Biochemical Pharmacology, Vol. 23, pp. 2727–2735. Pergamon Press, 1974. Printed in Great Britain.

INHIBITORS OF INOSINATE DEHYDROGENASE ACTIVITY IN EHRLICH ASCITES TUMOR CELLS *IN VITRO**

CAMILLA M. SMITH, LYDIA J. FONTENELLE,† HUONG MUZIK, ALAN R. P. PATERSON, HERTA UNGER, LARRY W. BROX and J. FRANK HENDERSON‡

University of Alberta Cancer Research Unit (McEachern Laboratory) and Department of Biochemistry, Edmonton, Alberta, Canada T6G 2E1

(*Received* 3 *November* 1973; *accepted* 1 *February* 1974)

Abstract—l-Amino guanosine, virazole and mycophenolic acid all inhibit inosinate dehydrogenase activity in intact Ehrlich ascites tumor cells *in vitro*, and exert 50 per cent inhibition at concentrations of 500, 50 and < 0·1 μM respectively. At these concentrations, the activity of guanylate synthetase was inhibited by 0, 20 and 10 per cent respectively. l-Amino 2′-deoxyguanosine was a less potent inhibitor of intracellular inosinate dehydrogenase activity than l-amino guanosine, and ten other l-substituted purines tested had little or no activity. l-Amino guansoine (400 μM) inhibited both the growth of cultured mouse leukemia L1210 cells and inosinate dehydrogenase activity by approximately 50 per cent.

INOSINATE dehydrogenase is required in animal cells for the synthesis of guanine ribonucleotides from adenine, adenosine and adenine ribonucleotides, from hypoxanthine, and from inosinate produced by the pathway of purine biosynthesis *de novo*. 6-Mercaptopurine, presumably as its ribonucleoside monophosphate derivative, is believed to inhibit inosinate dehydrogenase in intact animal and bacterial cells (review: Ref. 1), and such inhibition has also been observed in animal cells after treatment with 6-chloropurine.[2,3] Partially or highly purified preparations of inosinate dehydrogenase were inhibited by ribonucleotides of 6-mercaptopurine,[4–6] 6-thioguanine,[4] 6-chloropurine,[4,6,7] 8-azaguanine[8] and 2,6-diaminopurine;[8] 6-mercaptopurine ribonucleotide is also a substrate of this enzyme.[4,6,9]

Mycophenolic acid (Fig. 1) is also an inhibitor of inosinate dehydrogenase both in intact cells and in partially purified preparations.[10–14] Most recently, virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) (Fig. 1) has also been reported to be a potent inhibitor of inosinate dehydrogenase.[15] The structures of these two inhibitors less closely resemble that of the natural substrate, inosinate, than do those of the purine analog nucleotides mentioned above.

Animal cell[8] and bacterial (review: Ref. 16) inosinate dehydrogenases were inhibited by GMP and to a lesser degree also by GTP, and the activity of this enzyme in intact Ehrlich ascites tumor cells was inhibited when intracellular concentrations of GTP were elevated.[17]

* This work was supported by the National Cancer Institute of Canada.

† Present address: Department of Pharmacology, Idaho State University, Pocatello, Idaho.

‡ To whom reprint requests should be addressed.

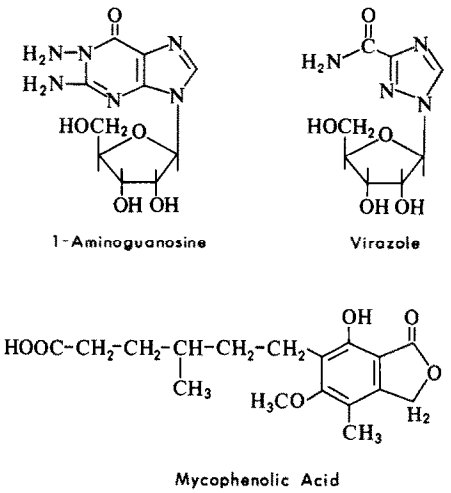

FIG. 1. Structures of l-amino guanosine, virazole and mycophenolic acid.

This paper reports results of studies of a new inhibitor of inosinate dehydrogenase, l-amino guanosine (Fig. 1), and compares some of its properties with those of virazole and mycophenolic acid.

MATERIALS AND METHODS

Adenine-8-^{14}C (39·3 mCi/m-mole) and guanine-8-^{14}C (41·8 mCi/m-mole) were obtained from New England Nuclear Corp., Boston, Mass.; hypoxanthine-8-^{14}C (49·4 mCi/m-mole) from Schwarz BioResearch, Orangeburg, N.Y.; non-radioactive purine bases, ribonucleosides and ribonucleotides from Sigma Chemical Co., St. Louis, Mo.; and Fischer's and RPMI* 1620 media and horse and fetal calf sera from Grand Island Biological Co., Grand Island, N.Y. Radioactive hypoxanthine and guanine contained impurities which were removed by column chromatography on Dowex-50-H$^+$.

Adenine-l-oxide was purchased from Sigma Chemical Co., mycophenolic acid was a gift to Dr. A. R. P. Paterson from Dr. T. Franklin, Imperial Chemical Industries, Alderly Park, Cheshire, England, and virazole was a gift of Dr. R. K. Robins, ICN Nucleic Acid Research Institute, Irvine, Calif. The following compounds were provided by the Drug Evaluation Branch, Drug Research and Development, National Cancer Institute, Bethesda, Md.: 2-amino-l-methyl purine-6-thione (NSC 43405), l-methyl purine-6-thione (NSC 54258), l-methyl adenine (NSC 70896), l-amino guanosine (NSC 112521), 1,2-diamino-9-β-D-ribofuranosyl purine-6,8-dione (NSC 112522), 2-amino-1-methyl-8-oxo-9-β-D-ribofuranosyl purine-6-thione (NSC 119845), 2-amino-1-methyl-9-β-D-ribofuranosyl purine-6, 8-dione (NSC 119846), 1,2-diamino-8-benzyloxy inosine (NSC 119848), l-amino-2′-deoxyguanosine (NSC 119850), l-amino inosine (NSC 119851), 1,2-diamino-7-methyl-9-β-D-ribofuranosyl purine-6,8-dione

* Abbreviations used: RPMI, Roswell Park Memorial Institute; AMPS, adenylosuccinate.

(NSC 127520), and 5,6-dimethyl-1-β-D-ribofuranosyl imidazo [4,5-*b*]pyrazine (NSC 136512).

Procedures for the maintenance, preparation and incubation of Ehrlich ascites tumor cells have been described previously, as have methods for the measurement of radioactivity in individual purine bases, ribonucleosides, ribonucleotides and nucleic acids.[14,17,18] Human leukemia RPMI 6410, human lymphoma P1R (Raji), and human lymphocyte RPMI 1788 and RPMI 6237 cells (obtained from Associated Biomedic Systems, Buffalo, N.Y.) were grown in RPMI 1620 medium containing 10% fetal calf serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin. Mouse lymphoma L5178Y (clone FF_D) and mouse leukemia L1210 cells were grown in Fischer's medium[19] containing 20% horse serum, 100 μg/ml of streptomycin and 100 units/ml of penicillin.[20]

The apparent activities of enzymes of purine metabolism were calculated essentially by the method of Snyder *et al.*[14] In general, this is done by calculating the amount of radioactive substrate metabolized by each enzyme; i.e. the radioactivity in all metabolites further along a pathway from the enzyme in question is summed. The original description of this procedure[14] applied only to the case in which radioactive hypoxanthine was used as precursor and in which nucleotides were the only metabolites whose radioactivity was measured. In this case, the apparent activity of inosinate dehydrogenase is equated to the sum of radioactivity in xanthylate, guanylate, GDP and GTP. In most of the present studies, the original method has been extended to the use of radioactive adenine and guanine as precursors and to the measurement of radioactivity in nucleotide, nucleoside and base metabolites. In this case, when adenine or hypoxanthine is used as precursor, the apparent activity of inosinate dehydrogenase is equated to the sum of radioactivity in xanthylate, guanylate, GDP, GTP, xanthosine, guanosine and guanine. When cells are incubated in the presence and absence of drugs, the apparent per cent inhibition of each enzyme can be calculated,[14] and the results may be slightly different depending on which summation is used.

Limitations and assumptions made in this procedure for the calculation of apparent enzyme activity have been discussed previously.[14] More recent studies[21] of the kinetics of purine metabolism in this system have shown that the conversion of 50 μM adenine, guanine and hypoxanthine to nucleoside triphosphates plus nucleic acids is pseudo-first order for at least 90 min, that nucleoside triphosphates are the major acid-soluble metabolites that accumulate, and that recycling of isotope does not occur to any appreciable extent.

Acid-soluble nucleotides were separated, and individual nucleotide concentrations were measured both by chromatography of cell extracts using a Varian-Aerograph LCS-1000 liquid chromatograph as described by Brown[22] and by chromatography on DEAE-Sephadex as described by Caldwell.[23]

RESULTS

Table 1 shows the effects of 1 mM 1-amino guanosine on the apparent activities of a number of reactions of purine metabolism in intact Ehrlich ascites tumor cells. Nucleotide synthesis from radioactive adenine, hypoxanthine and guanine was inhibited slightly, but the major effect of this compound was on the apparent activity

TABLE 1. EFFECTS OF 1-AMINO GUANOSINE ON PURINE METABOLISM*

Precursor	Reaction†	Apparent enzyme activity (% inhibition)
Adenine-^{14}C	Adenine phosphoribosyltransferase (27,530)	10·2
	AMP kinase (25,380)	0
	ADP kinase (24,620)	4·19
	RNA polymerase (531)	5·90
	AMP deaminase (2013)	2·03
Hypoxanthine-^{14}C	Hypoxanthine phosphoribosyltransferase (18,260)	17·3
	AMPS synthetase + lyase (14,850)	0
	AMP kinase (14,670)	0
	ADP kinase (13,800)	0
	IMP dehydrogenase (1802)	65·7
	GMP synthetase (1748)	4·6
	GMP kinase (1632)	16·7
	GDP kinase (1235)	14·9
	Xanthine oxidase (734)	8·9
Guanine-^{14}C	Guanine phosphoribosyltransferase (14,790)	19·2
	GMP kinase (13,080)	6·5
	GDP kinase (12,700)	0·1
	RNA polymerase (665)	11·9
	GMP reductase (1111)	0
	Guanine deaminase (7190)	0
	Xanthine oxidase (331)	0

* Ehrlich ascites tumor cells, 2% by volume, were incubated for 20 min at 37° with shaking in an atmosphere of air in 0·1 ml Fischer's medium containing 25 mM sodium phosphate buffer, pH 7·4, and 5·5 mM glucose, with and without 1 mM l-amino guanosine. Adenine-^{14}C, hypoxanthine-^{14}C or guanine-^{14}C was then added to a final concentration of 50 μM, and incubation continued for 60 min. Apparent enzyme activities were calculated,[14] typical control values (cpm) are given in parentheses.

† The "kinase" reactions refer to processes rather than to specific enzymes, as more than one enzyme may be involved in each case.

of inosinate dehydrogenase. Figure 2 shows the relation of l-amino guanosine concentration to inhibition of inosinate dehydrogenase activity under these conditions. Fifty per cent inhibition was achieved at approximately 500 μM l-amino guanosine, and the extent of inhibition was the same at 750 and 1000 μM; complete inhibition was not attained at any of the drug concentrations tested.

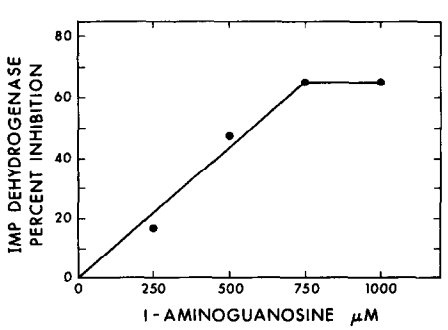

FIG. 2. Inhibition of inosinate dehydrogenase apparent activity by l-amino guanosine. Tumor cells were incubated as described in Table 1 with various concentrations of l-amino guanosine and 50 μM hypoxanthine-^{14}C.

In these studies, the apparent activity of inosinate dehydrogenase was equated to the sum of radioactivity in xanthylate, guanylate, GDP, GTP, xanthosine, guanosine and guanine. When the relationship, inosinate dehydrogenase = (xanthylate + guanylate + GDP + GTP) was used, the apparent per cent inhibition was approximately 80 per cent. To evaluate the possibility that the apparent inhibition might be due to dilution of the specific activity of the precursor hypoxanthine by non-radioactive hypoxanthine derived from the breakdown of inosinate, the time-course of the effect of 1 mM 1-amino guanosine on the metabolism of radioactive hypoxanthine was determined. At 15, 30, 45 and 60 min of incubation, the apparent per cent inhibitions of inosinate dehydrogenase were 85, 83, 81 and 79 respectively. A small degree of dilution thus may occur, but does not markedly affect the results. Since the concentration of inosinate in these cells is approximately 30 nmoles/g (compared, e.g., with > 3000 nmoles/g for ATP), the synthesis of inosinate from adenylate is very low, and purine biosynthesis is partially inhibited in the presence of hypoxanthine, only a small degree of dilution of radioactive hypoxanthine would be expected.

In an attempt to establish structure–activity relationships for inhibition of inosinate dehydrogenase activity by compounds related to 1-amino guanosine, a variety of other 1-substituted purines were tested in this system. Table 2 shows that only 1-amino 2′-deoxy guanosine was inhibitory, but that it was less potent than the parent ribosyl compound. 1-Amino inosine had no activity, nor did compounds which were further oxidized, substituted with large groups, or substituted with methyl instead of amino groups at the 1-position.

A range of concentrations of virazole and mycophenolic acid were tested both to

TABLE 2. INHIBITION OF INOSINATE DEHYDROGENASE ACTIVITY BY 1-SUBSTITUTED PURINE DERIVATIVES*

Compounds tested	Apparent activity of inosinate dehydrogenase† (% inhibition)
1-Amino guanosine	84·1
1-Amino 2′-deoxyguanosine	35·3
1-Amino inosine	0
1,2-Diamino-8-benzyloxy inosine	8·9
1,2-Diamino-9-β-D-ribofuranosyl purine-6,8-dione	0
1,2-Diamino-7-methyl-9-β-D-ribofuranosyl purine-6,8-dione	2·7
5,6-Dimethyl-1-β-D-ribofuranosyl imidazo[4,5-*b*]pyrazine	0
2-Amino-1-methyl-9-β-D-ribofuranosyl purine-6,8-dione	0
2-Amino-1-methyl-8-oxo-9-β-D-ribofuranosyl purine-6-thione	9·3
1-Methyl adenine	
Adenine-1-oxide	0
2-Amino-1-methyl purine-6-thione	5·8
1-Methyl purine-6-thione	10·0

* Tumour cells were incubated as described in Table 1 with and without 1 mM test compound and with 50 μM hypoxanthine-^{14}C.

† Control apparent activity: 1956 cpm. Enzyme activity equals radioactivity in xanthylate, guanylate, GDP and GTP.

compare the potency of their inhibition of inosinate dehydrogenase activity with that of 1-amino guanosine, and also to determine their effect, if any, on other reactions of purine metabolism. Table 3 shows that both virazole and mycophenolic acid were more potent inhibitors of inosinate dehydrogenase activity than 1-amino guanosine, 50 per cent inhibition being achieved at about 50 μM, $< 0{\cdot}1$ μM, and 500 μM (Fig. 2) respectively. Both virazole and mycophenolic acid also inhibited guanylate synthetase to a lesser extent.

TABLE 3. EFFECTS OF VIRAZOLE AND MYCOPHENOLIC ACID ON REACTIONS OF HYPOXANTHINE-^{14}C METABOLISM.*

Reaction	Apparent enzyme activity† % Inhibition by: Virazole (μM) 10	50	100	Mycophenolic acid (0·1 μM)
Hypoxanthine phosphoribosyl transferase	8·2	4·7	13·1	4·4
AMPS synthetase + lyase	0	0	0	0
AMP kinase	0	0	0	0
ADP kinase	0	0	0	0
IMP dehydrogenase	29·0	51·1	53·1	72·4
GMP synthetase	11·1	19·6	26·1	10·1
GMP kinase	6·6	9·7	11·2	7·6
GDP kinase	4·3	42·0	51·5	13·0

* Tumor cells were incubated as described in Table 1 with various concentrations of virazole and mycophenolic acid, and with 50 μM hypoxanthine-^{14}C.
† Control values are given in Table 1.

Although the data of Table 2 indicated that no other compound tested was a potent inhibitor of inosinate dehydrogenase activity, other effects of these l-substituted compounds on purine metabolism were detected. Thus, Table 4 shows that several compounds inhibited nucleotide formation from hypoxanthine, and in other experiments 2-amino-l-methyl purine-6-thione inhibited the accumulation of phosphoribosyl pyrophosphate by 46·2 per cent.

The effect of l-amino guanosine on the growth of human and mouse cells in tissue culture was tested. Figure 3 shows the inhibitory effect of 400 μM l-amino guanosine

TABLE 4. INHIBITION OF NUCLEOTIDE FORMATION FROM HYPOXANTHINE-^{14}C.*

Compounds tested	Nucleotides formed† (% Inhibition)
2-Amino-l-methyl purine-6-thione	73·9
l-Amino inosine	24·3
Adenine-l-oxide	18·9
l-Methyl purine-6-thione	15·2
l-Methyl adenine	14·3

* Tumor cells were incubated as described in Table 1 with and without 1 mM test compounds, and with 50 μM hypoxanthine-^{14}C.
† Control nucleotide radioactivity: 17,960 cpm.

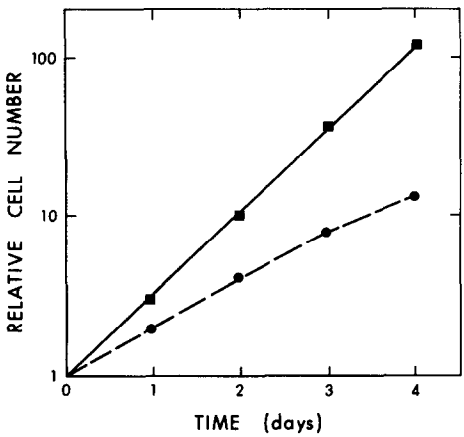

FIG. 3. Cell growth inhibition by l-amino guanosine. Mouse leukemia L1210 cells were grown in Fischer's medium containing 20% horse serum plus 100 μg/ml of streptomycin and 100 units/ml of penicillin, with (●--●) and without (■—■) 400 μM l-amino guanosine. The initial cell concentration was 100,000/ml.

on the growth of mouse leukemia L1210 cells; after 4 days the number of cell doublings was inhibited 45·6 per cent. In similar experiments, the growth of mouse lymphoma L5178Y cells was inhibited 16·4 per cent, and that of human lymphocyte RPMI 6237 cells was inhibited about 10 per cent; the growth of human leukemia RPMI 6410, human lymphoma PIR (Raji), and human lymphocyte RPMI 1788 cells was not significantly inhibited by 400 μM l-amino guanosine. When mouse leukemia L1210 cells (100,000 cells/ml) were incubated in complete growth medium plus 400 μM l-amino guanosine and 20 μM hypoxanthine-^{14}C and radioactivity in cellular nucleotides was measured, it was found that the apparent activity of inosinate dehydrogenase was inhibited 55 per cent and nucleotide formation from hypoxanthine was inhibited 34 per cent. Too little l-amino guanosine was available to evaluate its chemotherapeutic activity *in vivo*.

Preliminary studies of the metabolism of l-amino guanosine in Ehrlich ascites tumor cells were conducted. Paper chromatography and ultraviolet spectrophotometry of incubation media containing 1 mM l-amino guanosine, before and after a 60-min incubation with tumor cells, revealed no compounds other than l-amino guanosine.

Chromatography of acid-soluble nucleotides[22,23] from cells incubated with and without l-amino guanosine revealed in the latter case a peak of ultraviolet-absorbing material which was partially separated from GTP. Ultraviolet spectra of this fraction suggested that it contained a mixture of GTP and a l-amino guanosine metabolite, presumably the triphosphate. Paper chromatography also indicated that this fraction contained a mixture of GTP and another compound (not ATP). Because the presumptive l-amino guanosine metabolite was difficult to separate from GTP on ion-exchange chromatography and because it appeared to be unstable, further studies of l-amino guanosine metabolism await the availability of radioactive material. The concentration of GTP was not obviously elevated, and thus l-amino guanosine did not appear to be converted to guanine or its metabolites.

DISCUSSION

Inhibition of inosinate dehydrogenase activity by l-amino guanosine has not previously been reported. Although fairly high concentrations are required for inhibition, l-amino guanosine is a relatively specific inhibitor of this enzyme activity, since other reactions of purine metabolism were inhibited to a much smaller degree or not at all. Whether the low potency of l-amino guanosine is due to a low affinity of inosinate dehydrogenase for it or an active metabolite or to the presence of a relatively low intracellular concentration of drug or an active derivative, will require further studies both of l-amino guanosine metabolism and of its effects (and those of its metabolites) on a partially purified enzyme. The pharmacological relevance of the observed inhibition of inosinate dehydrogenase, even at relatively high l-amino guanosine concentrations, is suggested by the correspondence between the extent of enzyme inhibition and the extent of inhibition of growth of cultured mouse leukemia L1210 cells. Of course, much more work is required to establish definitively the mechanism of action of this drug. The preliminary studies of l-amino guanosine metabolism that were carried out suggest strongly that it is not being converted to guanosine or GTP, but that l-amino guanosine triphosphate may be a major metabolite. Thus the effect of l-amino guanosine (or a metabolite of it) may be direct and not mediated through conversion to guanine nucleotides, which have previously been shown to inhibit inosinate dehydrogenase.[8,16,17]

Of the limited number of l-substituted purine derivatives that were available for study of structure–activity relationships, only l-amino guanosine and l-amino 2′-deoxyguanosine had substantial activity as inhibitors of inosinate dehydrogenase activity, and it is particularly interesting that l-amino inosine was inactive. Whether activity in this system is related to specificity for drug activation or for interaction with inosinate dehydrogenase is not yet known. Previous studies have also investigated the effects of several of the other l-substituted purines studied here on adenine phosphoribosyltransferase,[24,25] adenosine kinase,[24–26] nucleoside uptake into human erythrocytes,[25,27] and purine biosynthesis *de novo*.[25]

It is interesting that inosinate dehydrogenase is inhibited by compounds of such diverse structure as mycophenolic acid, virazole and nucleosides or nucleotides containing l-amino guanine, 6-thioguanine, 6-mercaptopurine and 6-chloropurine moieties. This enzyme occupies a key position in purine metabolism, and potent and specific inhibitors of it might be useful chemotherapeutic agents.

Sweeney *et al.*[13] have reported that mycophenolic acid inhibited both inosinate dehydrogenase and guanylate synthetase equally in extracts of some tumors. However, this and an earlier[14] study using intact Ehrlich ascites tumor cells indicated that, over a wide range of mycophenolic acid concentrations, inosinate dehydrogenase is far more sensitive to inhibition than guanylate synthetase. The basis for this difference in results is not known.

REFERENCES

1. M. E. BALIS, *Antagonists and Nucleic Acids*, pp. 47–9. North-Holland, Amsterdam (1968).
2. A. C. SARTORELLI, J. R. AKERS and B. A. BOOTH, *Biochem. Pharmac.* **5**, 238 (1960).
3. A. C. SARTORELLI and B. A. BOOTH, *Biochem. Pharmac.* **5**, 245 (1960).
4. A. HAMPTON, *J. biol. Chem.* **238**, 3068 (1963).
5. M. R. ATKINSON, R. K. MORTON and A. W. MURRAY, *Biochem. J.* **89**, 167 (1963).

6. A. HAMPTON and A. NOMURA, *Biochemistry, N.Y.* **6,** 679 (1967).
7. L. W. BROX and A. HAMPTON, *Biochemistry, N.Y.* **7,** 2589 (1968).
8. J. H. ANDERSON and A. C. SARTORELLI, *Biochem. Pharmac.* **18,** 2747 (1969).
9. M. R. ATKINSON, G. ECKERMANN and J. STEPHENSON, *Biochim. biophys. Acta* **108,** 320 (1965).
10. T. J. FRANKLIN and J. M. COOK, *Biochem. J.* **113,** 515 (1969).
11. J. C. CLINE, J. D. NELSON, K. GERZON, R. H. WILLIAMS and D. C. DELONG, *Appl. Microbiol.* **18,** 14 (1969).
12. T. J. FRANKLIN and J. M. COOK, *Biochem. Pharmac.* **20,** 1335 (1971).
13. M. J. SWEENEY, D. H. HOFFMAN and M. A. ESTERMAN, *Cancer Res.* **32,** 1803 (1972).
14. F. F. SNYDER, J. F. HENDERSON and D. A. COOK, *Biochem. Pharmac.* **21,** 2351 (1972).
15. D. G. STREETER, J. T. WATKOWSKI, G. P. KHARE, R. W. SIDWELL, R. J. BAUER, R. K. ROBINS and L. N. SIMON, *Proc. natn. Acad. Sci. U.S.A.* **70,** 1174 (1973).
16. B. D. SANWAL, M. KAPOOR and H. W. DUCKWORTH, *Curr. Topics cell. Regulat.* **3,** 1 (1971).
17. F. F. SNYDER and J. F. HENDERSON, *Can. J. Biochem.* **51,** 943 (1973).
18. G. W. CRABTREE and J. F. HENDERSON, *Cancer Res.* **31,** 985 (1971).
19. G. A. FISCHER and A. C. SARTORELLI, *Meth. med. Res.* **10,** 247 (1964).
20. C. T. WARNICK, H. MUZIK and A. R. P. PATERSON, *Cancer Res.* **32,** 2017 (1972).
21. F. F. SNYDER and J. F. HENDERSON, *J. Cell. Physiol.* **82,** 349 (1974).
22. P. R. BROWN, *J. Chromat.* **52,** 257 (1970).
23. I. C. CALDWELL, *J. Chromat.* **44,** 331 (1969).
24. J. F. HENDERSON and R. E. A. GADD, *Cancer Chemother. Rep.* **1,** (Part 2) 363 (1968).
25. J. F. HENDERSON, A. R. P. PATERSON, I. C. CALDWELL, B. PAUL, M. C. CHAN and K. F. LAU, *Cancer Chemother. Rep.* **3,** (Part 2), 71 (1972).
26. I. C. CALDWELL and J. F. HENDERSON, *Cancer Chemother. Rep.* **2,** (Part 2), 237 (1971).
27. A. R. P. PATERSON and A. I. SIMPSON, *Cancer Res.* **27,** (Part 2), 353 (1967).