TABLE V. Reversal of ara-c Inhibition of "DNA Polymerase" by dCTP.[a]

ara-c (M)	TTP-2-^{14}C incorporated (mμmoles)		Molar ratio
	Control	$+ 1.75 \times 10^{-4} M$ dCTP	ara-c to dCTP
None	0.41	0.52	0.00:1
6.25×10^{-5}	0.22 (46)	0.46 (11)	0.36:1
1.25×10^{-4}	0.19 (54)	0.48 (8)	0.72:1
2.50×10^{-4}	0.17 (58)	0.50 (4)	1.43:1
5.00×10^{-4}	0.16 (61)	0.47 (10)	2.86:1

[a] The incubation tubes each contained TTP-2-^{14}C, 10 mμmoles; 3.31×10^6 cpm/μmole; Tris buffer, pH 7.8, 44 μmoles; $MgCl_2$, 6 μmoles; ATP, 1 μmole; creatine phosphate, 5 μmoles; creatine phosphokinase, 75 μg; dGTP, dATP, dCTP, each 20 mμmoles; denatured DNA, 100 μg, and Ehrlich enzyme extract, 1.72 mg protein; in a final volume of 0.80 ml. Ara-c, various molarities; dCTP, 120 mμmoles, or H_2O was preincubated with enzyme and all reagents except TTP-2-^{14}C, 10 mμmoles; dGTP, dATP, and dCTP, 20 mμmoles each at 37°C for 10 min, then these were added and incubations continued for 15 min. Numbers in parentheses refer to percentage inhibition.

peted with ara-c (or metabolite) for the same binding site on the DNA polymerase. (c) The binding of dCTP to the polymerase might have changed the conformation of the enzyme so that ara-c (or a metabolite) could not bind to an allosteric inhibitor site. These possibilities are subjects for future investigations.

The reversal of ara-c activity *in vivo* by deoxycytidine was first shown by Evans and Mengel (10). Our results may explain, in part, the results of Evans and Mengel (10).

Summary. β-D-Arabinosylcytosine was found to inhibit the utilization of thymidine for DNA synthesis in the Ehrlich ascites tumor. The DNA polymerase of the Ehrlich tumor was inhibited by β-D-arabinosylcytosine (or metabolite) and this inhibition could be reversed by deoxycytidine-5'-triphosphate.

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Formation of Inosine-5'-Monophosphate by a Kinase in Cell-Free Extracts of Ehrlich Ascites Cells *in Vitro** (32709)

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The effects of purine nucleosides upon the metabolism of normal and neoplastic tissues have led to increased investigations into the means by which these compounds exert their actions. Recent studies have shown that the active forms of the cytotoxic analogs of purine bases and nucleosides are the nucleotides, which are formed intracellularly by the action of pyrophosphorylases and kinases, respec-

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tively. The direct phosphorylation of a variety of nucleosides by enzymes obtained both from animal tissues and yeast have been reported (1–9), and the enzyme most commonly implicated is adenosine kinase. Despite the varied structures of nucleosides that have been phosphorylated, the existence of kinases that phosphorylate guanosine and inosine has long been questioned (10, 11). Recently, however, Tarr (12) has shown that salmon milt extracts contained enzymes that catalyzed the the conversion of both guanosine and inosine to the corresponding nucleotides, and LePage *et al.* (13,14) have demonstrated the phosphorylation of guanosine and some of its analogs by mammalian tissues. Studies by Brockman and Chumley (15) have also indicated that the metabolism of inosine and guanosine in intact cells and in cell-free extracts from cells devoid of inosine-5′-monophosphate–guanosine 5′-monophosphate (IMP-GMP) pyrophosphorylase activity suggested that a small conversion of these ribonucleosides to ribonucleotides had occurred. Subsequently Bennett *et al.* (8) obtained results which suggested the existence of a kinase other than adenosine kinase in cell lines resistant to methylthioinosine[2] and concluded that such a kinase, if it existed, must be closely linked with adenosine kinase.

This report describes the conversion of inosine to inosine 5′-monophosphate *in vitro* by a kinase in cell-free extracts of an Ehrlich ascites carcinoma which is resistant to thioguanine and lacks IMP-GMP pyrophosphorylase activity (16), so that the measurements were not compromised by nucleotide formation from free bases.

Materials and Methods. Materials. Inosine-8-^{14}C, adenosine-8-^{14}C, and hypoxanthine-8-^{14}C were purchased from Schwarz BioResearch, Inc., Orangeburg, New York. Creatine phosphokinase, the sodium salt of creatine phosphate, and adenosine 5′-triphosphate disodium salt were purchased from Sigma Chemical Company, St. Louis, Missouri.

Analytical methods. Paper chromatography was carried out by the descending technique, using the following solvent systems: A, 5% disodium hydrogen phosphate; B, aqueous propionic acid (44%)–aqueous *n*-butanol (93.8%) (1:1); C, saturated sodium borate–5 *M* ammonium acetate (pH 9.5)–ethanol–0.5 *M* EDTA (80:20:220:0.5); D, saturated ammonium sulfate–isopropanol–water (79-2-19); isobutyric acid–ammonium hydroxide–water (66:1:33). Radioactivity was measured in a liquid scintillation spectrometer with a toluene phosphor solution (17) or Bray's solution (18). For determining radioactivities on paper chromatograms the appropriate regions of the chromatograms were cut into strips (1 × 4 cm) and counted directly in the toluene phhosphor solution.

Tumors. The experiments were carried out with a thioguanine-resistant line of Ehrlich ascites carcinoma (referred to as Ehrlich TGRII) which lacked IMP-GMP pyrophosphorylase (16). Where other cell lines were used they are indicated by name. Maintenance of these tumors has been described (19). Cell-free extracts used to catalyze the phosphorylation were prepared as follows: Female Swiss mice were each inoculated with 10^6 cells and after 5–6 days of growth the ascites fluids were collected by flushing the peritoneal cavity with cold saline (0.9%) and collecting the suspension in ice-cold centrifuge tubes. The cells were packed for 2 min at 1470*g*, washed with cold saline (0.9%) to remove erythrocytes, and dispersed in two volumes of ice-cold distilled water. After standing for 2 min at 0°C, the swollen cells were homogenized in a Potter–Elvehjem homogenizer with a teflon pestle (20 passes). The suspension was centrifuged at 16,000*g* at 4°C for 90 min and the supernatant fluid was used as the soluble enzyme preparation (cell-free extract). The protein content of these preparations was approximately 11 mg/ml, estimated by the method of Lowry *et al.* (20).

Cell-free extracts retained full activity for about 6 days when stored at —20°C. Most of the experiments were carried out with freshly prepared extracts, the activities of which varied.

Cell-free extracts (0.2 ml) were incubated at 37°C with inosine (0.3–1 m*M*), magnesium chloride (5 m*M*); Tris buffer (10 m*M*), pH 7.4; ATP (1 m*M*), creatine phosphate (5

[2] The abbreviations used are: thioinosine, 9-(β-D-ribofuranosyl)-6-thiopurine; methylthioinosine, 9-(β-D-ribofuranosyl)-6-methylthiopurine.

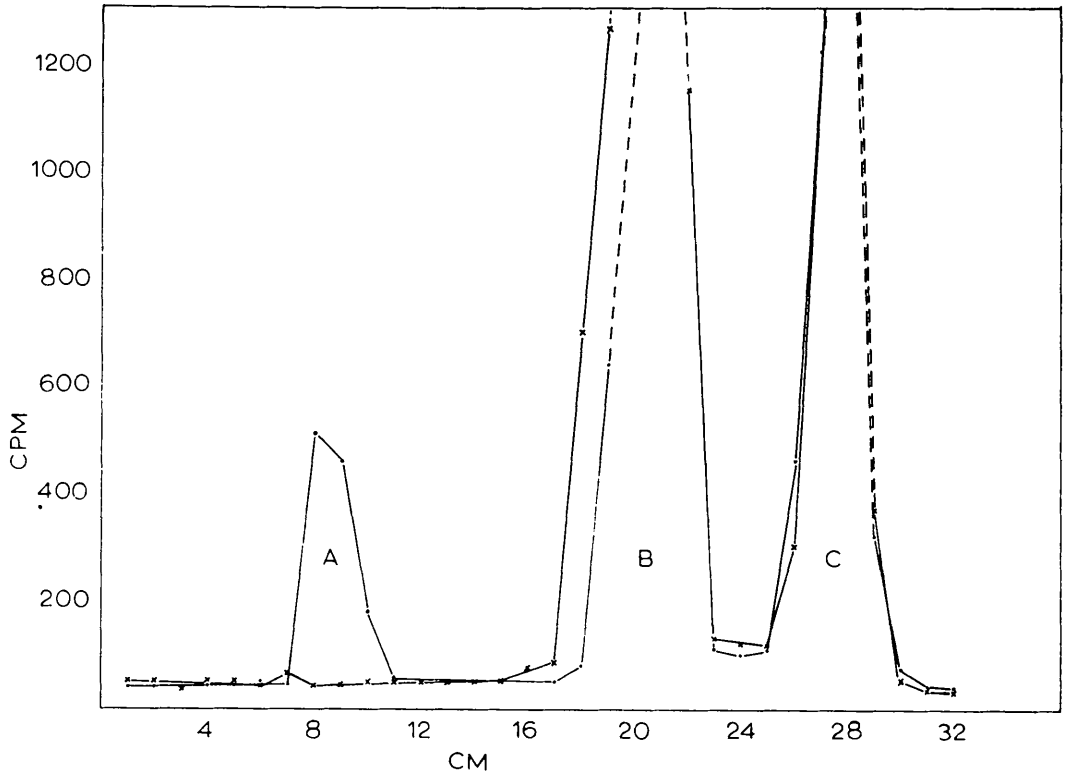

FIG. 1. Phosphorylation of inosine-8-^{14}C by Ehrlich TGRII cell-free extract. Paper chromotography in solvent B of Ehrlich TGRII cell-free extract incubated with inosine-8-^{14}C in the complete system (Table I). Portions (250 μl) were chromatographed after heat denaturation of the protein (•); treatment of an aliquot of the reaction mixture with snake venom followed by chromatography showed an elimination of the nucleotide peak (×). Peak A is inosine 5′-phosphate, B is inosine, and C is hypoxanthine.

m*M*) and creatine phosphokinase (78 μg). Total volume in all incubations was 1.0 ml. The reaction was stopped by heating the tubes for 3 min at 100°C. Control assays in which the protein was denatured immediately on addition of the substrate were carried out with each experiment. After removal of the denatured protein by centrifugation, aliquots (250 μl) of the supernatant solutions were analyzed for nucleotide formation by the paper chromatographic method.

Results and Discussion. Metabolism of inosine by cell-free extracts in vitro. A cell-free extract of Ehrlich TGRII tumor cells was incubated with inosine-8-^{14}C, in the presence of an ATP regenerating system. The reaction mixture, after heat denaturation of the protein, was examined by paper chromatography in solvents B and C. Three peaks of radioactivity were obtained when 1-cm strips of the chromatograms were counted in the toluene phosphor (Fig. 1). Co-chromatography of the reaction mixture with authentic inosine, hypoxanthine, inosine 5′-monophosphate, and inosine 5′-triphosphate in solvents B, C, and D indicated that the products were inosine 5′-monophosphate, inosine, and hypoxanthine. Solvent D was used to differentiate between inosine 5′-monophosphate and inosine 5′-triphosphate.

Treatment of a portion of the reaction mixture with crude snake venom (0.1 mg) (*C. adamanteus*) in 0.05 *M* Tris buffer (pH 8.0) for 60 min followed by paper chromatography in solvent B, resulted in the elimination of the nucleotide peak (A) with a simultaneous increase of the nucleoside peak (B).

Examination of cofactor requirements for

TABLE I. Requirements for the Phosphorylation of Inosine-8-^{14}C by Cell-Free Extracts of Ehrlich TGRII Ascites Cells.[a]

System	Inosine-8-^{14}C 5′-phosphate formed (mμmole/min)	Control (%)
Complete	7.40	100
Minus CFE[b]	0.0	0
Minus ATP	6.82	92
Minus creatine phosphate and creatine phosphokinase	0.89	13
Minus Mg^{++}	0.29	4
Plus EDTA[b] (10 μmoles)	0	0

[a] The complete reaction mixture contained 10 m*M* Tris buffer, pH 7.4; 1 m*M* ATP; 5 m*M* creatine phosphate; 78 μg creatine phosphokinase; 5 m*M* $MgCl_2$; 0.4 m*M* inosine-8-^{14}C (specific activity 1.25 × 10^6 cpm/μmole); 0.2 ml cell-free extract (2 mg protein); total volume 1 ml. The mixture was incubated at 37°C for 5 min, then the reaction stopped by heating the tubes for 3 min at 100°C. After removal of denatured protein the supernatant solution was analyzed by paper chromatographic methods for nucleotide formation.

[b] CFE, cell-free extract; EDTA, ethylenediaminetetraacetate.

the formation of inosine 5′-phosphate indicated that the reaction showed dependence upon the addition of enzyme, divalent cation and ATP regenerating system. The reaction did not show absolute dependence on ATP as long as the regenerating system was present (Table I). This was probably due to the presence of endogenous ATP in the extract.

The effect of pH (Fig. 2) upon the rate of inosine 5′-phosphate formation was examined in Tris-maleate and Tris-hydrochloride buffers. The pH optimum in either buffer was 7.4, with the rate in the latter being twice that in the former. This optimum pH of 7.4 for inosine 5′-phosphate formation differs from the value of 5.8 reported (9) for the liver adenosine kinase, but agrees with the value of 7.5 for the phosphorylation of methylthioinosine by the H.Ep No. 2 cells (8). Divalent cation was required for activity, and the curve for Mg^{++} dependence shows an optimum at 5 m*M*. The Mn^{++} was less effective (Fig. 3).

When the activity was measured as a function of ATP concentration, in the absence of the regenerating system, it was found that almost no phosphorylation of inosine occurred (Table II). Furthermore, when the ATP concentration was greater than 3 m*M*, the protein

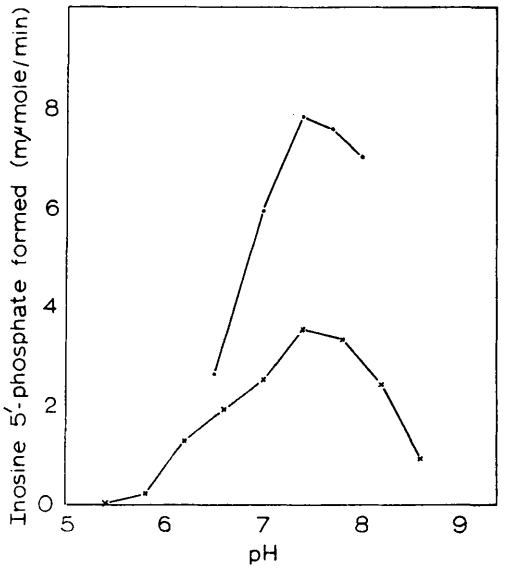

FIG. 2. Effect of pH on formation of inosine 5′-phosphate by Ehrlich TGRII cell-free extract. The reaction conditions were the same as in Table I, except that the incubation was for 10 min and the inosine-8-^{14}C had specific activity of 6.27 × 10^4 cpm/μmole. ●, Tris buffer; ×, Tris-maleate buffer.

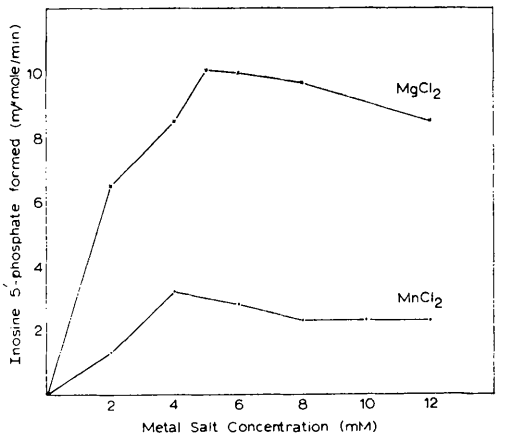

FIG. 3. Effect of metal salt concentration upon the rate of inosine 5′-phosphate formation. The reaction conditions were the same as in Table I, except inosine-8-^{14}C had specific activity of 6.27 × 10^4 cpm/μmole.

TABLE II. Effect of ATP Concentration on Phosphorylation of Inosine and Adenosine by Ehrlich TGRII Cell-Free Extracts.[a]

Substrate	ATP concentration (mM)	Nucleotide formed (mμmole per min per mg of protein)
Inosine-8-^{14}C	0	0.89
	1	0.29
	1.5	0.20
	2	0.0
	3	0.0
	0.5 + RS[b]	6.9
Adenosine-8-^{14}C	0	0.50
	1	0.91
	1.5	0.91
	2	1.61
	3	2.05
	0.5 + RS	5.25

[a] The reaction mixture consisted of 50 mM Tris buffer, pH 7.4; 0.5 mM inosine-8-^{14}C (6.27×10^4 cpm/μmole) or 0.5 mM adenosine-8-^{14}C (1.06×10^5 cpm/μmole), 5 mM $MgCl_2$ and 0.2 ml cell-free extract in total volume of 2 ml.

[b] Abbreviation: RS = ATP-regenerating system.

of the cell-free extract coagulated. This occurred even when the buffer strength of the Tris buffer was increased (40 mM). In the presence of the regenerating system, however, the activity was relatively high. A concurrent experiment, in which adenosine-8-^{14}C was used as substrate, showed that the rate of phosphorylation increased with ATP concentration up to 3 mM, which was the highest level tested. As was the case with inosine, the rate of phosphorylation of adenosine was much increased in the presence of the regenerating system. Varying the ATP concentration at various Mg^{++} concentrations in the presence of the regenerating system showed that the optimum ATP concentration was 0.5–1 mM at Mg^{++} concentration of 5 mM (Fig. 4).

The effect of inosine concentration on the rate of phosphorylation is shown in Fig. 5. When the data from Fig. 5 was plotted by the method of Lineweaver and Burke, extrapolation from the reaction rates observed at higher substrate concentrations gave V_m 5.8 mμM per min per mg protein and K_m of 7.8 $\times 10^{-5}$ M. Plotting the phosphorylation of inosine as a function of time showed that the rate was quite rapid over the first 5 min then fell off slightly with time (Table III). When inosine 5′-phosphate was added to the

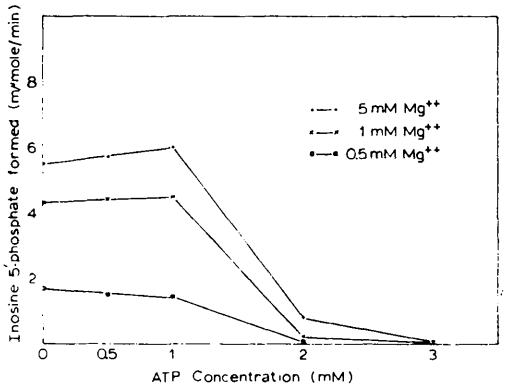

FIG. 4. Effect of ATP concentration on inosine 5′-phosphate formation at various Mg^{++} concentrations in the presence of an ATP regenerating system. The reaction conditions were the same as in Table I, except as indicated.

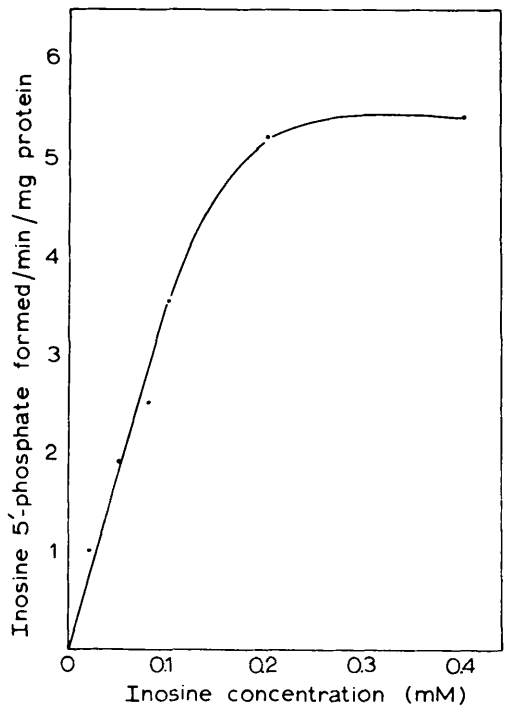

FIG. 5. Effect of substrate concentration upon the rate of phosphorylation of inosine by Ehrlich TGRII cell-free extracts. The reaction conditions were the same as those described in Table I, except as noted.

TABLE III. Phosphorylation of Inosine-8-^{14}C as a Function of Time by Cell-Free Extract of Ehrlich TGRII Ascites Cells.[a]

Substrate	Inosine 5'-phosphate (mM)	Time (min)	Nucleotide formed (mμmole)
Inosine-8-^{14}C	0	0	0
	0.5	5	98
	0.5	10	112
	0.5	15	135
	0.5	30	147
	0.5	60	167
	0	5	93
	0	10	129
	0	15	157
	0	30	201
	0	60	203
Hypoxanthine-^{14}C	0	0	0
	0	10	0

[a] The reaction conditions were the same as that described in Table I, except that 1 mM of inosine-8-^{14}C (specific activity 2.1 $\times$ 10^5 cpm/μmole) and 1 mM hypoxanthine-8-^{14}C (specific activity 2.27 $\times$ 10^6 cpm/μmole) were used. The amount of CFE used in this experiment was 0.3 ml ($\approx$ 3 mg protein).

reaction vessels it did not have any significant effect during the shorter periods but some inhibition seemed evident over the longer periods. During the incubation of inosine, cleavage occurred, giving rise to hypoxanthine. It was therefore necessary to have a hypoxanthine control to ascertain whether any nucleotide formation from free base occurred, through the nucleotide pyrophosphorylase reaction. The data in Table III show that hypoxanthine was not converted to nucleotide by the system.

To identify the nucleoside kinase involved, the rates of phosphorylation of inosine were measured in the presence of some other purine ribonucleosides with the aim of utilizing competitive effects. Velocities were determined for a series of concentrations of the nuclesides (inhibitors) at two different substrate concentrations. The data in Table IV indicate that thioinosine was a competitive inhibitor, but neither N^6-methyladenosine nor methylthioinosine had any effect. Adenosine inhibited the phosphorylation of inosine and when the data was plotted (Fig. 6) according

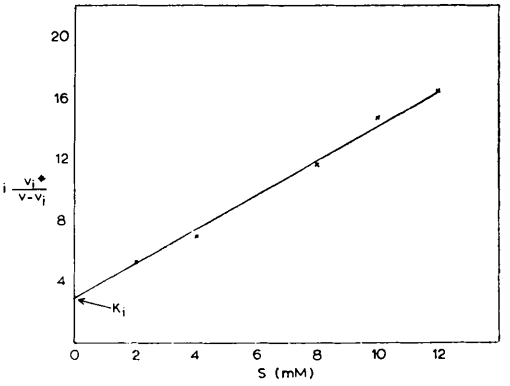

FIG. 6. Competitive inhibition of the phosphorylation of inosine by adenosine, plotted according to Hunter and Downs (21). Rates were observed at various concentrations of substrate with a constant adenosine concentration of 0.8 mM.

* V_i is velocity in the presence of inhibitor, and V the velocity in its absence with the same substrate concentration.

to the method of Hunter and Downs (21) it was found to be a competitive inhibitor. As shown in Table IV both N^6-methyladenosine and methylthioinosine inhibited the phosphorylation of adenosine whereas inosine had no effect.

Since the Ehrlich TGRII is devoid of IMP-GMP pyrophosphorylase it provided an excellent opportunity to study the effect of inosine on FGAR formation, because cleavage of the inosine to hypoxanthine was not a complicating factor. Using Henderson's method (22) it has been found that inosine did exhibit a slight inhibition of FGAR formation (Table V) of a magnitude similar to that previously reported (15).

The decrease of kinase activity with time at pH 7.4 and 37°C with either inosine or adenosine as substrate (Fig. 7) indicated that a rapid loss of activity occurred. The loss appeared to be less rapid after 30 min.

Phosphorylation of inosine has also been found to occur with cell-free extracts of Ehrlich ascites cells (sensitive), L1210/MP cells and L1210/MP/6MeMPR cells, though the activities in the latter two were relatively low (Table VI).

The studies with competitive substrates (Table IV) indicate that the inosine kinase is distinct from adenosine kinase. The failure of Meikle *et al.* (11) to obtain activity with in-

TABLE IV. Effects of Purine Nucleosides on the Phosphorylation of Inosine by Ehrlich TGRII Cell-Free Extracts.[a]

Substrate and concentration (m*M*)		Inhibitor	Molar ratio (subst.:inhib.)	Inhibition (%)
Inosine-8-^{14}C	0.3	Thioinosine	1:0	0
			1:1.33	29
			1:2	39
			1:2.66	46
			1:3.33	51
	0.6		1:0	0
			1:0.66	23
			1:1	32
			1:1.33	43
			1:1.66	48
Inosine-8-^{14}C	0.4	N^6-methyladenosine	1:0	0
			1:1	0
			1:2	0
			1:4	0
			1:6	0
	0.8		1:0	0
			1:0.5	0
			1:1	0
			1:2	0
			1:3	0
Inosine-8-^{14}C	0.4	Methylthioinosine	1:0	0
			1:0.5	0
			1:1	0
			1:2	0
			1:4	0
	0.8		1:0	0
			1:0.25	0
			1:0.5	0
			1:1	0
			1:2	0
Adenosine-8-^{14}C	0.5	Methylthioinosine	1:0	—
			1:1	20
			1:2	38
			1:4	58
			1:6	83
	1.0		1:0	—
			1:0.5	11
			1:1	20
			1:2	33
			1:3	45
Adenosine-8-^{14}C	0.5	N^6-methyladenosine	1:0	—
			1:1	28
			1:2	39
			1:4	49
			1:6	55

TABLE IV. Concluded.

Substrate and concentration (mM)	Inhibitor	Molar ratio (subst.:inhib.)	Inhibition (%)
1.0		1:0	—
		1:0.5	22
		1:1	30
		1:2	33
		1:3	45
Adenosine-8-^{14}C 0.5	Inosine	1:0	—
		1:1	0
		1:2	0
		1:4	0
		1:6	0
1.0		1:0	0
		1:0.5	0
		1:2	0
		1:3	0

[a] The reaction condition for inosine-8-^{14}C substrate was the same as in Table I except as indicated. The adenosine-8-^{14}C used as substrate had specific activity of 1.16×10^5 cpm/μmole. All other conditions were as in Table I except as indicated.

osine as a substrate, in an Ehrlich tumor line, can apparently be explained on the basis of the data presented herein. Our data indicate that the longer incubation time and high ATP concentration used in the experiments of Meikle *et al.* (11) would not permit the detection of inosine kinase activity.

Summary. Cell-free extracts from Ehrlich ascites tumor cells were found to convert inosine to inosine 5'-monophosphate at a rate comparable to the conversion of adenosine to adenosine 5'-monophosphate by the same extracts. This inosine kinase appeared to be distinct from adenosine kinase. Conditions necessary for optimum activity were studied. Magnesium ions were required, as was a relatively low level of ATP and an ATP-regenerating system. The pH optimum was approximately 7.4.

TABLE V. *In Vitro* Feedback Inhibition of *de Novo* Purine Synthesis Pathway in Ehrlich TGR11 Cells.[a]

Drug (2×10^{-3} M)	FGAR[b] (cpm $\times 10^3$)	Inhibition (%)
None (control)	10.50 ± 0.34	—
Adenosine	3.45 ± 0.12	67
Inosine	8.15 ± 0.21	23
Hypoxanthine	10.20 ± 0.46	3

[a] Tumor cells, 25 mg wet wt., were incubated in Krebs-Ringer phosphate buffer (no $CaCl_2$) pH 7.4, in air at 37°C with 1 μmole of glycine-2-^{14}C, 0.3 μC/μmole; 2 μmole of glutamine; 11 μmole of glucose, 2 μg of azaserine, and drugs 4 μmole. The total volume was 2 ml. Each value is an average of separate determinations on 3 flasks $\pm$ the average deviation from the mean.

[b] Abbreviation: FGAR = formylglycinamide ribotide.

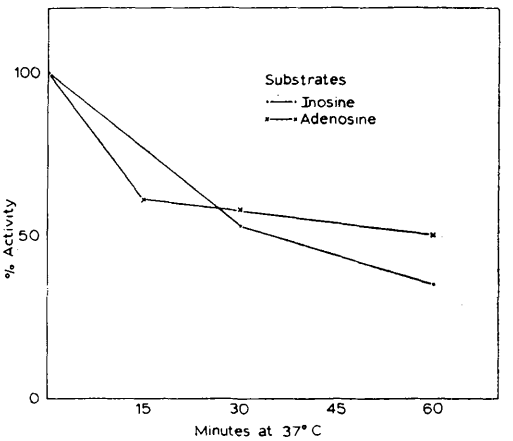

FIG. 7. Deterioration of enzyme activity. Cell-free extract was incubated at 37°C and at the time intervals indicated the activities with inosine and adenosine as substrates were measured.

TABLE VI. *In Vitro* Formation of Inosine 5'-phosphate by Cell-Free Extracts of Ascites Cells.[a]

Substrate	Tumor cell line	Nucleotide formed (mμmole per min per mg of protein)
Inosine-8-^{14}C	Ehrlich Sensitive	6.30
Inosine-8-^{14}C	L1210/MP/MeMPR[b]	0.55
Inosine-8-^{14}C	L1210/MP	0.45

[a] The reaction conditions were the same as those described in Table I.

[b] This subline is doubly resistant to 6-thiopurine and methylthioinosine.

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Lack of Directional Selectivity of Cassaine in Excitation of the Medullary Emetic Chemoreceptor Trigger Zone from Blood and Cerebrospinal Fluid in Cats*† (32710)

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The chemoreceptor trigger zone (CTZ), situated in area postrema of the medulla oblongata, has been proposed as the receptor site for the emetic action of a variety of drugs (1–3). Two major experimental bases for this proposal are first, that the emetic response to intravenous injection of a given agent is abolished by surgical ablation of the CTZ (1,2), and second, that local administration of the agent, as by injection into the cerebrospinal fluid (CSF), is a potent means of evoking emesis (3). In the case of cardiac glycosides, however, it was found in the cat that this class of drugs is ineffective in eliciting emesis through the intracerebroventricular route even though these agents satisfy the first criterion for an emetic stimulant action at the CTZ (4). It was suggested therefore

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