrated that all of the enzymes investigated which normally utilize uracil, uridine or UMP are capable of metabolizing the comparable thiouracil compound to form thiouridine, thio-UMP and other 2-thionucleotides and to incorporate thiouracil into RNA. This suggests that some degree of competition between uracil and thiouracil compounds must exist for each enzyme. Thiouracil effects on specific enzymes in the pyrimidine pathways received little attention in the past. However, Lindsay *et al.*[9] recently demonstrated that thiouracil was a potent inhibitor of uridine phosphorylase depressing uracil incorporation into rat liver RNA. Zamir and Ben-Ishai[10] also concluded that thiouracil, like uracil, might produce feedback inhibition of aspartate carbamylase synthesis in *Escherichia coli* altering pyrimidine nucleotide synthesis.

The primary objective of the present study was to carry out a systematic examination of the enzymes in the pyrimidine pathways to determine if thiouracil, thiouridine or thio-UMP are antimetabolites for normal substrates, which enzymes are most sensitive to their inhibitory effects and the degree of sensitivity of each enzyme. A demonstration of potent inhibition at one or more key enzyme sites in the pyrimidine pathways would greatly strengthen the hypothesis that thiouracil inhibition of pyrimidine nucleotide synthesis is the mechanism for some of the biological effects produced by thiouracil.

MATERIALS AND METHODS

Enzyme preparation and assay. Uridine phosphorylase (uridine:orthophosphate ribosyltransferase, EC 2.4.2.3) and uridine kinase (ATP:uridine 5′-phosphotransferase, EC 2.7.1.48) were prepared from fresh rat liver as previously described.[7] Uridine phosphorylase was assayed by the isotopic method of Lindsay *et al.*[11] and uridine kinase by the isotopic method described by Akamatsu *et al.*[12] Thymidine phosphorylase (thymidine:orthophosphate deoxyribosyltransferase, EC 2.7.7.8) was prepared from acetone powders of horse liver[7] and assayed according to a slight modification of the isotopic method of Krenitsky.[13] Thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) was prepared and assayed by slight modifications of methods described by Klemperer and Haynes.[14] UMP pyrophosphorylase (UMP:-pyrophosphate phosphoribosyltransferase, EC 2.4.2.9) was prepared from cultures of *Lactobacillus leichmannii* (ATCC 4749) grown in Micro Inoculum Broth (Difco) supplemented with 0·03% uracil by the method of Crawford *et al.*[15] The enzyme was assayed according to the isotopic procedure previously outlined.[6] OMP pyrophosphorylase (orotidine-5′-phosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.10) was prepared as crude extracts from autolysates of baker's yeast by the method of Lieberman *et al.*[16] and from calf thymus as described by Lindsay *et al.*[9] Assays were carried out according to the method of Lieberman *et al.*[16] measuring $^{14}CO_2$ release from carboxyl labeled orotic acid on conversion to UMP in the presence of excess OMP decarboxylase.

OMP decarboxylase (orotidine-5′-phosphate carboxylase, EC 4.1.1.23) was prepared from rat liver according to the procedure described by Creasey and Handschumacher[17] and assayed by a modification of the method of Appel.[18] The incubation medium contained 0·5 nmole [carboxy-^{14}C]OMP, 2 nCi; 12·5 μmoles Tris–HCl, pH 7·5; 50 μg enzyme protein, 1·5 mg albumin and usually various amounts of UMP or thio-UMP in a final volume of 0·5 ml. Incubation was carried out in air at 37°

for 15 min in a stoppered tube with a removable center well containing 1·0 ml Hyamine. The reaction was terminated by the injection of 0·3 ml of 4 N $HClO_4$. $NaHCO_3$ (0·1 μmole in 0·1 ml) was also injected into each vessel and the tubes were shaken for an additional 60 min. The Hyamine was then quantitatively transferred to counting vials and radioactivity determined in a Packard liquid scintillation spectrometer.

Protein determinations. Protein determinations were carried out by the method of Lowry *et al.*[19] using crystalline bovine albumin as the standard. Unless otherwise indicated, specific activities represent μmoles of product formed/mg protein/hr.

General. [2-^{14}C]uridine, [2-^{14}C]uracil, [2-^{14}C]thymidine, [2-^{14}C]thymine, [carboxy-^{14}C]orotic acid and [carboxy-^{14}C]OMP (triammonium salt) were obtained from New England Nuclear Corp. 2-Thiodeoxyuridine and 2-thio-UMP were prepared by enzymatic methods described by Lindsay *et al.*[20] and 2-thiouridine was prepared by a chemical method presented by Brown *et al.*[21] All determinations of radioactivity were made with a Packard Tri-Carb liquid scintillation counter.

RESULTS

The pertinent features of three of the pyrimidine pathways investigated are presented in the scheme shown in Fig. 1.

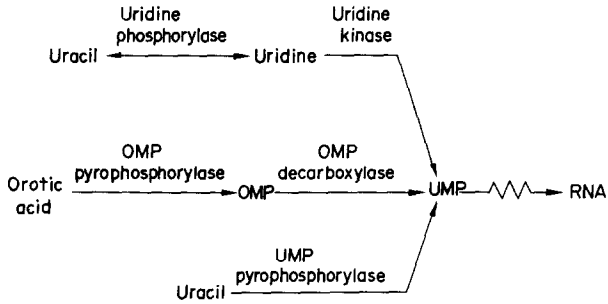

FIG. 1. Scheme showing pertinent pyrimidine pathways investigated.

TABLE 1. INHIBITORY EFFECTS OF 2-THIOURIDINE ON URIDINE PHOSPHORYLASE*

Thiouridine (μmoles)	Thiouridine/ uridine	Uracil formed (μmoles/hr/mg prot.)	Inhibition (%)
0	0	0·71 ± 0·03	0
0·46	4·7	0·67 ± 0·02	5·8
0·92	9·4	0·64 ± 0·03	10·4
2·3	24·0	0·62 ± 0·03	12·9
4·6	48·0	0·52 ± 0·02	27·0

*The incubation medium contained 0·097 μmole [2-^{14}C]uridine, 0·045 μCi; 25 μmoles potassium phosphate, pH 8·1; 20 μmoles Tris-HCl, pH 8·1; 82 μg enzyme protein and various amounts of 2-thiouridine in a final volume of 0·4 ml. After 15 min of incubation at 37°, the reaction was stopped by heating in a boiling water bath for 3 min. The values presented are means ± S.E. of six determinations.

Uridine phosphorylase. It was demonstrated previously that uridine phosphorylase catalyzed the reversible conversion of a small amount of thiouracil to thiouridine with the reaction occurring more readily in the reverse direction.[7] Since thiouracil was a potent inhibitor of this enzyme, the effects of thiouridine were also examined (Table 1). Chromatographically pure thiouridine produced little inhibition of the reaction, depressing only 27 per cent at a concentration 48 times greater than uridine. This is in marked contrast to the effects noted previously with thiouracil which produced an inhibition of 62 per cent at concentrations nearly equimolar to uridine.[9]

Kinetic studies were carried out with the enzyme using uridine as a substrate and Dixon plots showed that the inhibition produced by thiouridine was competitive and that the K_i for thiouridine was $1{\cdot}02 \times 10^{-2}$ M (data not shown).

Uridine kinase. Thiouridine utilization as a substrate by rat liver uridine kinase suggested that this nucleoside might also be an inhibitor of uridine metabolism by the enzyme. The effects of thiouridine were investigated and marked inhibition of enzyme activity was observed. Dixon plots of activity at various inhibitor concentrations (Fig. 2) indicated that the thiouridine inhibition was competitive and that the K_i for thiouridine was $6{\cdot}3 \times 10^{-5}$ M. This value is only slightly higher than the

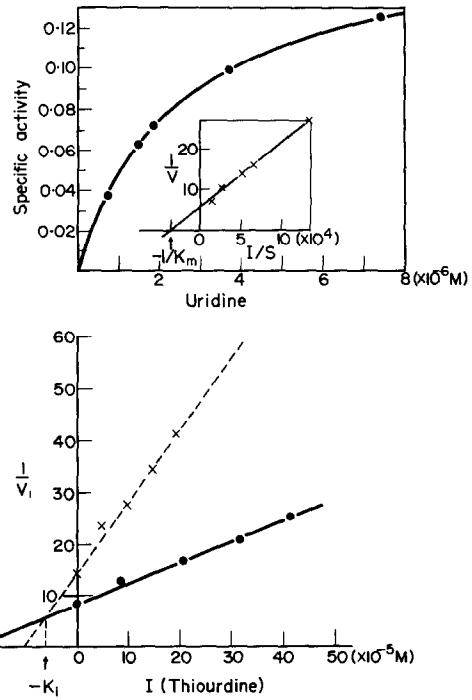

FIG. 2. Kinetics of uridine kinase utilization of uridine and inhibition by thiouridine. For determination of the K_m for uridine (top), the reaction medium contained various concentrations of [2-^{14}C]uridine, 10 μmoles $MgCl_2$, 15 μmoles ATP, 11·3 μmoles Tris–HCl, pH 7·4, and 13·3 μg enzyme protein in a final volume of 0·3 ml. Incubation was for 10 min at 37°. For determinations of the K_i value for thiouridine (bottom), the reaction medium was the same as above except that the concentration of [2-^{14}C]uridine was either 0·0224 μmole/0·2 μCi (solid line) or 0·0056 μmole/0·05 μCi (broken line) and various concentrations of thiouridine were added.

K_m of $2{\cdot}8 \times 10^{-5}$ M for uridine, demonstrating that thiouridine is a potent competitive inhibitor of rat liver uridine kinase.

The effects of thiouracil, propylthiouracil, methylthiouracil and methimazole on the activity or uridine kinase were also examined and compared with that of thiouridine (data not shown). At levels 20 times higher than the $5{\cdot}56 \times 10^{-5}$ M concentration of the uridine substrate, none of the drugs tested exerted any measurable inhibition on uridine kinase, whereas thiouridine under the same conditions inhibited the activity of the enzyme 80 per cent or more.

Pyrimidine or purine analogs may regulate nucleotide and RNA synthesis by product or feedback inhibition.[22] UMP is the normal product of uridine kinase action, and its inhibitory effect on this enzyme was compared to thio-UMP, the product formed from thiouridine (Table 2). Both thio-UMP and UMP exerted inhibitory effects, but neither was very potent. The inhibitory effects of thio-UMP at levels 5, 10 and 25 times the $3{\cdot}7 \times 10^{-5}$ M concentration of the uridine substrate were essentially the same, whereas at a concentration 50 times that of the substrate, depression increased to 33·8 per cent. UMP also produced little inhibition except at high UMP to uridine ratios but appeared to be slightly more potent as an inhibitor than thio-UMP. In contrast, thiouridine produced potent inhibition at relatively low concentrations.

OMP pyrophosphorylase. The effect of thiouracil on OMP pyrophosphorylase from bovine thymus was also investigated (data not shown). Levels of thiouracil up to 10 times the $2{\cdot}5 \times 10^{-5}$ M concentration of the orotic acid substrate had no effect on the enzyme. Similar negative results were obtained with an OMP pyrophosphorylase preparation from baker's yeast. Other antithyroidal drugs such as propylthiouracil, methylthiouracil and methimazole were also tried but no measurable inhibition was observed.

TABLE 2. INHIBITORY EFFECTS OF 2-THIO-UMP, UMP AND 2-THIOURIDINE ON URIDINE KINASE*

Inhibitor	Concn ($\times 10^{-4}$ M)	Sp. act.	Inhibition (%)
None		0·311 ± 0·018	
Thio-UMP	1·85	0·248 ± 0·013	20·3
	3·7	0·264 ± 0·008	15·1
	9·25	0·254 ± 0·018	18·3
	18·5	0·206 ± 0·010	33·8
UMP	1·85	0·265 ± 0·016	14·8
	3·7	0·247 ± 0·014	20·6
	9·25	0·212 ± 0·013	31·8
	18·5	0·179 ± 0·009	42·4
Thiouridine	0·925	0·204 ± 0·01	36·4
	1·85	0·147 ± 0·008	52·7

* The reaction mixture contained 0·0112 μmole [2-^{14}C]uridine, 0·1 μCi; 15 μmoles ATP; 10 μmoles $MgCl_2$; 11·3 μmoles Tris–HCl, pH 7·4; 29 μg enzyme protein and various amounts of thio-UMP, UMP and thiouridine in a final volume of 0·3 ml. The final concentration of uridine was $3{\cdot}7 \times 10^{-5}$ M. Total incubation period was 10 min. The values presented are means ± S. E. of six determinations.

OMP decarboxylase. OMP decarboxylase is one of the key enzymes in the *de novo* pathway of pyrimidine biosynthesis.[16] The effects of thio-UMP on rat liver OMP decarboxylase were compared with those of UMP. Both nucleotides inhibited the enzyme, with thio-UMP being less effective than UMP at all concentrations examined. The data obtained indicated that UMP was a competitive inhibitor with a K_i value of $7{\cdot}8 \times 10^{-5}$ M, as determined by the Dixon plot shown in Fig. 3. This is similar to the $1{\cdot}5 \times 10^{-4}$ M value previously reported for rat liver enzyme.[17] Results with thio-UMP indicated that this nucleotide was also a competitive inhibitor of the enzyme with a K_i of $2{\cdot}8 \times 10^{-4}$ M, demonstrating that the potencies of these two nucleotides as inhibitors of OMP decarboxylase are comparable.

UMP pyrophosphorylase. Lindsay *et al.*[6] showed that thiouracil could be utilized as a substrate by UMP pyrophosphorylase to form thio-UMP. In view of thiouracil's participation in the reaction, the possible competition between uracil and thiouracil was investigated and the influence of the product, thio-UMP, examined to determine if it exerted any "feedback" or product inhibition. The results are presented in Table 3. Thiouracil at levels up to 100 times the 1×10^{-5} M concentration of the uracil substrate had no detectable effect on enzyme activity. Inhibition of a modest 21 per cent was observed only after the thiouracil concentration was increased to a level 250 times that of uracil. UMP inhibition of the reaction was noticeable at a UMP to uracil ratio of 5 and increased to 79 per cent at a ratio of 100. Inhibition

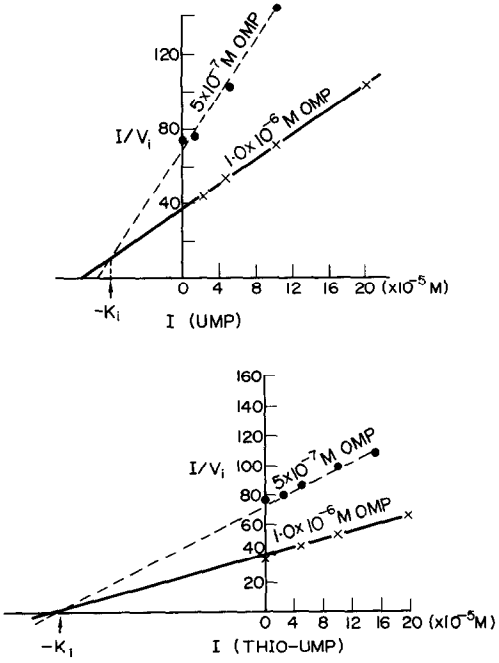

FIG. 3. Kinetics of UMP and thio-UMP inhibition of OMP decarboxylase. For determination of the K_i for UMP (top), the reaction medium contained either $5{\cdot}0 \times 10^{-4}$ μmoles/0·002 μCi (solid line) or $2{\cdot}5 \times 10^{-4}$ μmoles/0·001 μCi (broken line) [carboxy-^{14}C]OMP; 12·5 μmoles Tris–HCl, pH 7·5, 0·050 mg enzyme protein, 1·5 mg albumin and various concentrations of UMP in a final volume of 0·5 ml. Incubation was for 15 min at 37°. The K_i for thio-UMP (bottom) was determined under identical conditions with the concentrations of thio-UMP being varied as indicated.

TABLE 3. EFFECTS OF VARIOUS COMPOUNDS ON THE CONVERSION OF URACIL TO UMP BY UMP PYROPHOSPHORYLASE*

Compound/uracil	Inhibition of UMP formation (%)		
	Thiouracil	UMP	Thio-UMP
1	0	4 ± 0·7	0
5	0	20 ± 1·3	0
10	0	32 ± 1·3	0
50	0	64 ± 1·9	21 ± 1·3
100	0	79 ± 3·1	
250	21 ± 1·4		

* [2-^{14}C]uracil (0·025 μCi) was present in each vessel at a final concentration of 1×10^{-5} M. The specific activity of the enzyme in the absence of inhibitor was 0·488 ± 0·027. The values presented are means ± S. E. of six determinations.

with thio-UMP was less or completely absent at all comparable concentrations. In contrast to the extremely weak thiouracil inhibition of uracil metabolism, uracil at a level one-half the 1×10^{-5} M concentration of the substrate inhibited thiouracil conversion to thio-UMP more than 95 per cent (data not shown) as would be predicted from the K_m values of these two compounds for the enzyme.[6]

Thymidine phosphorylase. Both thiouracil and uracil are substrates for thymidine phosphorylase forming either the nucleoside or deoxynucleoside depending upon the pentose provided.[23,24] Thiouracil did not alter the enzymatic utilization of the natural substrate, thymine, at thiouracil levels as high as 40 times the $2{\cdot}5 \times 10^{-4}$ M concentration of thymine (data not shown). In addition, neither propylthiouracil nor methimazole showed any measurable effect on the enzyme even when the concentration of antithyroid drug was 80 times that of the substrate. From these results, thiouracil appears to have little inhibitory effect on the activity of thymidine phosphorylase.

Thymidine kinase. The effects of thiouracil and thiodeoxyuridine on thymidine kinase metabolism of thymidine were also determined (data not shown). Thiouracil, propylthiouracil and methimazole at levels up to 118 times the $3{\cdot}4 \times 10^{-6}$ M concentration of thymidine had no effect on thymidine utilization. In addition, thiodeoxyuridine at concentrations up to 140 times those of thymidine was also ineffective. The enzyme was also unaffected by uridine or thiouridine at concentrations 240 times those of thymidine. Since none of the compounds examined was an important inhibitor of the enzymes in the thymidine phosphorylase–thymidine kinase pathway, it appears that thiouracil, thiouracil metabolites and thiourea derivatives are not likely to inhibit this pathway sufficiently to alter DNA synthesis.

DISCUSSION

The data presented in this communication and elsewhere[9] demonstrate that the antithyroid drug thiouracil and its metabolites affect several enzymes in the pyrimidine pathways which have important roles in nucleotide synthesis. Three enzymes were identified as sites at which substantial inhibition might be exerted. These enzymes are uridine phosphorylase and uridine kinase, which are inhibited by thiouracil and thiouridine, respectively, and OMP decarboxylase which is inhibited

by thio-UMP. In each case, inhibition involved competition between the thiouracil derivative and a comparable uracil or orotic acid compound.

Skold[25] demonstrated a close correlation between the activities of uridine phosphorylase and uridine kinase and the growth rate in a number of systems, and suggested that inhibition of this pathway might be a mechanism for the inhibition of RNA and protein synthesis. Lindsay *et al.*[9] showed that uridine phosphorylase had a K_m value for uracil of $2{\cdot}6 \times 10^{-4}$ M and for uridine in the reverse reaction of $1{\cdot}2 \times 10^{-4}$ M. The K_i value for thiouracil, a competitive inhibitor in both directions was $1{\cdot}2 \times 10^{-4}$ M. These results suggest that the affinity of thiouracil for the enzyme is of the same order as that for uracil. Uridine kinase was not inhibited by thiouracil but was markedly inhibited in a competitive manner by thiouridine. Thiouridine had a K_i value of $6{\cdot}3 \times 10^{-5}$ M compared to a K_m of $2{\cdot}8 \times 10^{-5}$ M for uridine, indicating that the enzyme had an affinity for thiouridine which was only slightly less than that for the natural substrate. Thus, both enzymes in the two-step uracil pathway can be markedly inhibited by the corresponding thiouracil compound and represent sites at which utilization of uracil or uridine for RNA synthesis could be blocked.

The orotic acid pathway represents the exclusive pathway in most systems for the synthesis of pyrimidines from small molecular weight precursors. In this pathway, UMP inhibition of OMP decarboxylase by negative "feedback" inhibition is apparently involved in the regulation of pyrimidine nucleotide availability in the cell.[17,22] Neither thiouracil nor any other antithyroid drug tested produced measurable inhibition of OMP pyrophosphorylase; however, thio-UMP, like UMP, was an inhibitor of OMP decarboxylase. Decarboxylation of OMP to form UMP was not as sensitive to thio-UMP with a K_i value of $2{\cdot}8 \times 10^{-4}$ M as it was to UMP with a K_i of $7{\cdot}8 \times 10^{-5}$ M. However, the order of potency was not vastly different, and this could represent an important site at which inhibition by thiouracil metabolites could occur.

Although thiouracil or its metabolites *in vitro* have been shown to produce substantial inhibition of three important enzymes in the pyrimidine pathways, it is not known how effective these substances are in producing alterations *in vivo*. Inhibition *in vivo* would occur only if appropriate concentrations of thiouracil or its metabolites were achieved. Metabolism of thiouracil to inhibitory metabolites can occur by several pathways,[6–8] and the necessary enzymes are generally widely distributed in biological systems. The formation of thiouridine and thio-UMP occurs less efficiently than the comparable uracil compound, but further metabolism is also less efficient and accumulation might occur. This is especially true of thio-UMP which is a poor substrate for nucleoside mono- and diphosphokinase.[26]

Inhibition of specific enzyme sites in the pyrimidine pathways suggests that some biological effects of thiouracil may be related to the antimetabolic actions of thiouracil and its metabolites. Furthermore, the data available suggest that thiouracil metabolites may have a propensity to accumulate. However, the biological significance of thiouracil inhibition *in vivo* cannot be reliably assessed until more information is available concerning pool sizes of both the antimetabolites and the natural substrates *in vivo*.

REFERENCES

1. E. B. ASTWOOD, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. GOODMAN and A. GILMAN), 4th Edn, p. 1466. MacMillan, New York (1970).

2. G. Morreale de Escobar and F. Escobar del Rey, *Recent Prog. Horm. Res.* **23,** 87 (1967).
3. W. K. Paik and P. P. Cohen, *J. biol. Chem.* **236,** 531 (1961).
4. K. E. Paschkis, A. Cantarow and J. Stasney, *Science, N.Y.* **114,** 264 (1951).
5. R. W. Brockman and E. P. Anderson, in *Metabolic Inhibitors* (Eds. J. H. Quastel and R. M. Hochster), Vol. 1, p. 239. Academic Press, New York (1963).
6. R. H. Lindsay, C. R. Tillery and M. W. Yu, *Archs Biochem. Biophys.* **148,** 466 (1972).
7. M. W. Yu, J. Sedlak and R. H. Lindsay, *Proc. Soc. exp. Biol. Med.* **139,** 1292 (1972).
8. M. W. Yu, J. Sedlak and R. H. Lindsay, *Archs Biochem. Biophys.* **155,** 111 (1973).
9. R. H. Lindsay, C. J. Romine and M. Y. Wong, *Archs Biochem. Biophys.* **126,** 812 (1968).
10. A. Zamir and R. Ben-Ishai, *Nature, Lond.* **189,** 922 (1961).
11. R. H. Lindsay, M. Y. Wong, C. J. Romine and J. B. Hill, *Analyt. Biochem.* **24,** 506 (1968).
12. N. Akamatsu, R. H. Lindsay and P. P. Cohen, *J. biol. Chem.* **239,** 2246 (1964).
13. T. A. Krenitsky, *J. biol. Chem.* **243,** 2871 (1968).
14. H. G. Klemperer and G. R. Haynes, *Biochem. J.* **108,** 541 (1968).
15. I. Crawford, A. Kornberg and E. S. Simms, *J. biol. Chem.* **226,** 1093 (1957).
16. I. Lieberman, A. Kornberg and E. S. Simms, *J. biol. Chem.* **215,** 403 (1955).
17. W. A. Creasey and R. E. Handschumacher, *J. biol. Chem.* **236,** 2058 (1961).
18. S. H. Appel, *J. biol. Chem.* **243,** 3924 (1968).
19. D. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
20. R. H. Lindsay, M. W. Yu and J. Sedlak, *Ala J. med. Sci.* **9,** 70 (1972).
21. D. M. Brown, D. B. Parihar, A. R. Todd and S. Vanadarjan, *J. chem. Soc.* 3028 (1958).
22. P. Roy-Burman, in *Recent Results in Cancer Research* (Ed. P. Reutchnick), Vol. 25, p. 1. Springer, New York (1970).
23. M. Friedkin and D. Roberts, *J. biol. Chem.* **207,** 245 (1954).
24. W. E. Razzell and H. G. Khorana, *Biochim. biophys. Acta* **28,** 562 (1958).
25. O. Skold, *Biochim. biophys. Acta* **44,** 1 (1960).
26. M. W. Yu and R. H. Lindsay, *Proc. Soc. exp. Biol. Med.* **140,** 1089 (1972).