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THE UNIVERSITY OF ALBERTA

STUDIES ON THE CONTROL OF PURINE NUCLEOTIDE METABOLISM IN EHRLICH ASCITES TUMOR CELLS *IN VITRO*

by

© GERALD WINSTON CRABTREE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "STUDIES ON THE CONTROL OF PURINE NUCLEOTIDE METABOLISM IN EHRLICH ASCITES TUMOR CELLS IN VITRO" submitted by Gerald Winston Crabtree in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The reactions of purine ribonucleotide synthesis (from bases), transphosphorylation, interconversion and catabolism have been studied in Ehrlich ascites tumor cells *in vitro* in order to delineate some of the rate-controlling steps in these processes and to determine the factors responsible for the rate-limiting nature of such steps.

On examining the synthesis of purine nucleotides from preformed purine bases, it is apparent that the maximum percent incorporation of hypoxanthine and guanine into nucleotides is lower than that of adenine although the total percent metabolism of all three bases is similar. Adenine phosphoribosyltransferase is not saturated at the highest substrate level used, whereas hypoxanthine-guanine phosphoribosyltransferase appears to be saturated at substrate levels between 50 and 100 μM. Regardless of the base used, the rate of nucleotide synthesis decreases with increasing time of incubation; declining substrate levels, inhibition by nucleotides and limited availability of PP-ribose-P may be responsible for this observation.

Although the rate-limiting step in the conversion of AMP into ATP is difficult to elucidate, the formation of GTP from GMP appears to be limited by the rate of the nucleoside diphosphate kinase reaction. The factors responsible for the regulation of this reaction are not apparent, however.

The conversions of AMP into GMP and of GMP into AMP take place slowly and are limited by the rates of the reactions catalyzed by XMP aminase and GMP reductase, respectively. Concentrations of non-purine substrates such as L-aspartate and L-glutamine limit, under normal

conditions, the conversion of IMP into AMP and GMP, respectively. Analogs of these non-purine substrates, namely hadacidin and DON, inhibit the reactions catalyzed by AMPS synthetase and XMP aminase, respectively. Replacement of the normal incubation medium (Krebs-Ringer solution) by "Fischer's medium for leukemic cells of mice" or supplementation of the medium with 10% horse serum results in increased synthesis of both adenine and guanine nucleotides from hypoxanthine-^{14}C. Effects of the former treatment may be explained by the presence of non-purine substrates in the Fischer's medium; explanations for the effects of the latter treatment are obscure.

The major route of adenine nucleotide catabolism in Ehrlich ascites tumor cells <u>in vitro</u> is via the sequence: AMP → IMP → HR → H. The rate of the AMP deaminase reaction may limit this process although the rate of the reaction catalyzed by purine nucleoside phosphorylase is also low. The degradation of guanine nucleotides takes place via the sequence: GMP → GR → G → X, with the purine nucleoside phosphorylase reaction being the slowest step. Guanosine deaminase activity appears to be absent in these cells. With hypoxanthine as precursor, large amounts of xanthine accumulate, predominantly via the cleavage of xanthosine. Xanthine oxidase activity is low in these cells. Although utilization of Fischer's medium results in greatly reduced rates of nucleotide catabolism as compared to controls, supplementation of the medium with 10% horse serum has little effect on total nucleotide catabolism but may have specific effects on some of the reactions of nucleotide catabolism.

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I would like to express my deep appreciation to my supervisor, Dr. J. F. Henderson, for his invaluable aid and enduring patience during the course of this study. My thanks go to Dr. A. R. P. Paterson, Dr. I. C. Caldwell and other members of the McEachern Laboratory for their continued interest and helpful discussions.

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LIST OF ABBREVIATIONS

PP-ribose-P	α-D-1-pyrophosphorylribofuranosyl-5-phosphate
AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
dAMP	deoxyadenosine 5'-monophosphate
dADP	deoxyadenosine 5'-diphosphate
IMP	inosine 5'-monophosphate
IDP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
XMP	xanthosine 5'-monophosphate
XTP	xanthosine 5'-triphosphate
GMP	guanosine 5'-monophosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
dGMP	deoxyguanosine 5'-monophosphate
dGTP	deoxyguanosine 5'-triphosphate
UMP	uridine 5'-monophosphate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
dUMP	deoxyuridine 5'-monophosphate
CMP	cytidine 5'-monophosphate
CDP	cytidine 5'-diphosphate
CTP	cytidine 5'-triphosphate
dCMP	deoxycytidine 5'-monophosphate

dCTP	deoxycytidine 5'-triphosphate
TMP	thymidine 5'-monophosphate
TTP	thymidine 5'-triphosphate
AMPS	adenylosuccinic acid
P_i	orthophosphate
NAD	nicotine-adenine dinucleotide
NADH	reduced nicotine-adenine dinucleotide
NADP	nicotine-adenine dinucleotide phosphate
A	adenine
AR	adenosine
H	hypoxanthine
HR	inosine
G	guanine
GR	guanosine
X	xanthine
XR	xanthosine

I INTRODUCTION

Purine nucleotides are utilized by cells for a wide variety of metabolic processes. In addition to their important roles in energy production and storage and as components of nucleic acids, they are also involved as cofactors in almost every pathway of metabolism. Therefore, adequate control of the reactions of purine metabolism must be of great importance to the entire biochemical economy of the cell.

Purine nucleotides may be formed via three main pathways. In the first of these, the de novo route, purine nucleotides are synthesized from nonpurine precursors such as glycine, glutamine, formate, bicarbonate, aspartate and PP-ribose-P. In the other two pathways, preformed purine nucleosides and bases are utilized for nucleotide synthesis. Nucleosides may be phosphorylated to form nucleotides in reactions catalyzed by nucleoside kinases. In addition, purine phosphoribosyltransferases catalyze the direct conversion of purine bases into their respective nucleotides. Once formed, purine nucleotides may be interconverted at the monophosphate level, phosphorylated to the di, tri and even higher phosphates, converted to coenzymes, or degraded in various ways. The complexity of this interacting and interconnecting net of reactions is illustrated in Fig. 1, which includes only those reactions which are believed to occur in mammalian cells. Table I gives the names of the enzymes which catalyze the reactions numbered in Fig. 1.

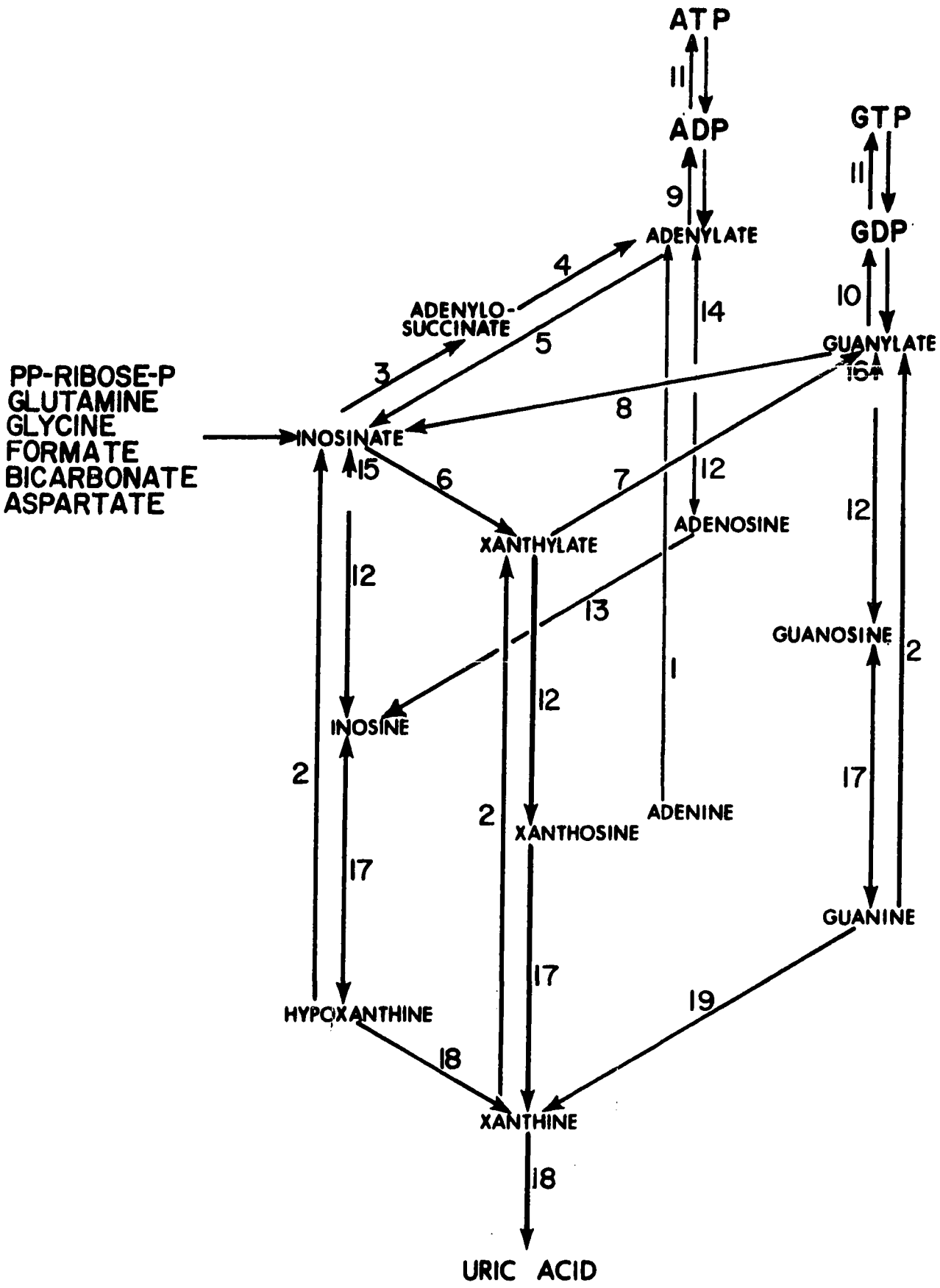


Figure 1. Outline of purine ribonucleotide metabolism.

TABLE I: Nomenclature of Enzymes of Purine Metabolism

Enzyme Number (Fig. 1)	Common Name	Systematic Name	E.C. Number
1	adenine phosphoribosyl-transferase	AMP:pyrophosphate phosphori-bosyltransferase	2.4.2.7
2	hypoxanthine-guanine phosphoribosyltransferase	IMP-GMP:pyrophosphate phosphoribosyltransferase	2.4.2.8
3	adenylosuccinate synthetase	IMP:L-aspartate ligase(GDP)	6.3.4.4
4	adenylosuccinate lyase	adenylosuccinate:AMP-lyase	4.3.2.2
5	AMP deaminase	AMP aminohydrolase	3.5.4.6
6	IMP dehydrogenase	IMP:NAD oxidoreductase	1.2.1.14
7	XMP aminase	XMP:L-glutamine amido-ligase(AMP)	6.3.5.2
8	GMP reductase	reduced-NADP:GMP oxido-reductase(deaminating)	1.6.6.8
9	adenylate kinase	ATP:AMP phosphotransferase	2.7.4.3
10	guanylate kinase	ATP:GMP phosphotransferase	2.7.4.8
11	nucleoside diphosphate kinase	ATP:nucleoside diphosphate phosphotransferase	2.7.4.6
12	5'-nucleotidase	5'-ribonucleotide phosphohydrolase	3.1.3.5

TABLE I continued

Enzyme Number (Fig. 1)	Common Name	Systematic Name	E.C. Number
13	adenosine deaminase	adenosine aminohydrolase	3.5.4.4
14	adenosine kinase	ATP:adenosine 5'-phospho-transferase	2.7.1.20
15	inosine kinase	ATP:inosine 5'-phospho-transferase	- - - -
16	guanosine kinase	ATP:guanosine 5'-phospho-transferase	- - - -
17	purine nucleoside phosphorylase	purine-nucleoside:ortho-phosphate ribosyltransferase	2.4.2.1
18	xanthine oxidase	xanthine:oxygen oxidoreductase	1.2.3.2
19	guanase	guanine aminohydrolase	3.5.4.3

Since this network of reactions is so complex, considerable difficulties are encountered in studying its regulation, and no complete picture of the control of these pathways in mammalian cells exists at the present time. Most of the early work on the control of these reactions has been concerned with the regulation of the de novo route of nucleotide synthesis. This pathway has been shown by many workers to be controlled by feedback inhibition mechanisms in bacteria, avian liver and mammalian cells and by repression in bacteria and in mammalian cells. In contrast, there is a paucity of information concerning the regulation of other pathways of purine nucleotide metabolism in intact cells.

Pertinent literature will be reviewed in the introduction to the appropriate chapter. At this point, however, some general comments on the control of enzyme activities will be presented.

In terms of whole cells, enzymes should be considered, not as individual catalysts, but as members of interacting multienzyme sequences. The rate of an overall sequence usually depends on the rate of the enzyme catalyzing the slowest step in the sequence. It is generally believed that rate-determining reactions appear at or near the beginnings of reaction sequences and at points where multienzyme sequences branch, intersect or interact by virtue of shared substrates or cofactors. Such rate-determining reactions may be regulated by mechanisms which involve kinetic properties and intracellular locations of the enzymes, and availability of substrates and cofactors. In addition, the intrinsic activity of such enzymes may be altered or the total amounts of these enzymes actually present may be regulated. Some examples of these possible control mechanisms are discussed below.

If a substrate is common to two or more reaction sequences, these sequences may compete for it and the relative affinities of the enzymes of the competing sequences for this substrate will determine its relative utilization by the different sequences. Many examples of this type of control may be involved in the reactions of purine metabolism. For example, PP-ribose-P is a substrate common to both the first reaction of purine synthesis de novo and to the reactions by which nucleotides are synthesized from purine bases. IMP is a common substrate for adenylosuccinate synthetase, IMP dehydrogenase and 5'-nucleotidase. Many other examples are evident in Fig. 1.

Individual reactions in different sequences may also compete for the use of the same cofactor. For example, many reactions in the cell utilize NAD and reduced NADP, cofactors for IMP dehydrogenase and GMP reductase, respectively.

Intracellular compartmentation of enzymes and substrates may play an important role in regulating the rates of multi-enzyme sequences. For example, Kandaswamy and Henderson (1962) obtained evidence which suggested that the pool of purine nucleotides made from preformed bases was in some way separate from that made de novo although nucleotides in these pools did become mixed before incorporation into nucleic acids occurred.

The activity of enzymes may also be regulated by allosteric mechanisms which may be stimulatory or inhibitory. Possibly the most common of these effects is feedback or end product inhibition in which the first enzyme of a particular reaction sequence is inhibited by an end product of that sequence. The inhibition of IMP dehydrogenase by

GMP is an illustration of this phenomenon. Such effects are usually reversible and as such are very sensitive to changes in the concentrations of end products.

Metabolic sequences may also be controlled by regulating the amounts of rate-determining enzymes; enzyme amount is controlled by the rate of gene expression and by the rate of enzyme catabolism. Although induction and repression are extremely important control processes in bacteria, they appear to be relatively minor control mechanisms in animal cells.

The availability of a substrate may limit the rate of a particular enzymic reaction and therefore the rate of an entire reaction sequence. If a substrate is synthesized at a low rate or, if its rate of degradation is high compared to its rate of synthesis, the concentration of this compound available for an enzymic reaction might be so low as to limit the rate of this reaction. This effect would be emphasized if the substrate is utilized in several metabolic reactions. As an example, since glutamine is required for many reactions of cellular metabolism and since this compound may be rapidly deamidated, its availability may limit the rate of the XMP aminase reaction.

Although all of the factors discussed above may play definite roles in the control of the reactions of purine nucleotide metabolism, the relative importance of these various factors for any particular cell type is uncertain.

Therefore, in this study we propose to examine purine nucleotide metabolism in intact cells in an effort to delineate the rate-controlling reactions of this particular area of cellular metabolism.

In addition, attempts will be made to elucidate the factors which are responsible for the rate-limiting nature of such reactions. For our work, we will utilize Ehrlich ascites tumor cells since they are relatively easily prepared and maintained and because much work has already been done with them. The type of experiments utilized in this study involve the measurement of the incorporation of radioactive purine bases into acid-soluble purine nucleotides, nucleosides and bases as functions of time and of base concentration. It should be emphasized that the results obtained in these studies reflect the synthesis of the various metabolites from the added precursors, but do not directly measure the concentrations of these metabolites which are present in the cells.

II EXPERIMENTAL PROCEDURES

A. Chemicals

Adenine-8-^{14}C (52.6 μc/μmole), hypoxanthine-8-^{14}C (49.0 and 50.0 μc/μmole) and guanine-8-^{14}C (31.7 μc/μmole) were obtained from Schwarz Bioresearch, Inc. Adenine, guanine, hypoxanthine, xanthine, adenosine, inosine, guanosine and xanthosine were purchased from Sigma Chemical Co. AMP, ADP, ATP, GMP, GDP, GTP, IMP, XMP were obtained from P-L Biochemicals, Inc. Fisher Scientific Co. supplied D-glucose, uric acid, inorganic salts, in addition to materials used for chromatographic solvents. L-glutamine and NAD were purchased from Calbiochem., L-aspartic acid was obtained from Mann Research Laboratories. Both hadacidin (N-formylhydroxyamino acetic acid) and 6-diazo-5-oxo-L-norleucine (DON) were supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute. "Fischer's medium for leukemic cells of mice" and horse serum were purchased from Grand Island Biological Co., the former as a dry powder.

B. Materials

Filter paper for chromatography and electrophoresis (Whatman No. 1) was obtained from Fisher Scientific Co., as was the thin-layer cellulose (Whatman CC41) utilized for making polyethyleneimine-cellulose. Polyethyleneimine (PEI) was purchased as a 50% aqueous solution from Chemirad Corp. The Mylar film (polyethylene terephthalate, 48 x 8 in, 0.0075 in thick) on which the layers were plated was obtained from Kensington Scientific Corp. Eastman chromagram sheets for thin-layer chromatography, 20 x 20 cm plastic sheets with unmodified cellulose as

the layer, were obtained from Fisher Scientific Co. Baker-Flex PEI-cellulose sheets (J.T. Baker Co.), plastic sheets with PEI-cellulose as the layer, were purchased from Canadian Laboratory Supplies, as was MN 300 cellulose powder. Dialysis tubing was purchased from Arthur H. Thomas Co. The unplasticized polyvinyl chloride which was used for the construction of chromatography apparatus was obtained from Johnston Industrial Plastics Ltd.

C. Analytical Procedures

The types of experiments utilized in this study and the complex nature of the products which resulted from these experiments dictated that the method of analysis be rapid, useful for large numbers of samples, applicable to small sample volumes, and suitable for resolving complex mixtures of products. In general, the separation procedures available at the time these experiments were begun failed to meet these criteria on one or more grounds. A good deal of this project was therefore devoted to the development of suitable methods. Methods not actually used to obtain the results presented below will not be discussed, however.

1. Thin-Layer Chromatography

Thin-layer chromatography possesses several advantages over paper chromatography or paper electrophoresis for the analysis of mixtures of nucleotides. Whereas analysis of complex mixtures by thin-layer chromatography can be carried out in just a few hours, the same analysis may take days to complete using paper chromatography. Much

more complex mixtures may be resolved by thin-layer chromatography than by paper chromatography or paper electrophoresis and the separations obtained are much sharper with the former technique. In addition, much smaller amounts of substances may be detected on thin-layer plates as compared to paper chromatograms when the same detection methods are used in each case. Generally, the same solvents used for paper chromatography may be used for thin-layer chromatography when unsubstituted cellulose is used as layer. As with paper chromatography, however, the resolution of complex mixtures of purine bases, nucleosides, and nucleotides is not possible. However, ion-exchange celluloses have been used with varying degrees of success for the resolution of nucleotide mixtures. ECTEOLA-cellulose (cellulose reacted with epichlorohydrin and triethanolamine) and DEAE-cellulose (diethylaminoethyl-cellulose) used to be the most common ion-exchangers, but E. and K. Randerath (1964) have more recently devised a method for the resolution of complex nucleotide mixtures which utilizes 2-dimensional thin-layer chromatography on PEI-cellulose, an anion-exchanger. More than 20 nucleotides could be separated in less than 3 hours using this procedure. This method has been modified for use in our experiments, and the details of this method are outlined in the following section.

A procedure has also been devised whereby mixtures containing adenine, guanine, hypoxanthine, xanthine, their respective nucleosides, and uric acid can be completely resolved on unsubstituted cellulose without interference from the nucleotides contained in the sample. This procedure will also be outlined in the following section.

In the early work which utilized thin-layer chromatography as an analytical technique, it was carried out on layers bound to

glass-plates. However, a major problem in the use of glass plates is that to quantitate the separation or to count radioactive material in the layer, the layer must be carefully removed from the glass plate and transferred to a suitable container for elution or for counting of radioactivity. This procedure almost always results in loss of material. A recent innovation to the field of thin-layer chromatography is the commercial availability of various support layers plated not on glass but on Mylar film, a very thin plastic material easily cut with scissors yet rigid enough to stand in developing tanks without support. This development reduces loss during quantitation since any particular area on the chromatogram may be simply cut out and then counted for radioactivity content, or the material contained in the area may be eluted from the adsorbant without prior removal of the adsorbant from the supporting material.

The early experiments reported in this dissertation were analyzed by using PEI-cellulose sheets prepared in this laboratory and utilized for 2-dimensional separations as per K. and E. Randerath (1964). Later experiments were analyzed by utilizing commercially available PEI-cellulose sheets in a 1-dimensional technique which results in adequate resolution. Both procedures are outlined in detail below, together with the procedure for the resolution of bases and nucleosides.

a. Preparation of PEI-Cellulose Sheets

A 1% aqueous solution of PEI is prepared as follows. Approximately 250 ml of distilled water are added to 100 g of 50% aqueous solution of PEI. The mixture is stirred until homogeneous. The PEI solution is adjusted to pH 6 with concentrated HCl and then made up to

500 ml with distilled water to give a 10% solution of PEI. Twenty ml of this material are placed inside dialysis tubing (1-3/4 x 12 in) and dialyzed for 24 hours against 4 l of distilled water. After dialysis, the tube is carefully cut open with scissors and the contents diluted to 200 ml with distilled water to give a final PEI concentration of 1%.

To a beaker containing 125 ml of 1% PEI solution are added 50 g of Whatman CC41 cellulose for thin-layer chromatography; the mixture is stirred well by magnetic stirrer. This material is placed in a high-speed electrical mixer and stirred for 30 seconds. The resulting homogeneous mixture is placed in a beaker and stirred slowly by magnetic stirrer to remove air bubbles.

The PEI-cellulose is then coated on a sheet of Mylar film in a thickness of 500μ by one of the usual methods. The layer is allowed to dry and the film is cut into 5-20 x 20 cm sheets. Before chromatography, the sheets are washed by developing them to a height of 5 cm with 10% NaCl, and then without intermediate drying, to the upper edge with distilled water. The layer is allowed to air-dry and is again developed with distilled water. When the layers are dried, they are stacked 5 high, wrapped in aluminum foil, and kept at -20° until used.

It should be noted that the PEI-cellulose did not bind well to the plastic film and sometimes flaked off during chromatography. Superior adhesion could be obtained by using MN 300 cellulose (Macheray, Nagel Co.) in place of Whatman CC41. However, the layers produced were much more impure and more extensive and therefore, time-consuming washing procedures had to be employed.

b. Two-Dimensional Chromatography of Nucleotides on PEI-Cellulose Sheets

This procedure is slightly modified from that outlined by E. and K. Randerath (1964). A sample (10 or 20 µl) of the cell-extract, together with the appropriate carrier compounds (about 30 nmoles of each) is spotted approximately 2 cm from the bottom and left side of the PEI-cellulose layer. The chromatogram is developed twice with methanol/water (1/1) with intermediate drying between developments. This preliminary development desalts the sample and removes interfering bases and nucleosides from the nucleotides; the latter are left as a streak near the origin, and the former migrate to the top of the layer where they are scraped off. The layer is then developed in the same direction, with LiCl solutions of increasing concentration as follows: 0.2 M LiCl for 2 min, 1.0 M LiCl for 6 min, and 2.6 M LiCl up to a line 13 cm above the origin. The plates are not dried between solvent changes. After development with 2.6 M LiCl, the layer is dried and areas of the layer not used for chromatography in the second direction are scraped off, that is, areas below the origin and at the top of the sheet above the 13 cm level. The layer is then washed with anhydrous methanol for 30 min to remove residual salt from the first chromatography. To do this, the sheet is placed in a flat dish containing 1000 ml of methanol and the dish is rocked slightly to ensure complete removal of salt. The layer is then dried and developed at 90° to the first development (origin at bottom right corner) with increasing concentrations of formic acid/sodium formate buffers, pH 3.4, as follows: 0.5 M formate for 30 sec, 2.0 M formate for 2 min, and 4.0 M formate to a line 15 cm above the origin, again without intermediate drying. After

the layer has dried, the plate is viewed under short-wave (2537 Å) ultraviolet light to visualize the nucleotide areas. A typical plate, showing the resolution of a mixture of authentic nucleotides, is represented by Fig. 2. The ultraviolet absorbing areas are outlined with pencil, cut out with scissors, and transferred to counting vials for radioactivity measurement. The total development time is approximately 4 hours.

It would have been advantageous for our studies to have included AMPS in the mixture of carrier nucleotides. However, the small amount of AMPS which was available to us was impure and multiple ultraviolet absorbing areas were found on two-dimensional thin-layer chromatograms; these interfered with the resolution of other nucleotides. Accordingly, AMPS was not included as carrier when samples of cell extracts were chromatographed.

The obvious drawback to the chromatographic procedure outlined above is that the number of samples which can be analyzed at one time is limited by the availability of chromatography tanks. Accordingly, we have designed apparatus with which it is possible to analyze up to 40 samples at one time. This apparatus was constructed of unplasticized polyvinyl chloride. Three developing tanks with lids were constructed from 1/4 in thick polyvinyl chloride in these dimensions: length, 18 in; width, 15 in; depth, 12 in. In addition, a wash tank with lid was constructed from the same material which was 15 in long, 12 in wide, 18 in deep. Two support racks were made from 8 sheets of polyvinyl chloride 1/8 in thick, each 8 x 11 in, and 2 sheets 1/4 in thick (end sheets) each 9 x 11 in. Holes were drilled near the four corners of each sheet and through these holes were passed 12 in lengths

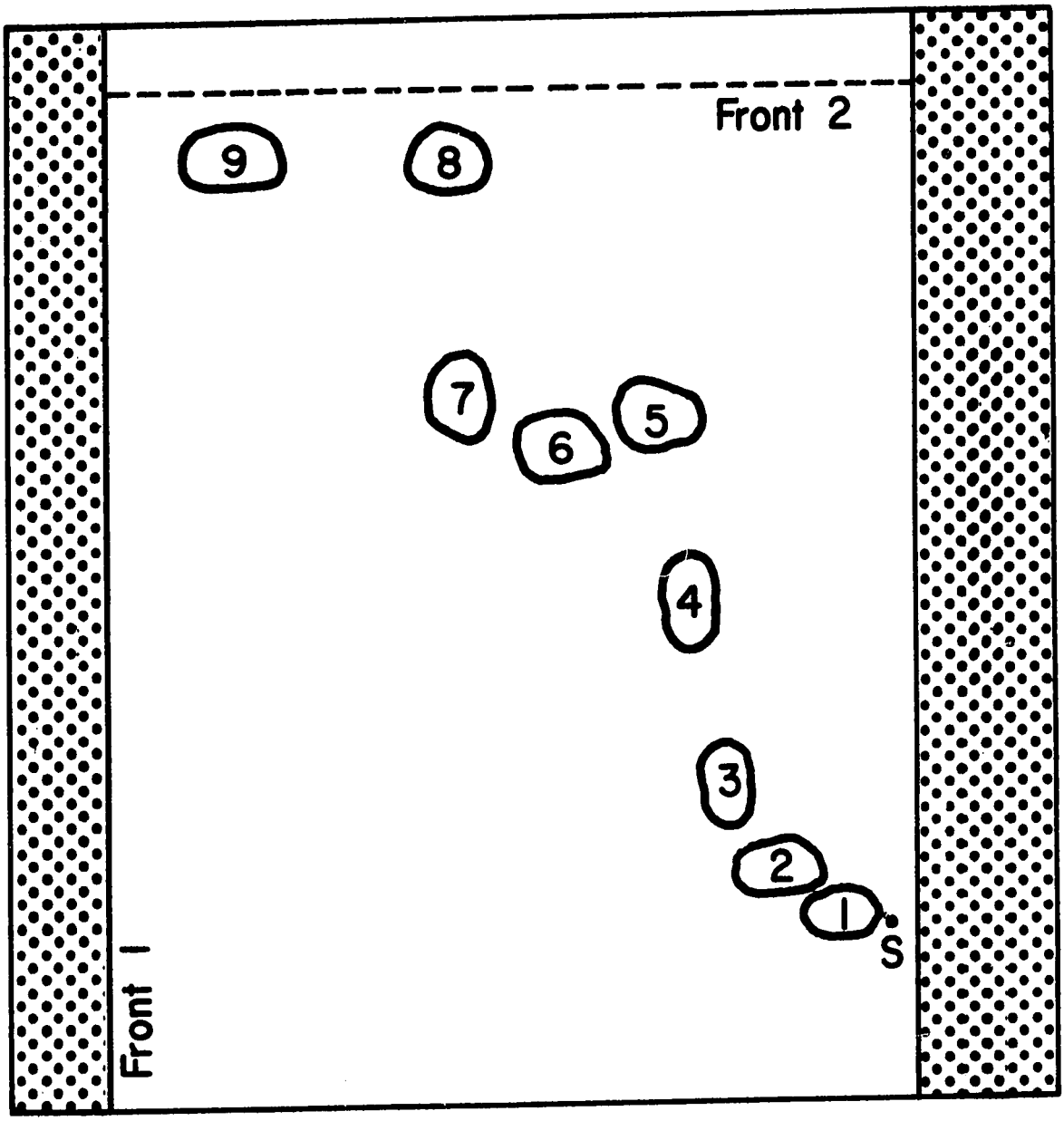


Figure 2. Two-dimensional chromatography of purine ribonucleotides on PEI-cellulose.

Authentic purine nucleotides were separated on a 0.5 mm layer of PEI-cellulose. Sample was spotted at S. First dimension chromatography: stepwise elution with 0.2 M LiCl (2 min), 1.0 M LiCl (6 min), 1.6 LiCl (to 13 cm above starting point). Second dimension chromatography: stepwise elution with 0.5 M sodium formate buffer pH 3.4 (30 sec), 2.0 M sodium formate buffer pH 3.4 (2 min), 4.0 M sodium formate buffer pH 3.4 (to 15 cm above starting point). Dotted areas were removed before chromatography in the second dimension. Ultraviolet absorbing areas are: 1, GTP; 2, ATP; 3, GDP; 4, ADP; 5, GMP; 6, XMP; 7, IMP; 8, AMP; 9, NAD.

of stainless steel rod (3/8 in diameter). Spacers made from polyvinyl chloride tubing (3/8 in I.D., 1/2 in O.D.) were placed on the rods to keep the sheets 1 in apart. The steel rods were threaded at the ends so that they could be fitted with washers and nuts to hold the entire rack rigid. This arrangement made the apparatus easy to dismantle for cleaning and storage. The two end sheets of each support rack were shaped so that the bottom corners of the sheets acted as "legs" in order that the interior sheets could be supported 1 in above the bottom of the developing tank. The plastic chromatography sheets were attached to the racks with letter clips so that the bottoms of the sheet would just touch the bottoms of the chromatography tanks. Twenty sheets could be supported by each rack. To move the sheets from solvent to solvent, the whole support apparatus plus chromatography sheets was simply moved from one tank to the next. For the intermediate methanol wash, the chromatography sheets were clipped top and bottom, and the racks were placed on end in the washing tank which contained enough methanol to cover all of the sheets. This apparatus and the 2-dimensional chromatographic technique made possible the simultaneous resolution of complex mixtures of purine nucleotides from 40 samples of cell extracts.

c. Chromatography on Baker-Flex PEI-Cellulose Sheets

The 2-dimensional technique outlined above was used throughout the early part of this work. However, when PEI-cellulose plastic chromatography sheets became commercially available, they were examined for their usefulness in separating complex nucleotide mixtures. The commercially available sheets were found to have several advantages over home-made sheets, the most important of which was that there was

very little sheet to sheet variation with respect to layer homogeneity, etc; this resulted in more constant flow rates. In addition, availability of these sheets eliminated the time needed to prepare the sheets in our laboratory. The initial cost of these sheets, however, is quite high and so we investigated the possibility of developing a 1-dimensional technique for resolving complex nucleotide mixtures which would allow us to apply more than one sample per sheet. Such a technique is outlined below.

Samples of cell extracts plus carriers are placed on a line about 1 cm from the bottom of the sheet. A wick of Whatman 3MM filter paper is attached with staples to the top of the sheet. The layer is developed with methanol/water (1/1), dried, and redeveloped for the same reasons as before. Bases, nucleosides, salts and layer impurities are absorbed onto the wick which is then discarded. The plate is dried and then developed with increasing concentrations of formic acid/sodium formate buffers, pH 3.4 as follows: 0.5 M formate to a line 2.5 cm above the origin, then 2.0 M formate to a line 7 cm above the origin, and finally to the top of the plate with 4.0 M formate. The plates are dried and visualized as before. A typical separation is shown in Fig. 3. As this figure illustrates, the separation is complete except for NAD and AMP. However, these compounds may be resolved by spotting other samples of the cell extracts on new plates, washing with methanol/water as before, and developing the plates with 0.2 M LiCl. In this solvent, NAD has R_f = 0.65, while AMP and all of the other nucleotides remain near the origin (Randerath, K. and Randerath, E., 1964).

One drawback to the use of the commercially available sheets

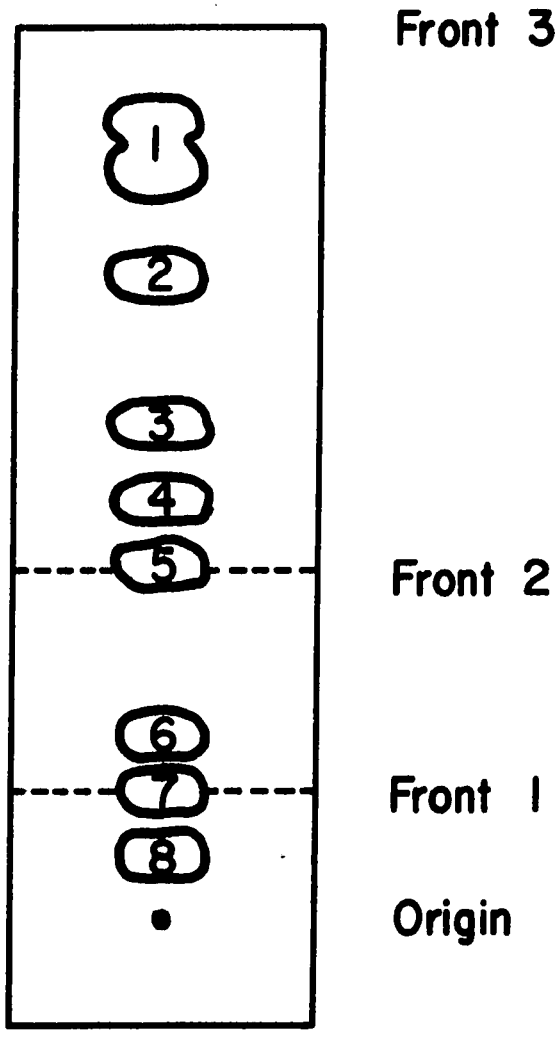


Figure 3. One-dimensional chromatography of purine ribonucleotides on PEI-cellulose.

Authentic purine nucleotides were separated on Baker-Flex PEI-cellulose sheets. The sample was applied at origin spot. The plate was developed to Front 1 with 0.5 M sodium formate buffer pH 3.4, to Front 2 with 2.0 M sodium formate buffer pH 3.4, and then to Front 3 with 4.0 M sodium formate buffer pH 3.4. Front 1 is 2.5 cm above X, Front 2 is 7.0 cm above X, and Front 3 is approximately 17 cm above X. Ultraviolet absorbing areas are: 1, NAD + AMP; 2. IMP; 3, XMP; 4, GMP; 5, ADP; 6, GDP; 7, ATP; 8, GTP.

is that samples containing high amounts of protein and/or salt are difficult to wet with developing solvent; solvent flows "around" the origin spot rather than "through" it. This difficulty may be overcome in two ways: (1) the developing solvent is allowed to flow up to the origin line, the sheets are quickly removed and the origin spots are immediately wetted with a small amount (2 or 3 μl) of methanol/water (1/1); development is then continued, or (2) the samples are applied as streaks approximately 1/2 in long rather than as spots; this procedure has the effect of diluting the protein and/or salt concentration at any point on the origin line.

This 1-dimensional technique was used during the latter portion of these studies and resulted in a significant reduction of analysis time and in much more reproducible separations over the previous 2-dimensional procedure.

d. Resolution of Mixtures of Purine Nucleosides and Bases

Two-dimensional thin-layer chromatography is utilized to effect complete separation of adenine, adenosine, guanine, guanosine, hypoxanthine, inosine, xanthine, xanthosine, and uric acid without interference from the nucleotides also contained in the cell-extracts. Samples of cell extracts plus appropriate carriers are spotted on commercially available cellulose thin-layer plastic sheets, 2 cm from the bottom and left edges. The layer is developed in the first direction for about 50 min with a solvent composed of acetonitrile/ ammonium acetate (0.1 M, pH 7)/ammonia (60/30/10). After the plates are dried, areas below the origin and 1-1/2 in from the top of the plate are scraped off and discarded. The plates are rotated 90° (origin spot now in lower right corner) and developed in the second

direction with 1-butanol/methanol/water/ammonia (60/20/20/1). The layers are dried and redeveloped with the second solvent in the same direction. After the layers are dried, they are visualized under short wave ultraviolet light as before. The appearance of the developed sheet is shown in Fig. 4. Nucleotides migrate somewhat in solvent 1 but not in solvent 2, and therefore remain as a streak along the bottom of the sheet. Total development time is about 6 hours.

e. Determination of Total Nucleotide Synthesis

A rapid thin-layer chromatographic system was used to measure total nucleotide synthesis from preformed purine bases. Samples of cell extracts plus appropriate carriers are spotted onto commercially available thin-layer plastic sheets with cellulose as adsorbant on a line approximately 2 cm from the bottom of the sheet. Up to 10 samples may be spotted per sheet. The layer is then developed with 95% ethanol/ 1.0 M ammonium acetate, pH 7.5 (75/35). The nucleosides and bases migrate up the plate whereas nucleotides remain at the origin. Development time is about 2 hours. The plate is dried and visualized as before. Xanthine, the slowest migrating base is about 2 cm above the nucleotide area after development.

2. Determination of PP-ribose-P

PP-ribose-P was measured by the method of Henderson and Khoo (1965a) as follows. Samples (1.0 ml) of the incubation mixtures are removed at appropriate times and placed in 12 ml conical centrifuge tubes standing in a boiling water bath. The contents of the tubes are heated for 20 seconds and the tubes are then plunged immediately into an ice-water bath to cool. After centrifugation at top speed in a clinical centrifuge for 5 minutes to sediment denatured protein, 0.05 ml

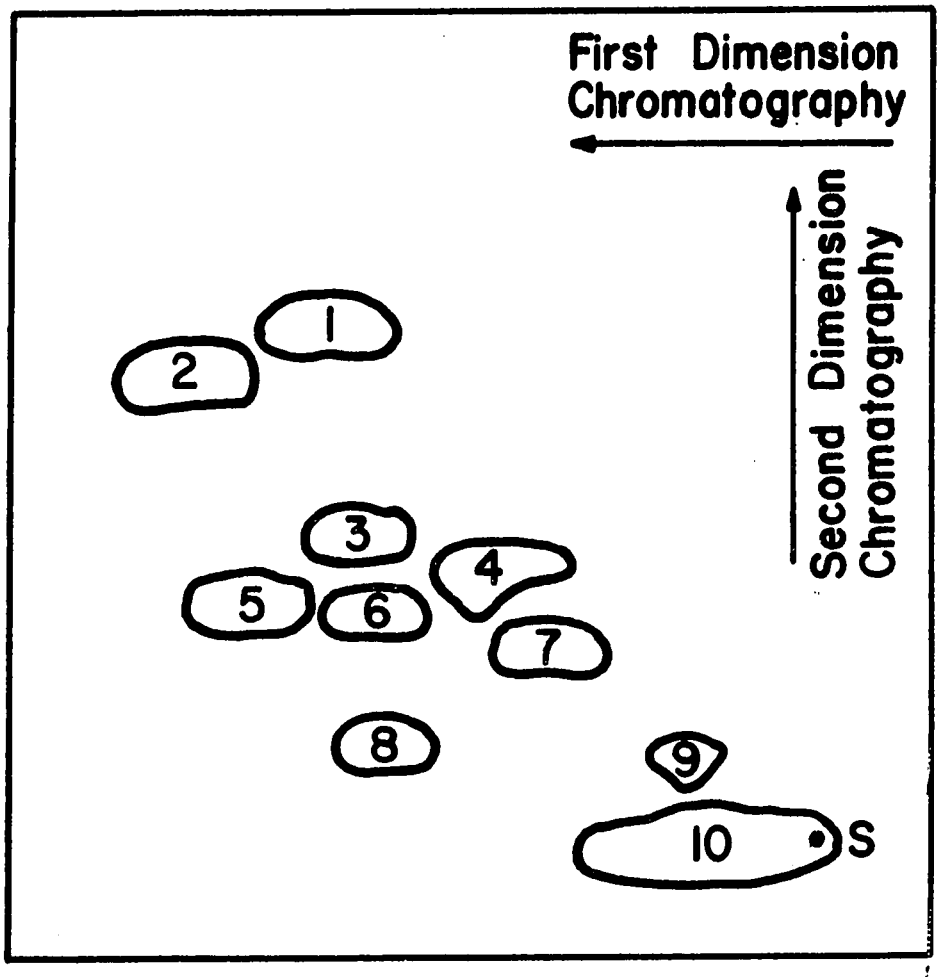


Figure 4. Two-dimensional chromatography of purine bases and ribonucleosides on cellulose.

Authentic purine bases, nucleosides and nucleotides were separated on Eastman Kodak cellulose sheets. Sample was applied at S. For chromatography in the first dimension the solvent was acetonitrile/ammonium acetate (0.1 M, pH 7)/ ammonia (60/30/10). For chromatography in the second dimension the solvent was 1-butanol/methanol/water/ammonia (60/20/20/1). After the sheets were dried, second dimension chromatography was repeated. Ultraviolet absorbing areas are: 1, adenine; 2, adenosine; 3, hypoxanthine; 4, guanine; 5, inosine; 6, guanosine; 7, xanthine; 8, xanthosine; 9, uric acid; 10, purine nucleotides.

of a crude preparation of adenine phosphoribosyltransferase (see below) is added to each tube followed by 0.10 ml of adenine-^{14}C (1 mM; 10.8 μc/μmole). The contents of the tubes are mixed carefully to avoid resuspension of the precipitate and are then incubated in a shaking water bath (about 60 strokes per minute) at 37° for 30 minutes. At the end of the incubation period, 0.1 ml of a solution of 0.2 M perchloric acid containing 10% $ZnSO_4$ (w/v) is added and the tubes are heated for 3 min in a boiling water bath. After chilling, the mixtures are centrifuged to sediment denatured protein and potassium perchlorate. 50 μl of the supernatant solutions are then spotted on strips of Whatman No. 1 paper with carrier adenine and AMP and the chromatograms are developed in a descending direction with 5% (w/v) aqueous Na_2HPO_4 for about 6 hrs. After drying, the AMP spots are visualized under short-wave ultraviolet light, cut out, and placed in counting vials. Radioactivity is determined after addition of toluene phosphor solution.

A standard curve for PP-ribose-P determinations is established by preparing a series of 12 ml conical centrifuge tubes containing various amounts of PP-ribose-P in pH 7.4 Krebs-Ringer solution containing 10^{-4}M adenine-^{14}C (total volume is 1 ml). After addition of 0.05 ml of the crude enzyme preparation (see below), PP-ribose-P is determined as outlined above. This standard curve shown in Fig. 5 illustrates the linear relationship between PP-ribose-P concentration and radioactivity incorporated from adenine-^{14}C into the total acid-soluble ribonucleotide fraction.

To obtain the crude enzyme preparation used in these determinations, Ehrlich ascites tumor cells are removed from one mouse with a Pasteur pipet, and placed in a 12 ml graduated centrifuge tube

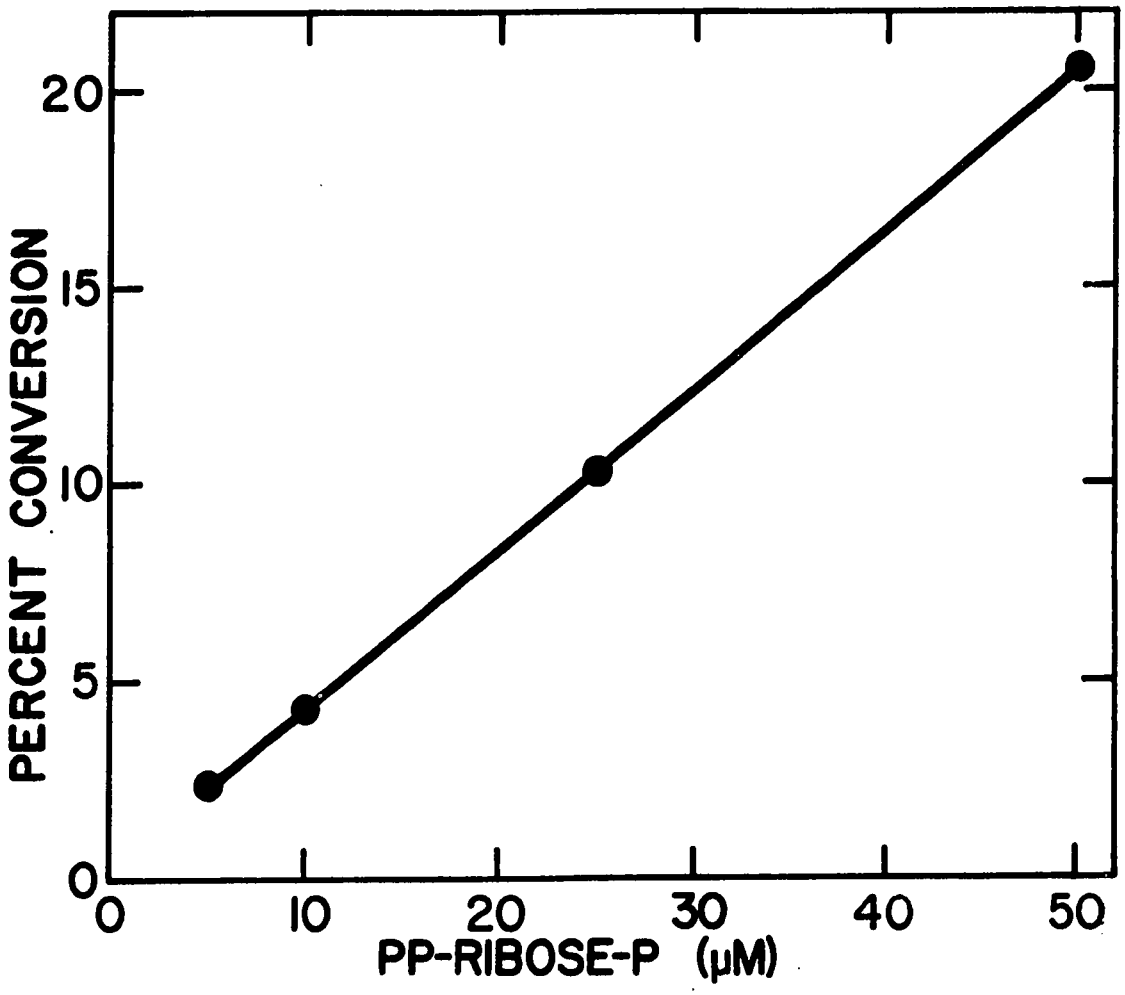


Figure 5. Standard curve for PP-ribose-P assay.

Various concentrations of PP-ribose-P were incubated for 30 min with 50 µl of crude adenine phosphoribosyltransferase (see text for preparation) and 10^{-4}M adenine-^{14}C. Results are expressed as the percentage of adenine-^{14}C converted into total nucleotides.

containing 0.9% NaCl solution. The cell suspension is centrifuged for 3 min in a clinical centrifuge and the supernatant fluid is discarded. The cells are resuspended in 9 volumes of 0.2 M Tris buffer (pH 7.4) and are then disrupted by sonication. The resulting material is centrifuged at 10,000 x g for 20 min and samples of the supernatant fluid are used as the source of adenine phosphoribosyltransferase. All manipulations are performed in the cold.

D. Radioactivity Measurements

Ultraviolet-absorbing areas are cut out of the Mylar thin-layer chromatography sheets and placed directly in counting vials so that the layers face the top of the counting vials. Eighteen ml of toluene phosphor solution is added to each vial and radioactivity is measured in a Nuclear Chicago Liquid Scintillation Spectrometer Model 724. The counting efficiency is approximately 72%. Quenching is measured by the channels ratio method. Quenching is observed when both cellulose and PEI-cellulose layers are present in the counting vials; the latter exhibit a higher degree of quenching. However, the channels-ratios for all samples counted on cellulose layers are similar, as are those for samples counted on PEI-cellulose, so that individual samples counted on the same layers can be directly compared without further corrections. The composition of the toluene phosphor solution is: 2,5-diphenyloxazole, 4 gm; 1,4-bis-[2-(5-phenyloxazolyl)] benzene, 0.1 gm; toluene, 1 liter.

E. Preparation and Handling of Tumor Cells

The Ehrlich ascites tumor cells used in these experiments have been maintained since 1956 by weekly transplantation in ICR Swiss

mice. Six days after transplantation, cells are removed with a Pasteur pipet through an abdominal incision and placed in 12 ml graduated centrifuge tubes containing heparin (approx. 20 μg) and buffered saline plus glucose (1 mg/ml). The composition of buffered saline is: NaCl (0.14 M), pH 7.4 Tris buffer (0.01 M) and pH 7.4 sodium phosphate buffer (0.004 M). The collection of cells and subsequent manipulations are carried out at room temperature since alternate chilling and warming of Ehrlich ascites carcinoma cells is known to result in disturbances in electrolyte transport (Chance and Hess, 1959).

Cells are sedimented by centrifugation and the ascitic fluid is discarded. The cells are then resuspended in 4 volumes of buffered saline plus glucose by gentle aspiration with a Pasteur pipet (Laws and Stickland, 1963). Erythrocytes are removed by repeated centrifugation of cell suspensions for 30 sec at top speed in a clinical centrifuge; supernatants containing red blood cells are discarded each time and the cells resuspended in 4 volumes of buffered saline plus glucose. Three washings are routinely used. The cells are packed by centrifugation at top speed in the clinical centrifuge for 3 min, packed cell volumes are determined, and the cells are gently suspended in 4 volumes of the appropriate incubation medium to give a final cell concentration of 20%.

F. Incubation Conditions

All incubations are carried out in a water bath at 37° with shaking at 80 oscillations per min. Concentrations of components in the reaction mixture are as follows: Ehrlich ascites carcinoma cells, 2%; glucose, 5.5 mM; radioactive purine base, 10^{-6} to 10^{-4} M; and other compounds as specified in individual experiments; the volume is made up

to 5 ml (for most experiments) with incubation medium. The usual incubation medium is modified Krebs-Ringer solution, which contains 4.9 mM KCl, 1.2 mM $MgSO_4$, 110 mM NaCl and 25 mM sodium phosphate. The pH of this medium is 7.4.

In most cases, incubations are carried out in 25 ml Erlenmeyer flasks but 10 ml Erlenmeyers are used when the volume of incubation mixture is 2 ml. All incubations are carried out with air as the gas phase. In all experiments, cells are incubated with glucose for 20 minutes (see Chapter III) before radioactive precursor is added.

G. Termination of Incubations and Extraction of Cells

At appropriate times after addition of radioactive precursor, 0.5 ml samples of the incubation mixture are pipetted into chilled 12 x 75 mm plastic tubes containing 25 µl of cold 42% perchloric acid; the final acid concentration is 4%. The contents of the tubes are mixed well and immediately chilled. As soon as possible, the acidic mixtures are adjusted to pH 7-8 by the addition of 25 µl of 4.42 M potassium hydroxide. This procedure results in a constant 10% volume change for all samples. Denatured protein and potassium perchlorate are sedimented by centrifugation. Samples of the supernatant are then analyzed, without further manipulation, by one of the methods described in a preceding section.

III CONTROL OF PURINE RIBONUCLEOTIDE SYNTHESIS FROM BASES

A. Introduction

The first aspect of this study of the control of purine nucleotide metabolism in Ehrlich ascites tumor cells incubated *in vitro* is the synthesis of purine nucleotides from exogenously supplied purine bases by the purine phosphoribosyltransferases.

That some control of the synthesis of purine nucleotides from bases does exist in intact cells is evident from the earlier work of Ellis and Scholefield (1962) with Ehrlich ascites tumor cells *in vitro*. They observed that the incorporation of adenine-^{14}C into total acid-soluble nucleotides was markedly stimulated by the addition of glucose to the incubation medium. However, they also noted that the rate of nucleotide synthesis in the presence of glucose decreased continuously throughout the incubation period. Non-linear time-courses of nucleotide synthesis from preformed purine bases were also observed by Henderson and Khoo (1965b) even in the presence of high concentrations of purine base. These observations suggested that some modulation of the phosphoribosyltransferase reactions might be taking place.

1. Product and Feedback Inhibition of Enzyme Activity

The activity of hypoxanthine-guanine phosphoribosyltransferase is potentially regulated by inhibition by purine nucleotides. IMP and GMP inhibit the enzyme from *Bacillus subtilis* (Berlin and Stadtman, 1966), Ehrlich ascites tumor cells (Murray, 1966) and human erythrocytes (Henderson *et al.*, 1968; Krenitsky and Papiaonnou, 1969) in a manner which is competitive with PP-ribose-P. Although XMP is a very

weak inhibitor of human erythrocyte hypoxanthine-guanine phosphoribosyltransferase, GMP, GDP, and GTP are strong inhibitors (Krenitsky and Papiaonnou, 1969). 6-Mercaptopurine ribonucleotide is a weak inhibitor of the Ehrlich ascites tumor cell enzyme (Murray, 1967).

Adenine phosphoribosyltransferase from many sources is subject to product inhibition by AMP; this inhibition is competitive with respect to PP-ribose-P (Savel and Handschumacher, 1965; Berlin and Stadtman, 1966; Hori and Henderson, 1966; Murray, 1966). GMP has been reported to be a competitive inhibitor of this enzyme as well (Murray, 1967). However, other workers (Hori et al., 1967) believe that inhibition by GMP is quite complex and may be "allosteric" in nature. Adenine phosphoribosyltransferase is also inhibited by dAMP, apparently by "dead-end" inhibition (Hori et al., 1967). Murray (1966), observed inhibition of this enzyme by ADP and ATP; these nucleotides presumably act as analogs of the product AMP and thereby compete with PP-ribose-P for binding to free enzyme. However, at low concentrations (less than 250 μM), ATP stimulates adenine phosphoribosyltransferase activity and can reverse inhibition by AMP and GMP. Stimulation by ATP varies with pH and temperature with respect to both extent of stimulation and the range of stimulatory concentration of ATP. Murray believes that at least three different forms of the enzyme exist, each with a different affinity for ATP. The hypothesis that more than one form of adenine phosphoribosyltransferase exists is supported by the kinetic data of Hori et al. (1967) and by physical studies (Kelley et al., 1967).

2. Availability of Substrates

PP-ribose-P, the ribose-phosphate donor in the purine phosphoribosyltransferase reactions, is also required for the syntheses of purine and pyrimidine nucleotides via the de novo pathways, and all of these biosynthetic pathways are known to compete for this substrate. Therefore, purine nucleotide synthesis from bases might be limited by the availability of PP-ribose-P.

Early support for this suggestion was given by the observation that addition of glucose to Ehrlich ascites tumor cells in vitro resulted in a seven-fold increase in the incorporation of adenine-^{14}C into the total acid-soluble nucleotide fraction (Ellis and Scholefield, 1962). This observation led to the hypothesis that nucleotide synthesis was being controlled by the presence of an adequate supply of glucose which served as a source of ribose for nucleotide synthesis. In addition, these workers believed that when sufficient glucose was present to maintain maximum rates of nucleotide synthesis, the availability of purine base became the limiting factor.

However, in 1965, Henderson and Khoo (1965b) obtained evidence which indicated that the rate of PP-ribose-P synthesis in Ehrlich cells in vitro was sufficiently rapid to support the maximum rate of nucleotide synthesis from a single purine base even when it was present at high concentration (1 mM), but that PP-ribose-P levels were not sufficient to give maximum rates of synthesis from two bases simultaneously if both were present in high concentrations. Moreover, these workers found that nucleotide synthesis from adenine-^{14}C (1 mM) or guanine-^{14}C (1 mM) was independent of glucose concentration above 2.75 mM. Therefore,

these workers believed that the rate of nucleotide synthesis from a single purine base was limited by the amount or kinetic properties of the particular purine phosphoribosyltransferase involved in the reaction, rather than by the availability of PP-ribose-P or purine base.

Hershko et al. (1967) observed that in rabbit erythrocytes, cells which do not synthesize purines de novo, the incorporation of purines into ribonucleotides in vitro was markedly stimulated by increasing the P_i content of the incubation medium up to levels of 20-30 mM. The increasing concentrations of P_i were attended by parallel increases in the intracellular pool of PP-ribose-P. Other workers had previously obtained evidence that PP-ribose-P synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase, E.C. 2.7.6.1.) has a P_i requirement (Kornberg et al., 1955; Remy et al., 1955; Preiss and Handler, 1958).

Recently, Murray and Wong (1969) reported the partial purification of PP-ribose-P synthetase from Ehrlich ascites tumor cells. The reaction was absolutely dependent on the presence of P_i, and half-maximal rates were obtained with a concentration of 3.3 mM. PP-ribose-P synthetase was inhibited strongly by AMP and ADP, weakly by IMP, GMP, GDP, CDP, UDP, GTP, ITP, XTP, UTP, CTP, TTP, dGTP, and dCTP, whereas XMP, UMP, dUMP, dGMP, CMP, dCMP, and TMP did not significantly inhibit.

Obviously then, mammalian PP-ribose-P synthesis is potentially tightly controlled by feedback mechanisms. Atkinson and Fall (1967), and Switzer (1967) have indicated that bacterial PP-ribose-P synthetase is also controlled in this manner, and the former workers have shown that the activity of this enzyme is regulated by the relative

concentrations of AMP, ADP, and ATP (Klungsoyr et al., 1968).

That PP-ribose-P concentrations control the rate of purine biosynthesis de novo is suggested by the work of Rajalakshmi and Handschumacher (1968) which showed that purine biosynthesis de novo is markedly reduced in the livers of rats fed orotic acid, presumably because of depletion of PP-ribose-P supplies by the orotic acid phosphoribosyltransferase reaction. In addition, Rosenbloom et al. (1968) observed increased rates of purine biosynthesis de novo in cells lacking hypoxanthine-guanine phosphoribosyltransferase. In this case, PP-ribose-P concentrations are elevated since an alternate pathway of PP-ribose-P utilization (hypoxanthine-guanine phosphoribosyltransferase) is non-operative. However, it appears that purine biosynthesis de novo might be more influenced by fluctuations in PP-ribose-P concentrations than is nucleotide synthesis from preformed bases because the relative affinities of the purine phosphoribosyltransferases (Atkinson and Murray, 1965; Hori and Henderson, 1966) and of PP-ribose-P amidotransferase (Wyngaarden and Ashton, 1959; Caskey et al., 1964) for PP-ribose-P suggest that the former enzymes would have a competitive advantage for this substrate.

3. Entry of Bases Into Cells

Since purine nucleotide synthesis takes place intracellularly, the synthesis of nucleotides from exogenously supplied purine bases may be governed by the rates at which the bases enter cells. Studies by several workers imply that the uptake of purine bases by cells is simply a passive process. For example, adenine is thought to enter Ehrlich ascites tumor cells by passive diffusion (Jacquez and Ginsberg,

1960); 6-mercaptopurine enters human leukocytes (Kessel and Hall, 1967) and 6-thioguanine enters Sarcoma 180 cells (Bieber and Sartorelli, 1964) in the same manner. In contrast, the transport of purine bases appears to be carrier-mediated in human erythrocytes (Lassen, 1967), in choroid plexus of rabbits (Berlin, 1969) and in polymorphonuclear leukocytes (Hawkins and Berlin, 1969).

Evidence has also been obtained which has been interpreted as indicating that rates of purine uptake are controlled by the activities of the phosphoribosyltransferases in resting cell suspensions of B. subtilis (Berlin and Stadtman, 1966), in yeast cells (Cummins and Mitchison, 1967; Bergmann et al., 1969) and in human blood platelets (Holmsen and Rozenberg, 1968). It has been postulated (Cummins and Mitchison, 1967) that the phosphoribosyltransferases may form integral parts of the transport complexes at the cell surface rather than simply catalyzing distal rate-limiting reactions.

It is not certain that the phosphoribosyltransferases play an important role in the entry of purine bases into Ehrlich ascites tumor cells or that the entry of bases is limiting for nucleotide synthesis in these cells. However, a study of these factors is beyond the scope of the present investigation.

B. Results and Discussion

1. Determination of Optimum Incubation Conditions

As noted above, the availability of PP-ribose-P may be limiting for the synthesis of purine nucleotides from bases. The synthesis of this compound in intact cells is known to be dependent on both the length of time cells are incubated with glucose before

precursor base is added and on the concentration of P_i in the incubation medium. Accordingly, the incubation conditions used in our studies were decided on following an investigation of the effects on both PP-ribose-P and nucleotide synthesis of incubating the cells with glucose for various times before addition of the purine base and of varying the P_i concentration of the incubation medium.

a. Effect on PP-ribose-P and Nucleotide Synthesis of Incubating Cells with Glucose Before Addition of Base.

Cells are incubated with 5.5 mM glucose and, at appropriate times, the amounts of PP-ribose-P formed and the amounts of nucleotides synthesized from a preformed purine base are determined. This particular concentration of glucose is chosen for these studies since it has been observed (Henderson and Khoo, 1965a) that the initial rate of PP-ribose-P synthesis in Ehrlich cells is independent of glucose concentration above 0.55 mM.

The formation of PP-ribose-P was determined as outlined in Chapter II. The effect of varying incubation times in the presence of glucose on nucleotide synthesis from bases was ascertained in the following manner. After the cells had been incubated for appropriate times in the presence of 5.5 mM glucose, adenine-^{14}C was added to each flask to a final concentration of 10^{-4} M (final volumes were 1.0 ml). The mixtures were incubated with shaking at 37° for 5 minutes, 0.2 ml of 12% perchloric acid was added to terminate the reaction, the tubes were cooled, and the mixtures were neutralized with KOH. After the mixtures were centrifuged to sediment denatured protein, 50 µl of the supernatants were spotted on Whatman No. 1 filter paper strips with appropriate carriers and the chromatograms were developed with 5%

Na_2HPO_4. The total nucleotide areas were removed from the strips and counted in a liquid scintillation system using toluene phosphor solution.

Fig. 6 illustrates the effects of incubation of Ehrlich ascites carcinoma cells with glucose on PP-ribose-P formation and on nucleotide synthesis from adenine-^{14}C. Nucleotide synthesis reaches a plateau after an incubation period of 20 min. However, PP-ribose-P synthesis exhibits a linear relationship with incubation time and does not apparently reach a plateau even after 60 minutes of incubation with glucose. The latter finding is in contrast to the results obtained by Henderson and Khoo (1965a) who found that PP-ribose-P concentration did reach a definite plateau after about 30 minutes of incubation with glucose. The reasons for the discrepancy of our results and those of Henderson and Khoo (1965a) have not been examined. However, in the light of their results on PP-ribose-P synthesis and our results on nucleotide synthesis, a 20 minutes incubation period with glucose before addition of base was chosen for all future experiments.

b. Effect of P_i Concentration on PP-Ribose-P and Nucleotide Synthesis

The P_i concentration of the medium was varied between about 5 and 50 mM by changing the concentration of sodium phosphate buffer present in the Krebs-Ringer solution and, at the same time, altering the NaCl concentrations in the medium in order to keep the total Na^+ concentration in the media at a constant value. Cells were incubated for 20 minutes with glucose (5.5 mM) in all cases and then PP-ribose-P determinations were made or total nucleotide synthesis was measured after a further 5 minute incubation in the presence of adenine-^{14}C.

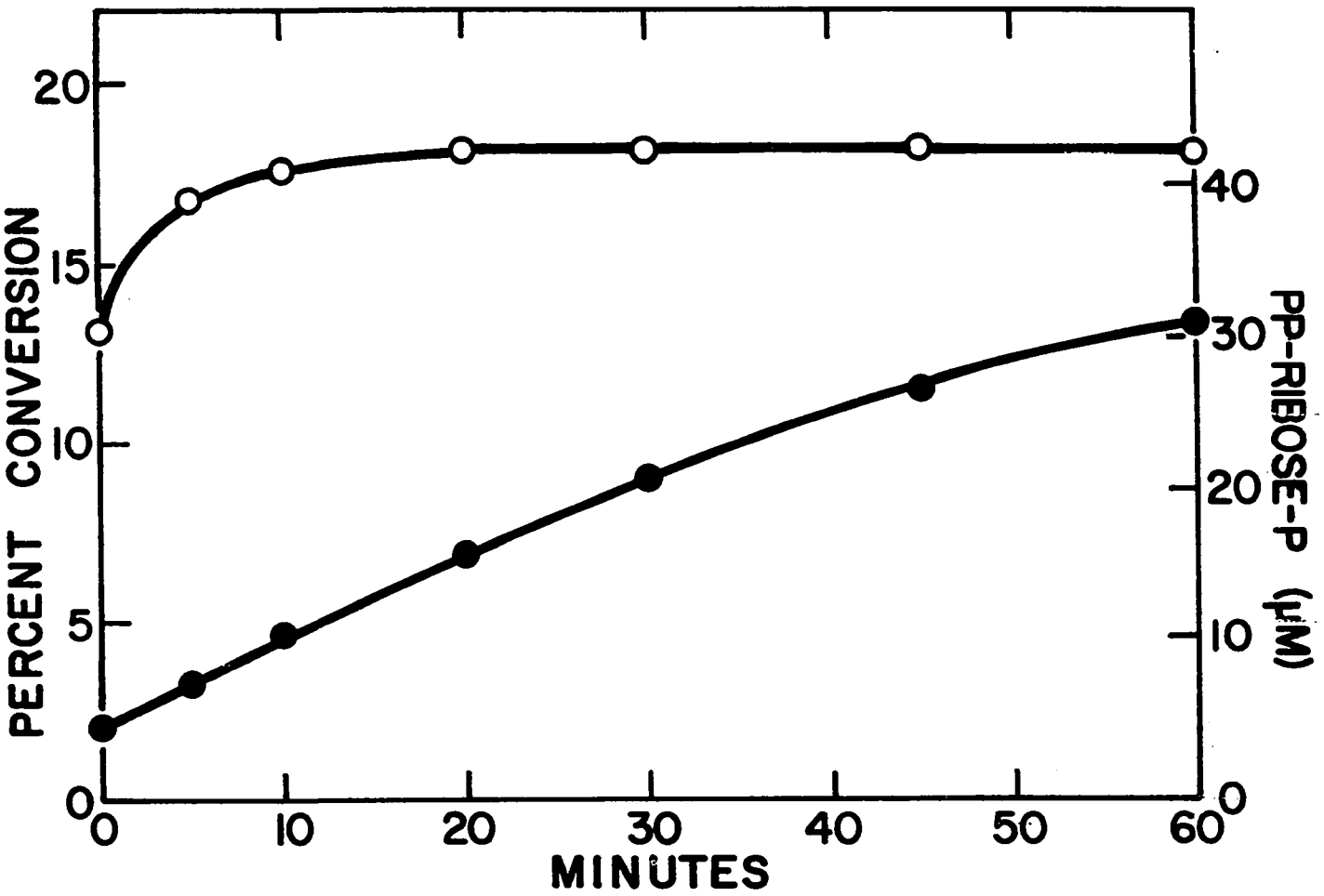


Figure 6. Effect of time of incubation with glucose on PP-ribose-P concentration and apparent initial rate of nucleotide synthesis from adenine.

PP-ribose-P was determined as described in the text on samples removed from the incubation mixture at the times indicated. For nucleotide synthesis, cells were incubated with 5.5mM glucose for the times indicated and adenine-^{14}C was added (final concentration 10^{-4}M). Total nucleotide synthesis was measured after a further 5 min. incubation period and is expressed as the percentage of adenine-^{14}C converted into total nucleotides. P_i concentration in the incubation medium was 25 mM. PP-ribose-P, ● ; total nucleotides, O .

Fig. 7 illustrates that PP-ribose-P synthesis appears to increase as the P_i content of the medium increases. Similar results are observed for total nucleotide synthesis. Although no definite plateaux are reached in the two curves, the slopes do decrease markedly at about 20 mM P_i. Accordingly, we have chosen to use an incubation medium containing 25 mM P_i for all future studies in place of the normal Krebs-Ringer solution, which is 16 mM in P_i.

Hershko et al. (1969) feel that this P_i effect is due to stimulation of the P_i-dependent PP-ribose-P synthetase, and they believe that the intracellular concentration of P_i may be the rate-limiting factor for PP-ribose-P synthesis in human erythrocytes. Recent work (Murray and Wong, 1969) on the PP-ribose-P synthetase from Ehrlich cells also shows it to be absolutely dependent on the presence of P_i and, therefore, the concentration of P_i may be rate-limiting for the synthesis of PP-ribose-P in our studies as well.

2. Nucleotide Synthesis with Adenine-^{14}C as Precursor

a. Time Course Studies

In this section, the data are presented in two ways. First, the amounts of nucleotides formed from different concentrations of precursor base are presented as functions of time. Second, the percentages of precursor base incorporated into the total nucleotide fraction at different base concentrations are given as functions of time.

When the data obtained using adenine-^{14}C at concentrations between 1 and 100 μM are plotted to show the amounts of nucleotides formed, the results are as shown in Fig. 8. The maximum amounts of nucleotide synthesis (after 90 min) are directly related to the initial

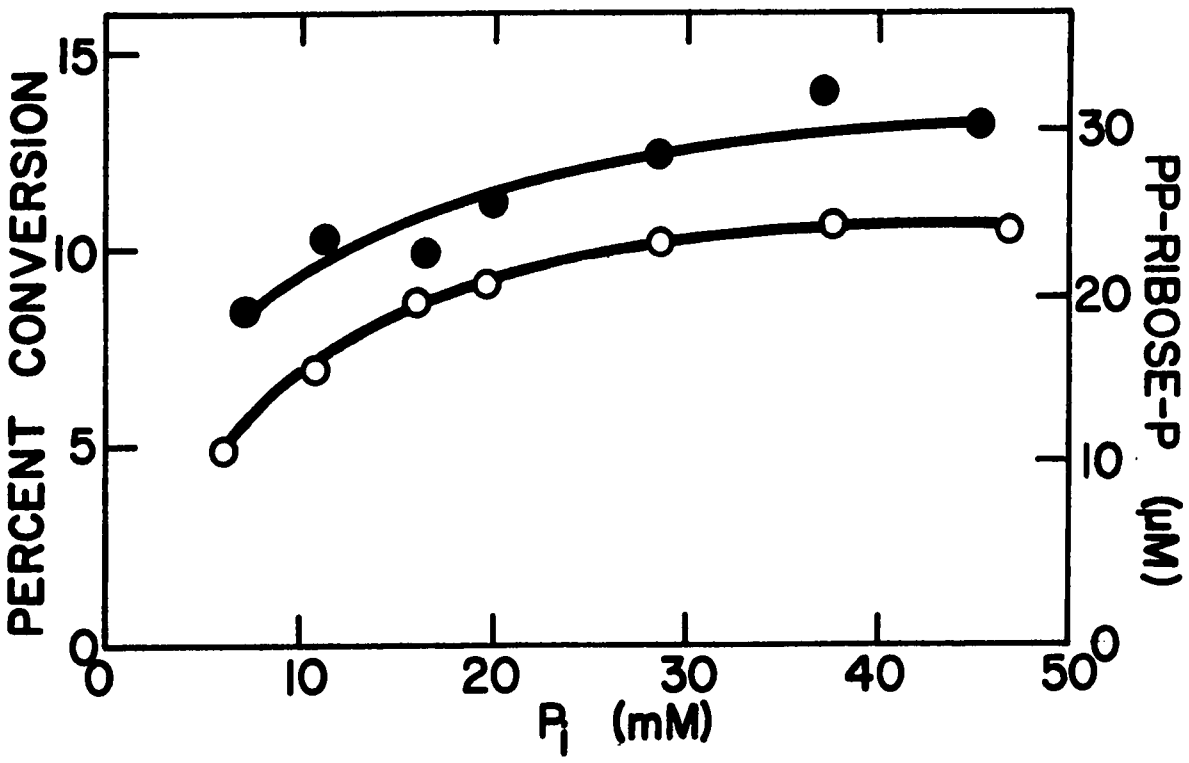


Figure 7. Effect of P_i on PP-ribose-P concentration and apparent initial rate of nucleotide synthesis from adenine.

Cells were incubated for 45 min. in the presence of glucose (initial concentration 5.5mM) before measurements were made. PP-ribose-P was determined as outlined in the text. Total nucleotide synthesis was measured after 5 min. incubation with adenine-^{14}C ($10^{-4}M$) and is expressed as the percentage of adenine-^{14}C converted into total nucleotides. Total nucleotide synthesis, ● ; PP-ribose-P synthesis, ○ .

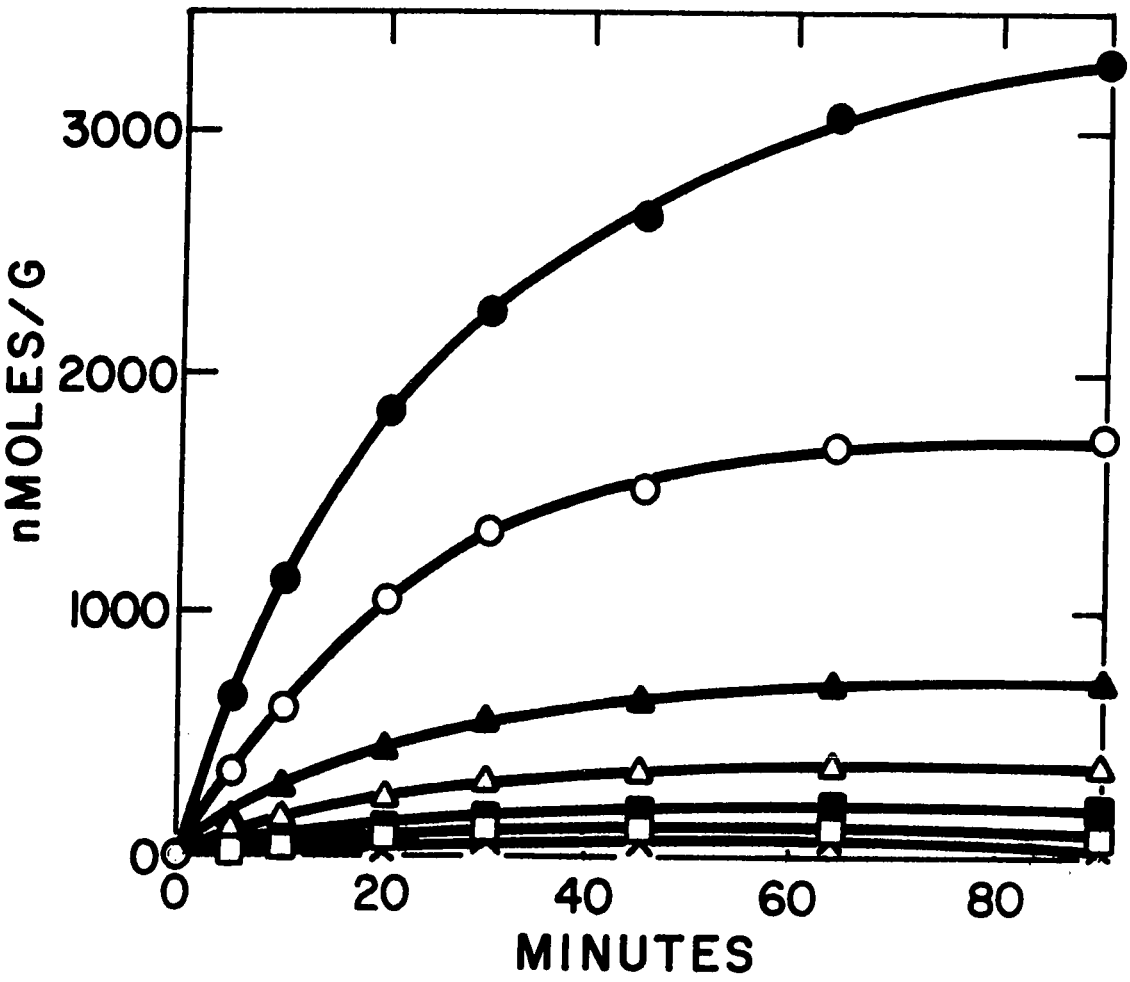


Figure 8. Incorporation of adenine-^{14}C into total acid-soluble nucleotides.

Adenine-^{14}C concentrations were: 1μM, X ; 2μM, □ ; 5μM, ■ ; 10μM, △ ; 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

concentration of precursor present in the incubation medium. The decline in rate of nucleotide synthesis in the course of the incubation may be due to declining substrate levels, to inhibition of adenine phosphoribosyltransferase by the nucleotides which are being synthesized, or combinations of these factors. At high adenine concentrations, the availability of PP-ribose-P may also play a role.

When the percentage of adenine-^{14}C incorporated into total nucleotides is studied as a function of time and of base concentration, the results are as shown in Fig. 9. The final percent incorporation (after 90 minutes) is independent of base concentration since at all concentrations of adenine-^{14}C used, between 74 and 80% of the base is converted into nucleotides after 90 minutes.

About 20% of the base appears not to be incorporated into nucleotides regardless of initial base concentration. However, since nucleotides formed from adenine-^{14}C may be degraded to nucleosides and other bases, especially at longer incubation times, the total conversion of radioactivity from adenine-^{14}C into other metabolites was determined. The results obtained for the three highest concentrations of base used (20, 50 and 100 μM), as presented in Fig. 10, illustrate that more than 90% of the base is metabolized at the highest concentration used, and that almost 100% is metabolized at 20 μM adenine and presumably at still lower concentrations.

b. Initial Rate Studies

The amount of nucleotide synthesized in the first five minutes after addition of the radioactive base is taken as the apparent initial rate of nucleotide synthesis. When these apparent initial rates are plotted as a function of adenine-^{14}C concentration, an essentially

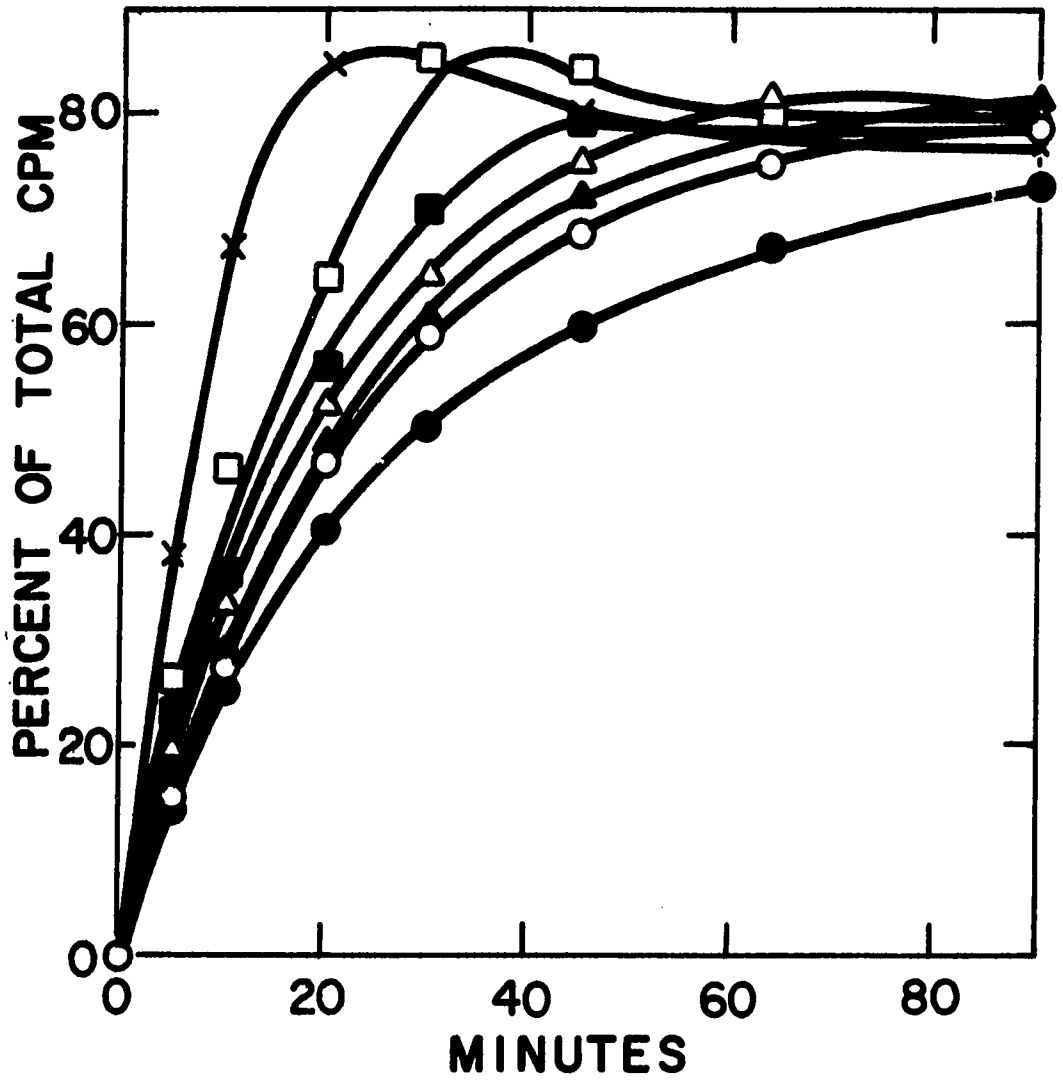


Figure 9. Percent incorporation of adenine-^{14}C into total acid-soluble nucleotides.

Adenine-^{14}C concentrations were: 1μM, ×; 2μM, □; 5μM, ■; 10μM, △; 20μM, ▲; 50μM, ○; 100μM ●.

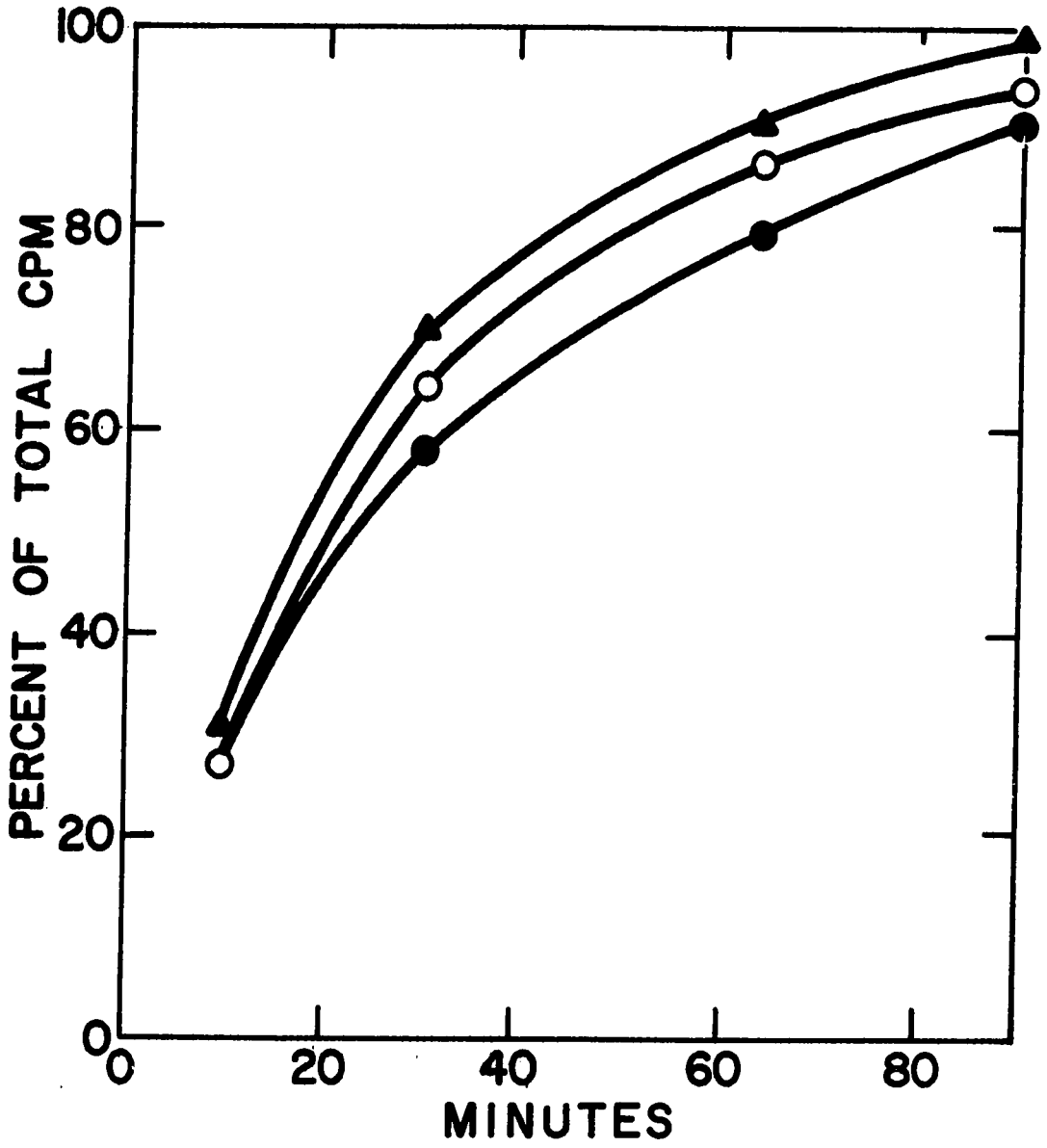


Figure 10. Percent total metabolism of adenine-^{14}C.
Results expressed as percent of total radioactivity present in purine nucleotides plus nucleosides plus bases (other than adenine). Adenine-^{14}C concentration: 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

linear relationship is observed (Fig. 11).

The concentration of adenine-^{14}C present at any time in the incubation period may be calculated when the initial precursor concentration is 20, 50 or 100 μM. To do this, the initial adenine-^{14}C concentration is multiplied by the percentage of precursor remaining at the particular time in question (obtained from Fig. 10). The rates of nucleotide synthesis in the periods of 10-15, 20-25, 30-35, 40-45, 50-55, 60-65, 70-75, 80-85 min after addition or precursor are taken from Fig 8, and are presented in Fig. 11 as functions of the concentration of base present at the start of these 5 min periods. As Fig. 11 shows, when the initial adenine-^{14}C concentrations are 50 and 100 μM, these data fall well below the curve for apparent initial rates.

c. Changes in Intracellular Levels of Nucleotides

Inhibition of purine phosphoribosyltransferases by nucleotides synthesized from the precursor base may play a role in controlling the rates of purine nucleotide synthesis. Since the major portion of the adenine-^{14}C incorporated into total nucleotides is present as ATP at all incubation times examined (see Chapter IV) and since ATP is known to be an inhibitor of adenine phosphoribosyltransferase, we have calculated the changes in ATP levels which occur during the incubation period when the concentrations of adenine-^{14}C are 20, 50 and 100 μM. The calculations were made in the following manner.

It has been determined that approximately 75% of the wet weight of packed Ehrlich ascites tumor cells is contributed by water and that about 45% of this water is intracellular (Wang, 1965). Since a cell concentration of 2% is used in the present studies and since the total incubation volume is 5 ml, then 2/100 x 5 = 0.1 ml of the total

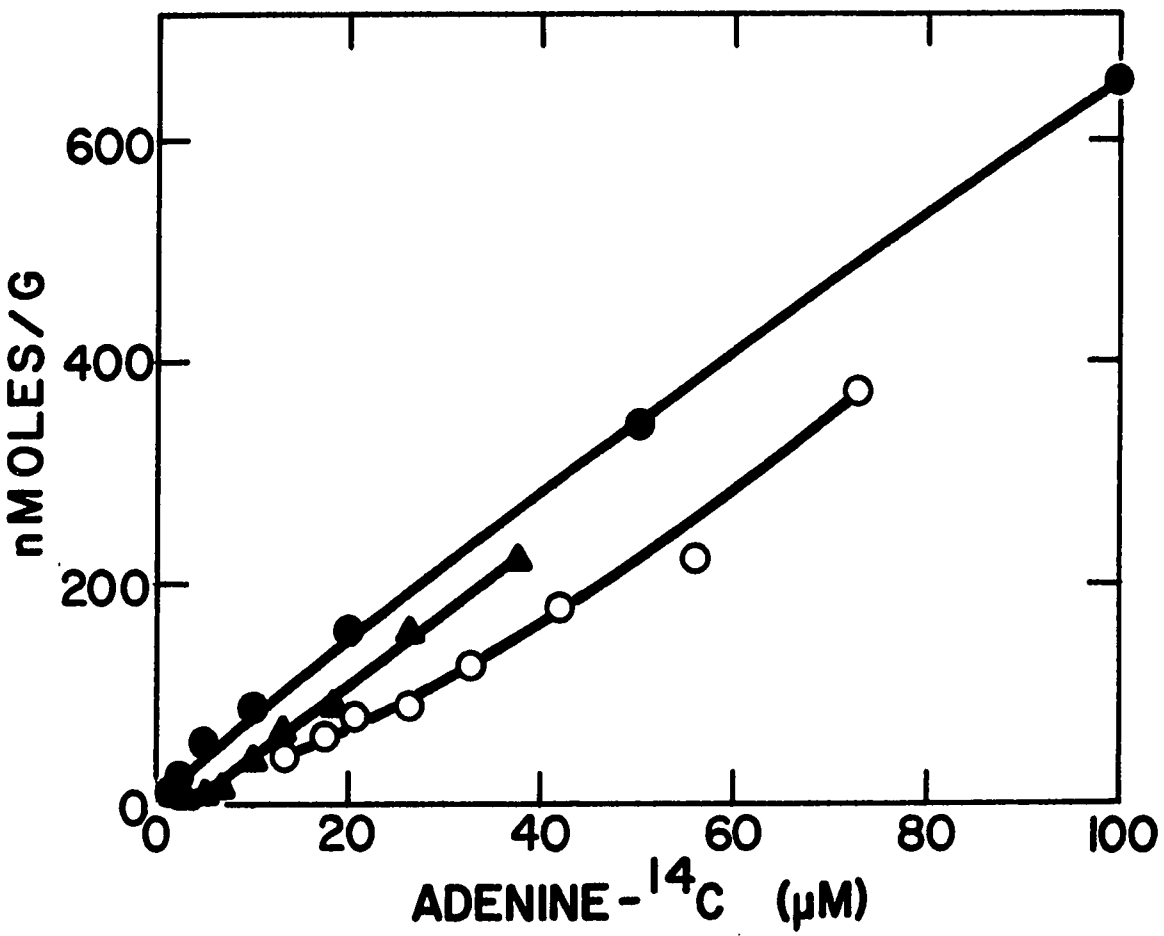


Figure 11. Relationship between adenine-^{14}C concentration and nucleotide synthesis.

Total nucleotide synthesis measured 5 min. after the addition of adenine-^{14}C was taken as the apparent initial rate of nucleotide synthesis. Initial rates of nucleotide synthesis, ● ; 5 min. rates of nucleotide synthesis with initial adenine-^{14}C concentration of 50μM, ▲ ; 5 min. rates of nucleotide synthesis with initial adenine-^{14}C concentration of 100μM, ○ .

incubation volume is occupied by packed tumor cells, and the volume occupied by intracellular water is therefore 0.1 x 75/100 x 45/100 = 0.034 ml. It is possible to calculate the amount of any particular nucleotide formed from the precursor base at any time in the incubation period given the initial precursor concentration. For example, when 100 μM (0.50 μmole/5 ml) adenine-^{14}C is used, about 50% of the base, or 0.25 μmoles/5 ml of reaction mixture, is incorporated into total nucleotides after 30 minutes of incubation. Nucleotides formed from the precursor base are presumed to be contained in the intracellular water and therefore, the total nucleotide concentration in these cells under the incubation conditions outlined above is 0.25 μmole/0.034 ml or 7.35 μmole/ml (7.35 mM). Of the total nucleotide fraction, approximately 70% is contributed by ATP under these conditions. Therefore, the amount of ATP formed during the reaction is 7/10 x 7.35 = 5.14 μmole/ml (5.14 mM). Since the normal ATP concentration in Ehrlich ascites tumor cells is about 5 mM (I.C. Caldwell, personal communication), the ATP concentration is increased by 5.14/5 x 100 = 103%.

Fig. 12 illustrates the change in ATP concentrations as a function of the 5 min rates of nucleotide synthesis at three initial concentrations (20, 50 and 100 μM) of adenine-^{14}C. For all three concentrations of adenine-^{14}C, a direct relationship between the change in ATP levels and the rate of nucleotide synthesis is apparent over the time period examined.

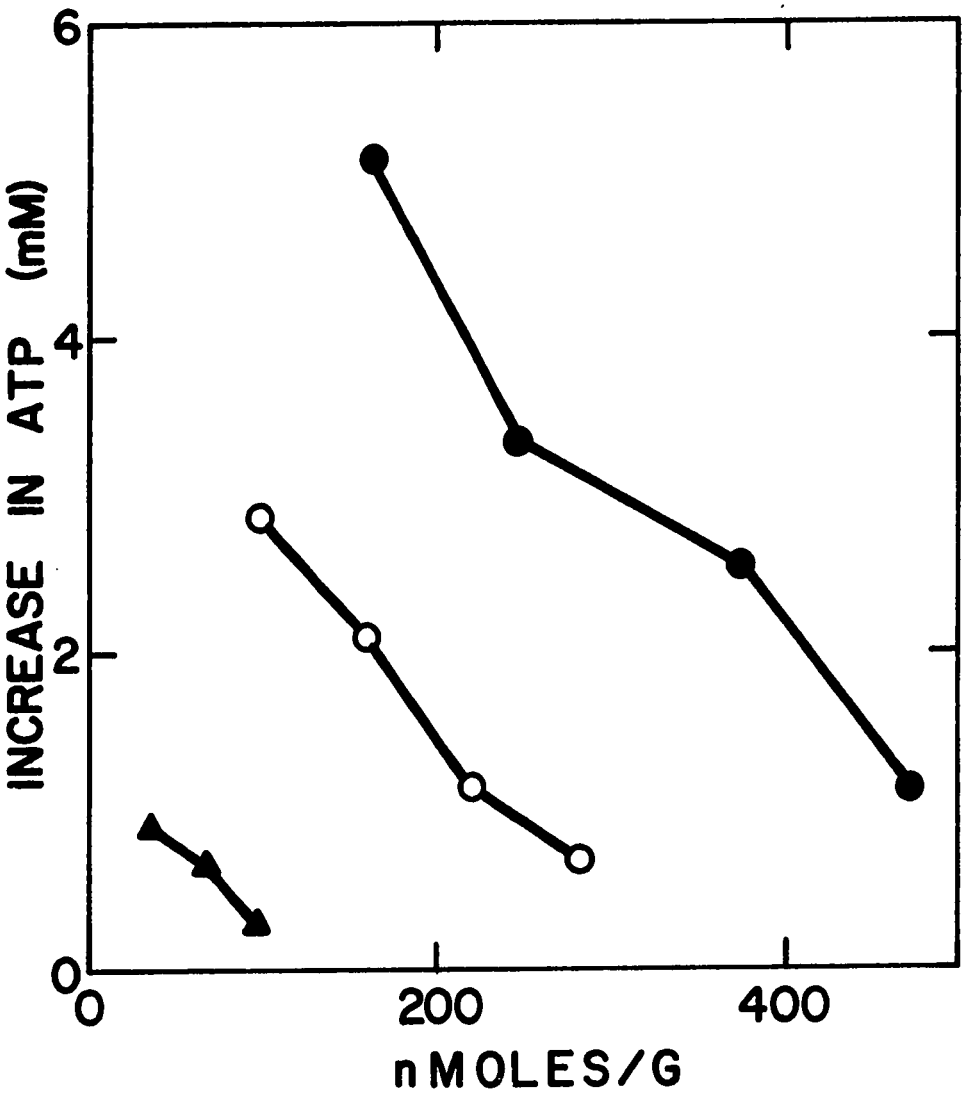


Figure 12. Relationship between apparent initial rate of nucleotide synthesis from adenine-^{14}C and change in ATP concentration.

Total nucleotide synthesis measured 5 min. after the addition of adenine-^{14}C was taken as the apparent initial rate of nucleotide synthesis. The intracellular concentration of ATP at the time adenine-^{14}C was added was assumed to be 5mM. Adenine-^{14}C concentration: 20μM, ▲ ; 50μM, ○ ; 100μM, ●.

The types of metabolic control which might be responsible for these observations are difficult to ascertain. In addition to declining substrate concentrations, inhibition of adenine phosphoribosyltransferase and/or inhibition of PP-ribose-P synthetase by nucleotides, may play a role. The results of these experiments and calculations indicate that the metabolic controls of purine nucleotide synthesis are complex.

d. Application of the Data Obtained for the Design of Other Experiments

As a corollary to these studies, it is interesting to consider how the data obtained from the above experiments could be utilized to design experiments to study other aspects of purine metabolism. Other than providing a sound basis for studies to be discussed later in this dissertation, an interesting possibility is to utilize these data to design experiments of the "pulse-chase" type. The criteria for such experiments may be obtained from the data outlined above.

Of prime importance for such studies is the assurance that the "pulse" will be diluted sufficiently by the "chase" that only insignificant amounts of metabolites would be synthesized from the radioactive precursor during the chase period. The amount of nucleotides synthesized during the pulse period for any concentration of adenine-^{14}C as pulse can be obtained from Fig. 8. It is necessary to calculate (1) the specific activity of the chase compound and (2) the amount of adenine-^{14}C incorporated into nucleotides during the chase period. The specific activity of the chase may be calculated as follows:

$$\text{specific activity of chase} = \frac{\text{concentration of adenine-}^{14}\text{C at end of pulse period} \times \text{initial specific activity of adenine-}^{14}\text{C pulse}}{\text{concentration of non-radioactive adenine added as chase}}$$

with the concentration of adenine-^{14}C remaining at the end of the pulse period being determined from Fig. 9. The amount of radioactivity from adenine-^{14}C appearing as nucleotides during the chase period is derived from:

$$\text{nucleotide cpm formed during chase} = \text{nucleotide cpm formed in control experiment from adenine-}^{14}\text{C present at chase concentration in time equal to duration of chase} \times \frac{\text{specific activity of chase}}{\text{specific activity of control}}$$

Fig. 13 shows a representative set of data which has been calculated assuming a pulse of 20 minutes duration with varying concentrations of adenine-^{14}C followed by a chase with 10^{-2} M non-radioactive adenine of 60 minutes in duration.

As an example of the use of these data, it is apparent that under these conditions, when 50 μM adenine-^{14}C is used as pulse, about 12,000 cpm will appear in the total nucleotide fraction at the end of the pulse period and only 75 cpm will appear during the chase period (about 0.6% of that appearing during the pulse). Therefore, under these conditions, reactions which take place during the chase period may be examined without interference from metabolism of the radioactive precursor itself. The possibility of using other concentrations of base for pulse and chase may be examined in the same way.

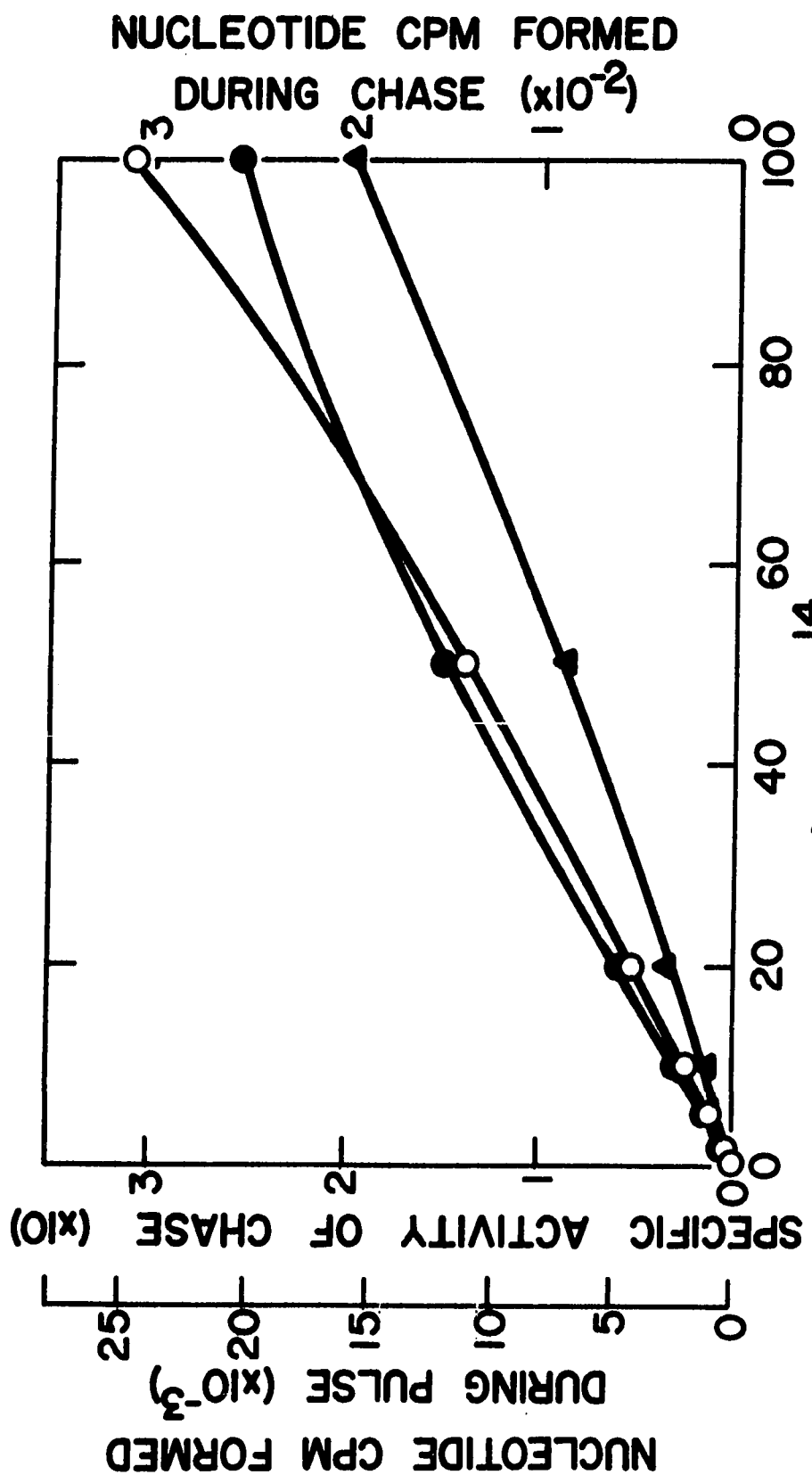


Figure 13. Hypothetical pulse-chase experiments with adenine-^{14}C. Pulse is 20 min. with various concentrations of adenine-14C (52.6μC/μmole). Chase is 60 min. with non-radioactive adenine (100μM). Total nucleotide synthesis during pulse period, ● ; specific activity of chase, ○ ; total nucleotide synthesis during chase period, ▲ .

3. Nucleotide Synthesis with Hypoxanthine-^{14}C as Precursor

a. Time Course Studies

The amount of nucleotide synthesized from hypoxanthine-^{14}C after 90 minutes of incubation is directly related to the initial base concentration (Fig. 14), except that the synthesis of nucleotides from 100 μM base is not increased over that from 50 μM base. Hypoxanthine-guanine phosphoribosyltransferase may become saturated with hypoxanthine when this base is present at concentrations exceeding 50 μM.

When the data are plotted to show the percent incorporation of the precursor into total acid-soluble nucleotides (Fig. 15), it is apparent that when the initial concentration of hypoxanthine is 20 μM or less, the maximum percent incorporation (measured after 90 minutes of incubation) is between 57 and 62%. However, when the initial base concentrations are 50 and 100 μM, the maximum percent incorporations are 47 and 20%, respectively.

The rates of nucleotide synthesis from hypoxanthine-^{14}C decrease with time (Fig. 14), for probably the same reasons as do those when adenine-^{14}C is used as precursor. With low initial hypoxanthine-^{14}C concentrations, probably the main factor contributing to the decreased rates is diminished levels of substrate. These effects would be most noticeable at longer incubation times. At higher precusor concentrations, however, inhibition of the phosphoribosyltransferase by nucleotides may play a role. Fig. 16 shows that significant amounts of hypoxanthine-^{14}C are not converted to other compounds even after 90 min at the three highest levels of precursor used. For example, at an initial concentration of precursor of 20 μM, more than 14% of the hypoxanthine (2.8 μM) remains unincorporated at 90 minutes.

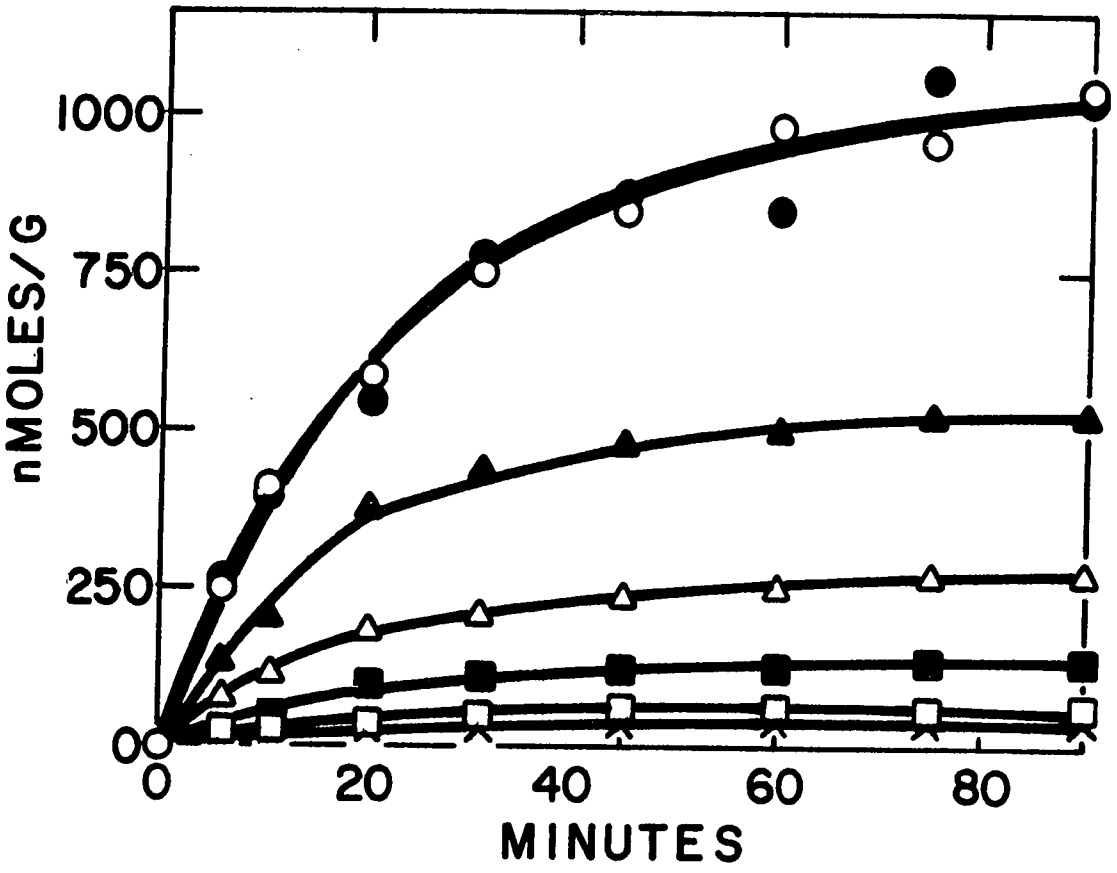


Figure 14. Incorporation of hypoxanthine-^{14}C into acid-soluble nucleotides.

Hypoxanthine-^{14}C concentration: 1μM. ✕ ; 2μM, □ ; 5μM, ■ ; 10μM, △ ; 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

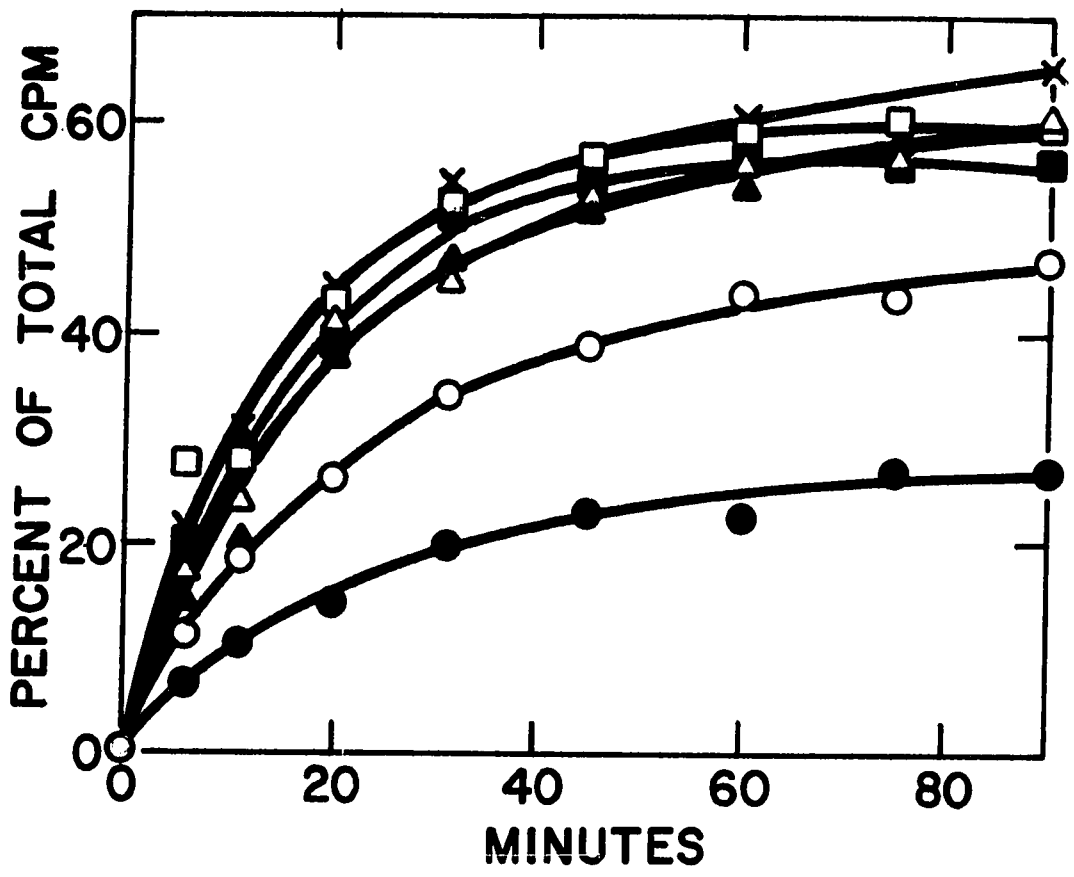


Figure 15. Percent incorporation of hypoxanthine-^{14}C into acid soluble nucleotides.

Hypoxanthine-^{14}C concentration: 1μM, ✕ ; 2μM, □ ; 5μM, ■ ; 10μM, △ ; 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

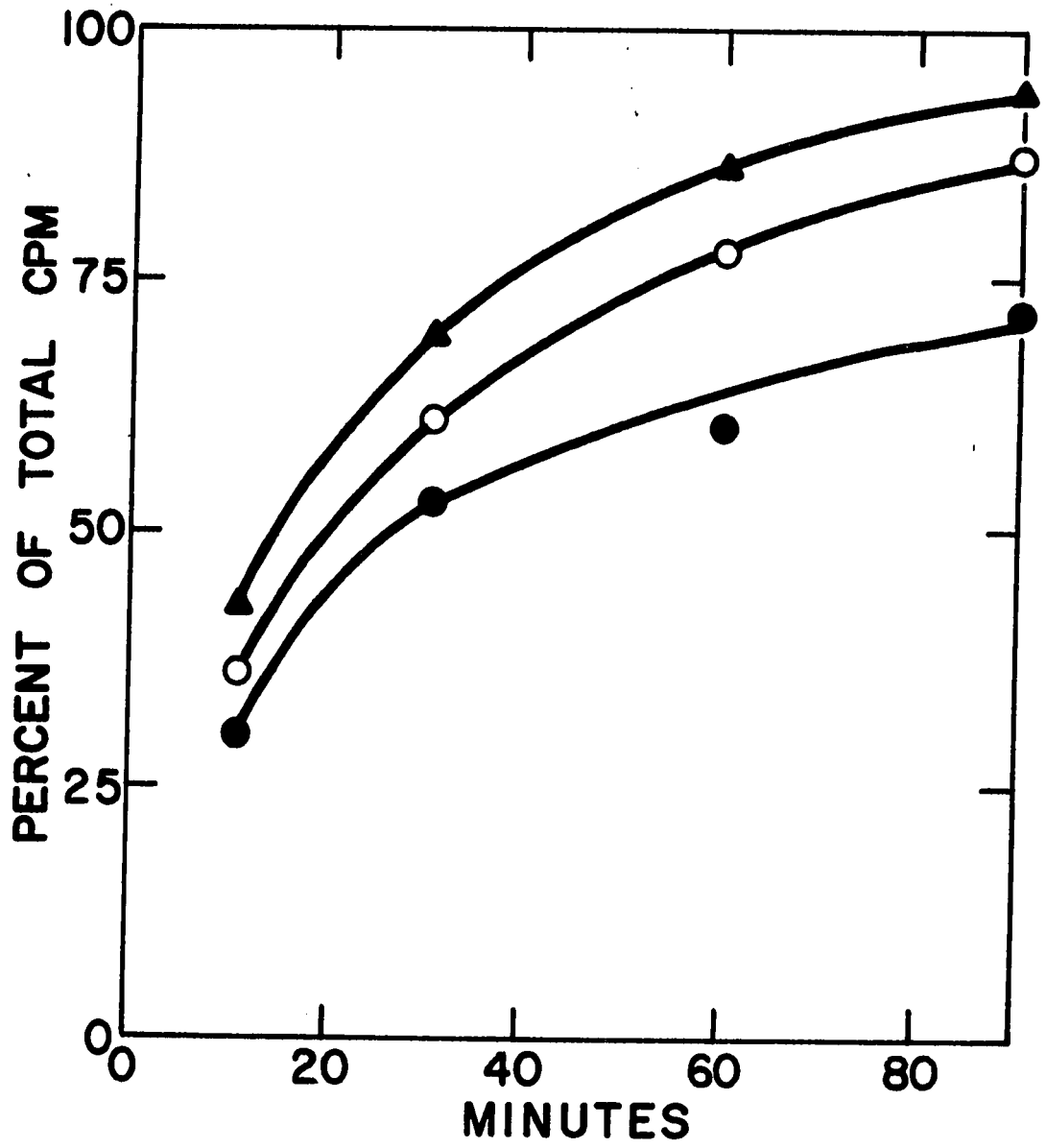


Figure 16. Percent total metabolism of hypoxanthine-^{14}C.
Results expressed as percent of total radioactivity present in purine nucleotides plus nucleosides plus bases (other than hypoxanthine). Hypoxanthine-^{14}C concentration: 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

At the same time in the incubation period, the apparent rate of nucleotide synthesis is almost zero (Fig. 14).

b. Initial Rate Studies

When the apparent initial rates of nucleotide formation from hypoxanthine-^{14}C are plotted against precursor concentration (Fig. 17), the resulting curve approximates a rectangular hyperbola which exhibits a maximum apparent rate of nucleotide synthesis at a base concentration of 50 μM. The curve may reach a plateau because hypoxanthine-guanine phosphoribosyltransferase may be saturated with substrate.

Also included in Fig. 17 are the 5 minute rates of nucleotide synthesis after various incubation times (10, 20, 30, 40, 50, 60, 70 and 80 minutes) when the initial precursor concentrations are 50 and 100 μM. The precursor concentrations present at the start of the 5 minute periods are deduced by referring to Fig. 16. The divergance of the curves obtained in this manner from the apparent initial rate curve again indicates the complexity of the metabolic control exerted over the synthetic pathways by which hypoxanthine is incorporated into nucleotides in Ehrlich ascites tumor cells. When the rates of nucleotide synthesis for 5 minute periods starting at 1, 2, 3, 4, 5, 6, 7, 8 and 9 minutes after addition of isotope are also plotted, the resultant curves appear to become sigmoid in shape.

3. Nucleotide Synthesis with Guanine-^{14}C as Precursor

a. Time Course Studies

The amount of nucleotides synthesized from various concentrations of guanine-^{14}C are illustrated in Fig. 18. By analogy with the results obtained with hypoxanthine-^{14}C as precursor, it appears

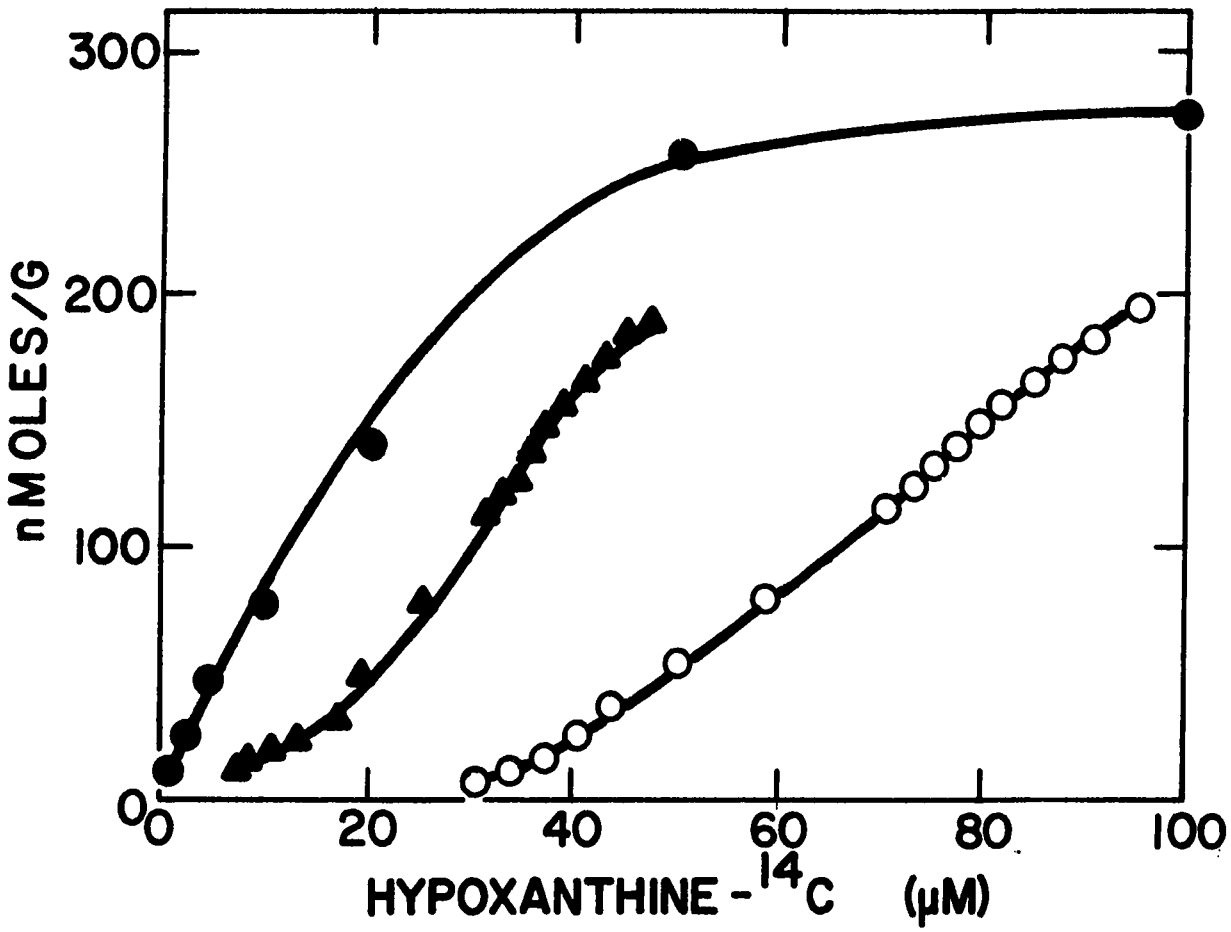


Figure 17. Relationship between hypoxanthine-^{14}C concentration and nucleotide synthesis.

Total nucleotide synthesis measured 5 min. after the addition of hypoxanthine-^{14}C was taken as the apparent initial rate of nucleotide synthesis. Initial rates of nucleotide synthesis, ● ; 5 min. rates of nucleotide synthesis with initial hypoxanthine-^{14}C concentration of 50μM, ▲ ; 5 min. rates of nucleotide synthesis with initial hypoxanthine-^{14}C concentration of 100μM, ○ .

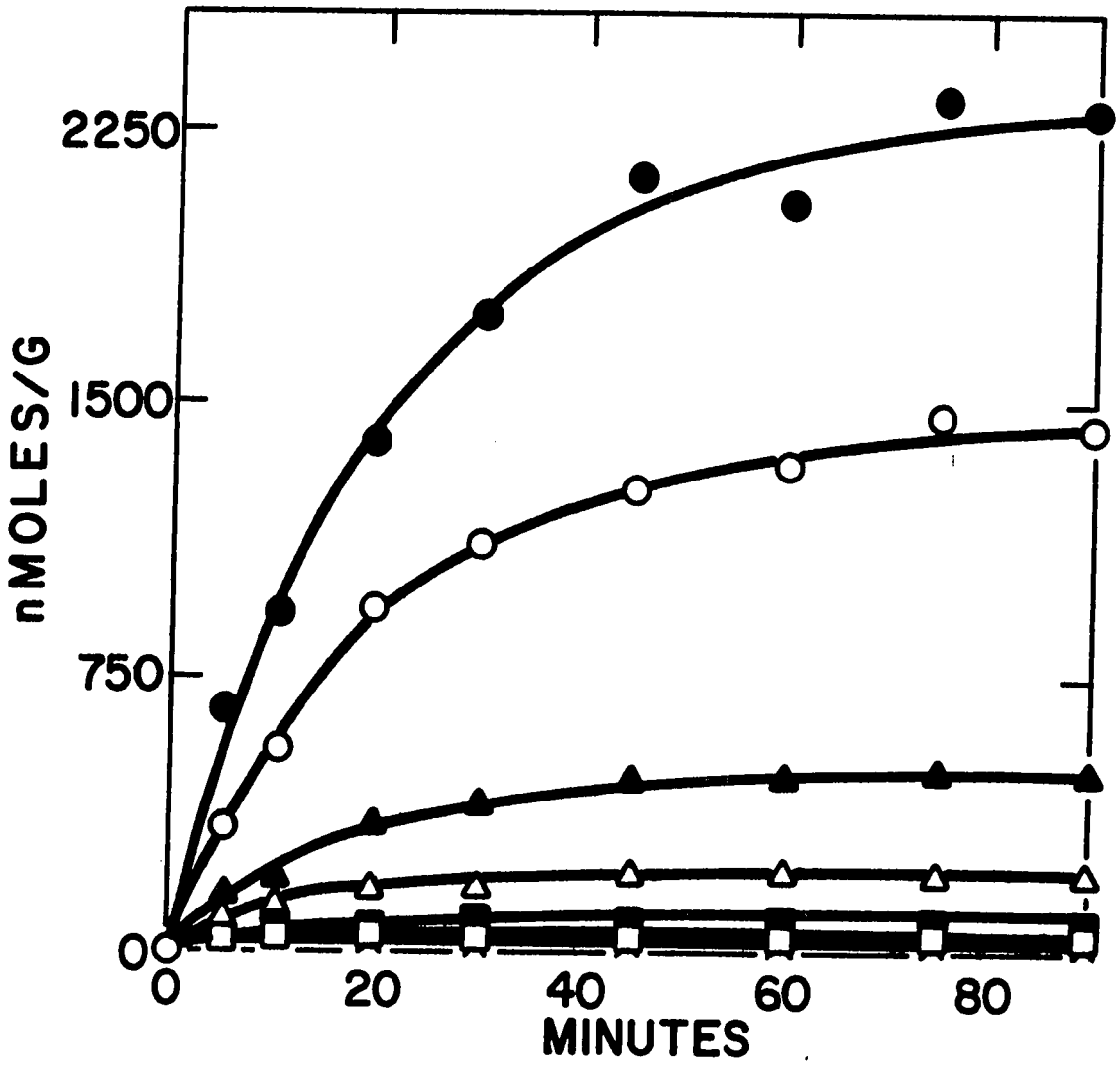


Figure 18. Incorporation of guanine-^{14}C into acid-soluble nucleotides.

Guanine-^{14}C concentration: 1μM, X ; 2μM, □ ; 5μM, ■ ; 10μM, △ ; 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

that the phosphoribosyltransferase may be saturated with substrate when the initial guanine-^{14}C concentration is 100 μM since the increase in amount of nucleotide synthesis from 50 to 100 μM guanine-^{14}C is only about 1.6-fold after 90 minutes of incubation.

The percent of the guanine-^{14}C incorporated into total acid-soluble purine ribonucleotides is given by Fig. 19. In general, this figure shows that the maximum conversion of this base is strictly related to the concentration of base used, with one notable exception. It is apparent that the lowest maximum percent incorporation (about 35%) is given when the precursor concentration is 1 or 2 μM, whereas the highest maximum (62%) is exhibited by the 50 μM curve. When guanine-^{14}C is present at 100 μM however, the maximum percent conversion is only about 50%. Again, this observation indicates that hypoxanthine-guanine phosphoribosyltransferase may be saturated with substrate at a guanine-^{14}C concentration of 100 μM.

When the total conversion of guanine-^{14}C to metabolites is examined (Fig. 20), efficient metabolism of this base is evident at precursor concentrations of 50 μM or less. After 60 minutes of incubation, less than 10% of the precursor is not metabolized when guanine-^{14}C concentrations are 20 and 50 μM. In fact, after 90 minutes, almost all (98%) of the base is converted. Therefore, the dominant factor responsible for markedly decreased rates of incorporation observed at longer incubation times is probably the very low substrate concentrations remaining in the reaction mixtures when guanine concentrations are 50 μM or less. In the case of the 100 μM precursor concentration, Fig. 20 shows that almost 20% of the base remains unincorporated even after 90 minutes of incubation. This large amount of precursor

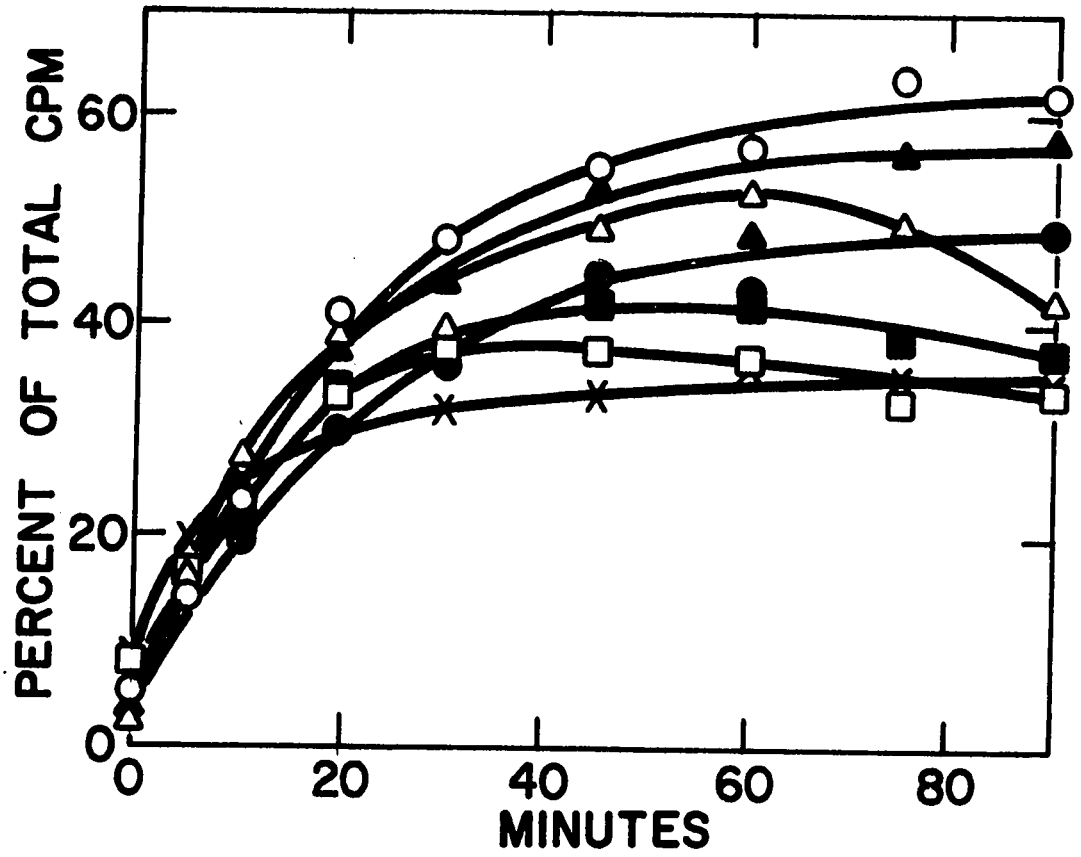


Figure 19. Percent incorporation of guanine-^{14}C into acid-soluble nucleotides.

Guanine-^{14}C concentration: 1μM, ✕ ; 2μM, □ ; 5μM, ■ ; 10μM, △ ; 20μM, ▲ ; 50μM, ○ ; 100μM, ● .

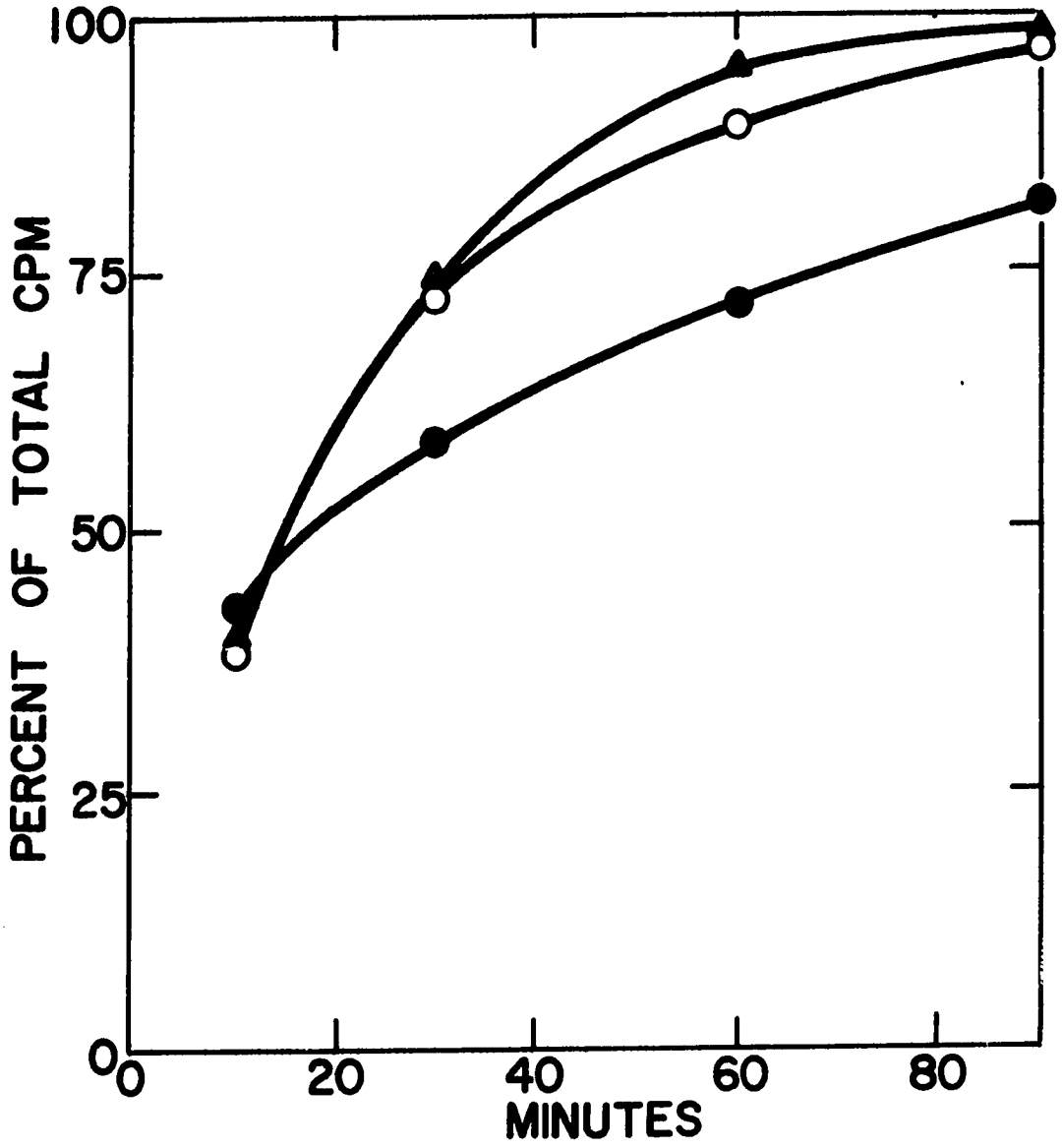


Figure 20. Percent total metabolism of guanine-^{14}C. Results expressed as percent of total radioactivity present in purine nucleotides plus nucleosides plus bases (other than guanine). Guanine-^{14}C concentration: 20μM, ▲ ; 50μM, ○; 100μM, ● .

remaining may reflect inhibition of hypoxanthine-guanine phosphoribosyltransferase by nucleotide metabolites.

b. Initial Rate Studies

The apparent initial rate plots for guanine-^{14}C (Fig. 21) show a linear relationship with base concentration. That controls of nucleotide synthesis from guanine do exist however, is apparent from the results obtained when succeeding 5 minute periods are examined at the 50 and 100 μM concentrations of base since these curves do not follow the initial rate curve exactly.

C. Summary

The maximum percent incorporation of adenine into acid-soluble purine ribonucleotides exceeds that of either hypoxanthine or guanine. However, the total percent metabolism of the latter two bases is not significantly different from that of adenine both because they are substrates for other pathways, and because nucleotides formed from them are catabolized faster than those formed from adenine. Adenine phosphoribosyltransferase is not saturated by the highest substrate level used, whereas hypoxanthine-guanine phosphoribosyltransferase appears to be saturated at substrate levels between 50 and 100 μM.

Regardless of the base used, the rate of nucleotide synthesis decreases with increasing time of incubation. This may be due to declining substrate concentration, inhibition by nucleotides, or limited availability of PP-ribose-P. At the present time, it is not possible to evaluate the contribution made by each of these processes.

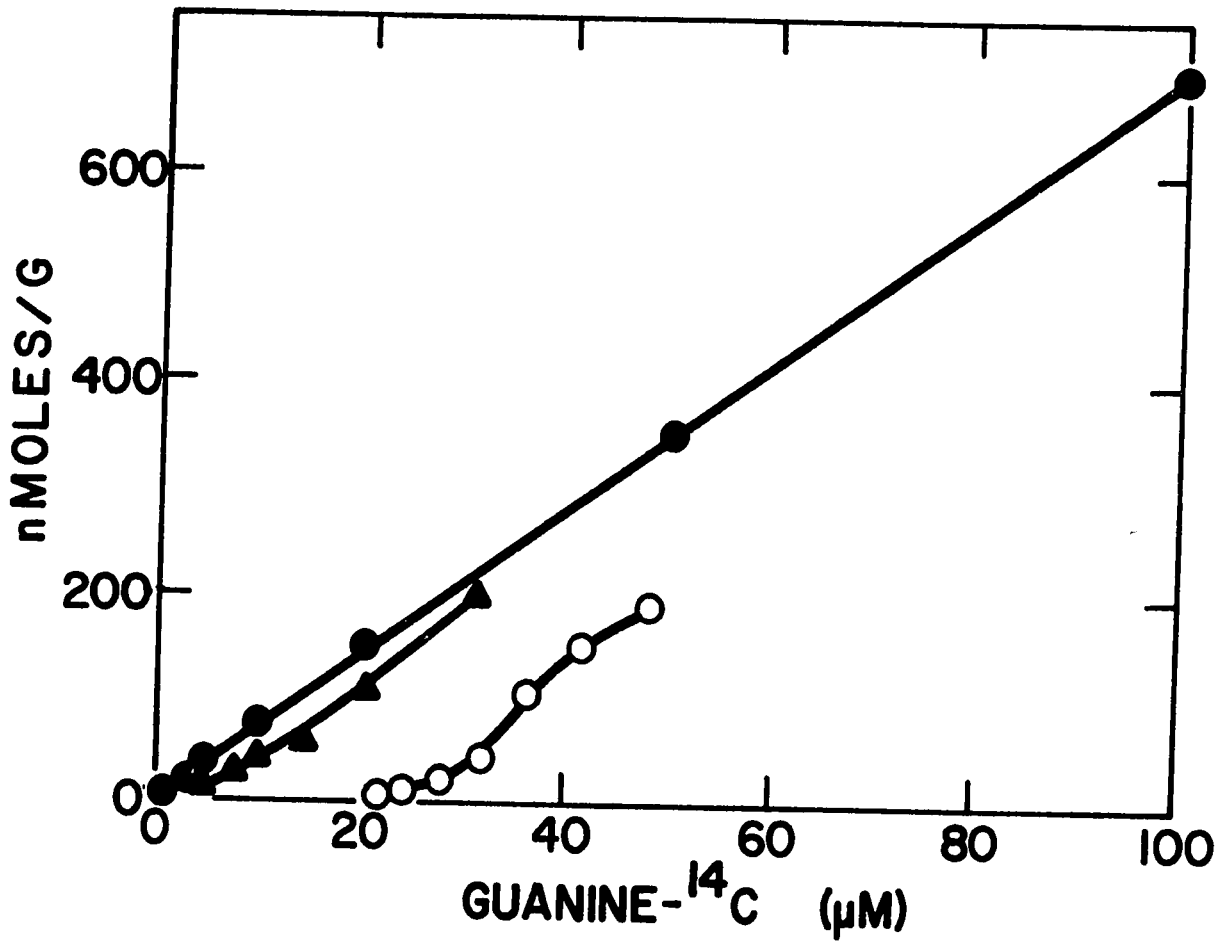


Figure 21. Relationship between guanine-^{14}C concentration and nucleotide synthesis.

Total nucleotide synthesis measured 5 min. after addition of guanine-^{14}C was taken as the apparent initial rate of nucleotide synthesis. Initial rates of nucleotide synthesis, ● ; 5 min. rates of nucleotide synthesis with initial guanine-^{14}C concentration of 50μM, ▲ ; 5 min. rates of nucleotide synthesis with initial guanine-^{14}C concentration of 100μM, ○ .

IV CONTROL OF PURINE RIBONUCLEOTIDE TRANSPHOSPHORYLATIONS

A. Introduction

Before purine nucleotides can be utilized for nucleic acid synthesis, for energy metabolism and for many other diverse reactions in which they participate, they must be converted to the triphosphate level. The conversion of nucleoside monophosphates to the corresponding triphosphate derivatives is a two-step process. In the first step, catalyzed by nucleoside monophosphate kinases, monophosphates are converted to diphosphates, and in the second step, catalyzed by nucleoside diphosphate kinases, diphosphates are converted to triphosphates. Although these kinases have been isolated from many sources, few details are available concerning the regulation of their activities even in isolated systems. Some of this information is outlined below.

1. Nucleoside Monophosphate Kinases

The general reaction catalyzed by these enzymes is:

$$XTP + YMP \rightleftharpoons XDP + YDP$$

These kinases can be divided into three subclasses according to whether (a) ATP is the phosphate donor, (b) AMP is the phosphate acceptor or (c) the same base moiety is present in both donor and acceptor molecules. It is generally believed the nucleoside monophosphate kinases require an adenine nucleotide as one of the substrates since no monophosphate kinases have been found in which both substrates may be replaced by nucleotides which do not contain adenine as the base moiety.

Undoubtedly, the most widely studied enzymes of this class of kinases are those which catalyze ATP-AMP transphosphorylation

(adenylate kinases). Adenylate kinase is the only enzyme so far found which fits all three subclasses (above). This enzyme has been highly purified from many tissues. In most cases, divalent cations are required for activity and accordingly the true substrates are believed to be AMP and Mg-ATP (Noda, 1958; Callaghan and Weber, 1959). In support of this hypothesis it has been shown that the maximum enzymic activity occurs when the ratio Mg^{2+}/ATP approaches unity and that ADP inhibits competitively with ATP whereas Mg-ADP competes with Mg-ATP (Noda, 1958).

Guanylate kinase catalyzes the phosphorylation of GMP (or dGMP) by ATP to form GDP (or dGDP). As for adenylate kinase, Mg-ATP is thought to be the true substrate for guanylate kinase from rat liver (Buccino and Roth, 1969). Whereas guanylate kinase from E. coli is inhibited by AMP, IMP, dXMP and guanosine (Oeschger and Bessman, 1966), the preparation from hog brain is not (Meich and Parks, 1965). Both the hog brain and Sarcoma 180 preparations (Meich and Parks, 1969) are strongly inhibited by the antitumor agent 6-thio-GMP, however.

Non-specific ATP-NMP phosphotransferase activities have been detected in calf liver (Strominger et al., 1959) and in pea seeds (Kirkland and Turner, 1959), but Strominger et al. (1959) believe that these activities are due to many separate enzymes with narrow specificities rather than a few enzymes with wide specificities. In addition, nucleoside monophosphate kinases which are specific for AMP as the phosphate acceptor but non-specific for the phosphate donor (NTP-AMP phosphotransferases) have been detected in many animal tissues. No information is available concerning the regulation of either ATP-NMP or NTP-AMP phosphotransferases, however.

In summary, enzymes which are capable of converting nucleoside monophosphates into their corresponding diphosphates are present in many different types of cells. Such enzymes are quite numerous and may possess rigid specificities for both donor and acceptor molecules. As far as this reviewer is aware, mammalian cells do not possess an enzyme which is capable of forming IDP from IMP. Little, if any, work has been done on the regulation of the activity of nucleoside monophosphate kinases in intact cells.

2. Nucleoside Diphosphate Kinases

Many plant, animal and bacterial cells possess enzymes which catalyze the reaction

$$XTP + YDP \rightleftharpoons XDP + YTP.$$

These nucleoside diphosphate kinases have wide specificities with respect to both the phosphate donor and phosphate acceptor since they may be either purines or pyrimidines and either ribo- or deoxyribonucleotides. This apparent lack of specificity has led most workers to postulate that, unlike the nucleoside monophosphate kinases, these activities are properties of one or two enzymes with wide substrate specificities rather than many enzymes with rigid substrate specificities. In general, alternative substrates prove to be competitive inhibitors indicating that they compete for the same sites on the enzymes (cf. Weaver, 1962).

Mg^{2+}-nucleotide complexes are thought to be utilized as substrates for these enzymes (Goffeau et al., 1967). In the case of the beef heart preparation, MgATP but not free ATP is the phosphate donor, and both free ADP and MgADP can be utilized as phosphate acceptors although free ADP is the preferred substrate (Colomb et al., 1969). In

fact, MgADP appears to be inhibitory since with the bovine liver enzyme, the saturation curve for MgATP is sigmoid in the presence of high concentrations for MgADP (Goffeau et al., 1967) and MgADP may form abortive complexes with the enzyme. In addition to the MgADP inhibition, many other Mg-complexed nucleoside diphosphates will inhibit these enzymes, but the extent of the inhibition appears to depend on the identity of the Mg-complexed nucleoside triphosphate partner. Goffeau et al. (1967) believe that the nucleoside diphosphate kinase from beef liver has the properties of a regulatory enzyme and has two or more binding sites for substrates which may show site-site interactions. The bovine liver preparation is, incidentally, the only nucleoside diphosphate kinase so far examined which appears to possess the characteristics of a regulatory enzyme.

3. Possible Control Points

Although no work has been reported which attempts to designate the rate-limiting reactions in the conversion of ribonucleoside monophosphates into ribonucleoside triphosphates, the reactions catalyzed by the nucleoside monophosphate kinases have been postulated to be a more likely point of control than the reactions catalyzed by the nucleoside diphosphate kinases for the following reasons (Mourad and Parks, 1966). First, many workers have shown that nucleoside diphosphate kinase activities are much higher than those of the nucleoside monophosphate kinases in many of the tissues in which these enzymes have been detected. Second, whereas nucleoside monophosphate kinases have rigid substrate specificities, the diphosphate kinases appear to catalyze transphosphorylations between a wide variety of substrates. It is interesting to note, however, that the only enzyme implicated to have a regulatory

role up to the present time is the nucleoside diphosphate kinase from bovine liver (Goffeau et al., 1967).

Since mammalian cells contain enzymes which are capable of phosphorylating nucleoside monophosphates to the di- and triphosphate levels when adenine or guanine, but not hypoxanthine, is the base moiety, and since studies with hypoxanthine as precursor would be complicated by the reactions of purine nucleotide conversion if such was not the case, only experiments in which adenine or guanine was the preformed base are reported in this chapter. In addition, the interpretation of the data obtained in these experiments is facilitated by the observation that the rates of interconversion of adenine and guanine nucleotides are quite low in Ehrlich ascites tumor cells (see Chapter V).

Accordingly, with adenine-^{14}C and guanine-^{14}C as precursors, we have examined the time-courses of the incorporation of these bases into their respective ribonucleoside mono-, di-, and triphosphates in order to determine the rate-limiting step in these reaction sequences. Although precursor concentrations ranged between 1 and 100 μM, no data are reported when the base concentration is lower than 10 μM since the low amounts of radioactivity incorporated into individual metabolites at low precursor concentrations makes accurate determinations and comparisons quite difficult.

B. Results and Discussion

1. Adenine-^{14}C as Precursor

AMP synthesized from adenine-^{14}C by adenine phosphoribosyltransferase is readily converted into ADP and ATP by reactions catalyzed by adenylate kinase and nucleoside diphosphate kinase. Although adenylate kinase can catalyze the formation of ATP as well as ADP since:

$$2ADP \rightleftharpoons ATP + AMP,$$

nucleoside diphosphate kinase is also thought to be involved in the formation of ATP in order to account for the high levels of ATP, relative to ADP and AMP, found in these cells. The participation of both enzymes in the formation of ATP from AMP creates difficulties when attempting to establish the rate-controlling step in these phosphorylation reactions.

Fig. 22 illustrates the time courses of AMP, ADP and ATP formation from adenine-^{14}C at three concentrations (10, 50 and 100 μM) of precursor. The rates of ATP synthesis, regardless of precursor concentration, are far greater than those of AMP and ADP formation. In addition, the rates of ATP synthesis do not appear to decrease as incubation time increases to the same extent as do those of AMP and ADP formation. It is interesting to note that at the two highest precursor concentrations, 50 and 100 μM, the synthesis of AMP appears to plateau at a value of about 92 nmoles/g. This value may represent the steady state level of AMP in the cells which is maintained by the adenylate kinase reaction. The large excess of ATP (compared to AMP) which accumulates might then reflect the contributions made to ATP synthesis by the nucleoside diphosphate kinase reaction.

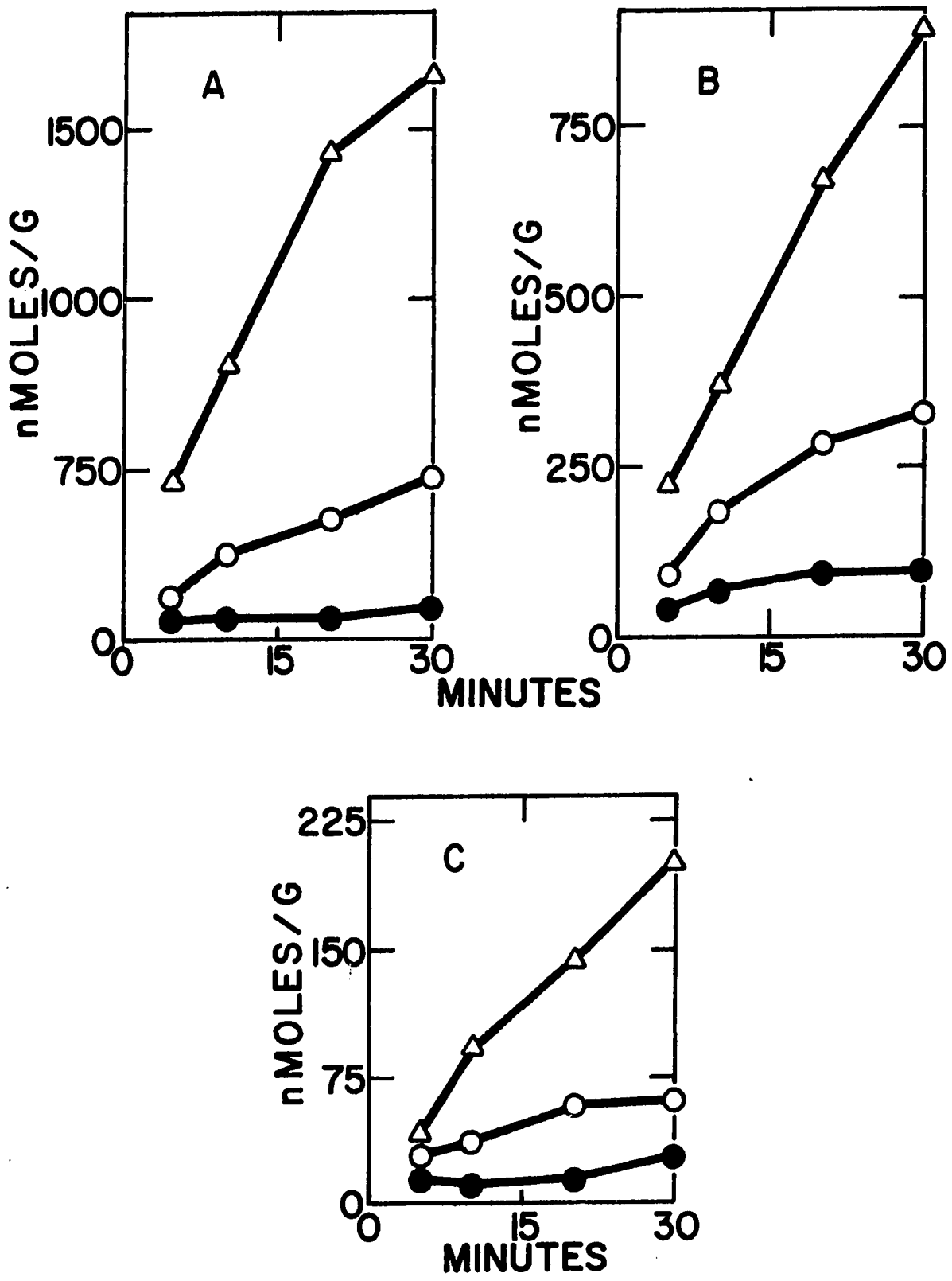


Figure 22. Incorporation of adenine-^{14}C into AMP, ADP, and ATP. Adenine-^{14}C concentration: Frame A, 100μM; Frame B, 50μM; Frame C, 10μM. AMP, ● ; ADP, ○ ; ATP, △ .

The formations of AMP, ADP and ATP are given in Fig. 23 as percentages of the total acid-soluble purine ribonucleotide fraction. When the two highest levels of adenine-^{14}C are utilized, the contributions made by each of the three adenine nucleotides to the total nucleotide fraction are fairly constant at all incubation times examined. For example, when the precursor concentration is 100 μM, the values for ATP range between 68 and 71%, those for ADP between 22 and 20% and those for AMP between 7 and 4%. With the lowest concentration of precursor utilized, the values show somewhat greater variation since after 5 minutes of incubation, the values for ATP, ADP and AMP are 44, 30 and 18%, but after 30 minutes, these values become 66, 20 and 8%, respectively. From 30 minutes until the termination of the incubation period, the values for ATP, ADP and AMP become more stabilized and fall in the ranges 66-71%, 20-24% and 4-8%, respectively.

The fact that both AMP and ADP contribute significantly to the total acid-soluble nucleotide fraction at all times and the fact that the contributions of these nucleotides to this fraction are quite constant throughout the incubation period may indicate the important role played by the adenylate kinase reaction in maintaining a particular relationship between the concentrations of ATP, ADP and AMP in Ehrlich ascites tumor cells <u>in vitro</u>.

It is extremely difficult to define the rate-controlling step for the synthesis of ATP from AMP since (a) two different enzymes are responsible for the synthesis of ATP from ADP and (b) the reaction catalyzed by adenylate kinase may be responsible for maintaining ADP and AMP at certain levels in the cell. Furthermore, at the time that the radioactive precursor is added to the cells, the levels of ATP may

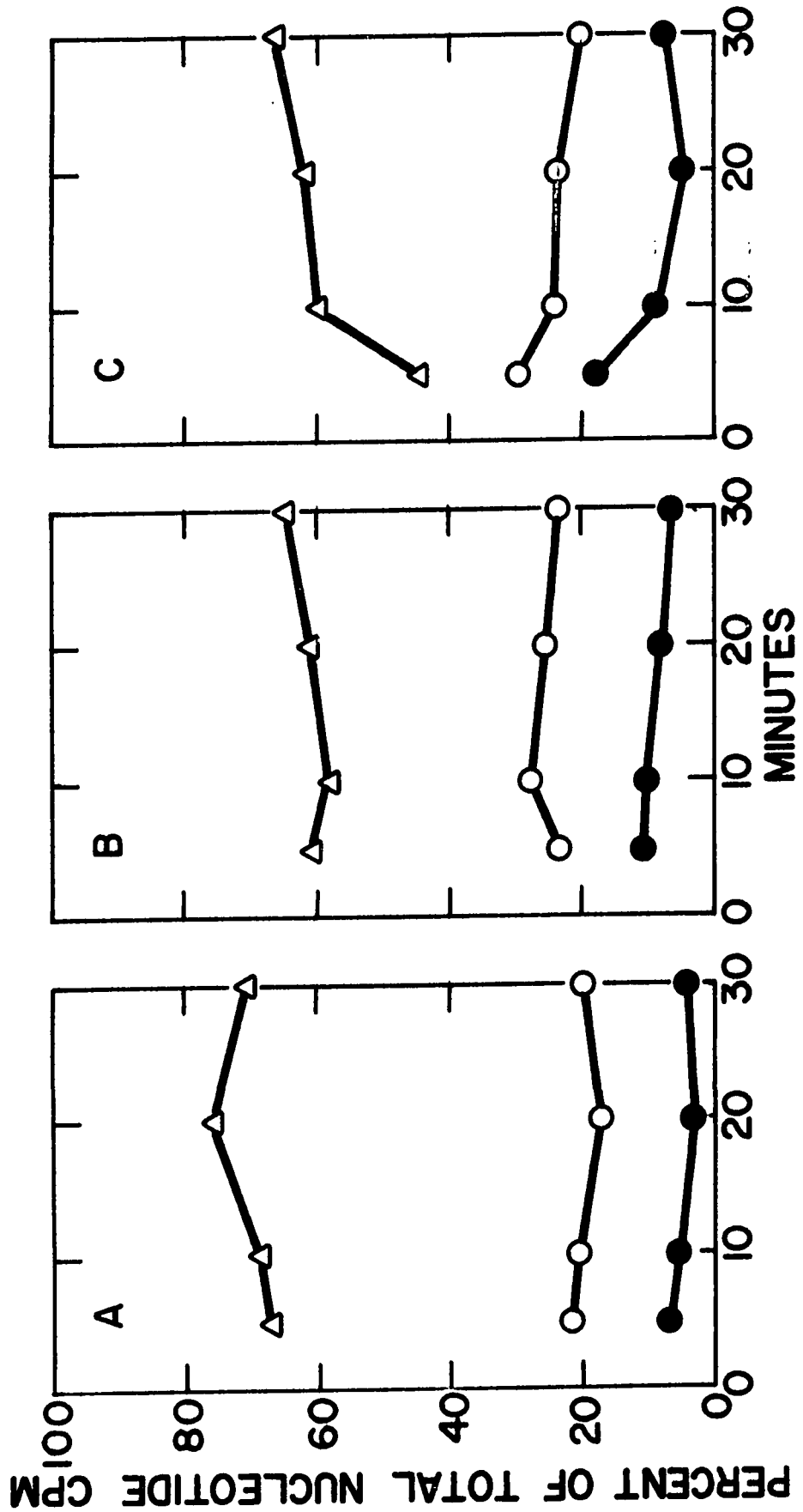


Figure 23. Synthesis of AMP, ADP and ATP from adenine-^{14}C as percent of total nucleotide synthesis.
Adenine-^{14}C concentration: Frame A, 100μM; Frame B, 50μM; Frame C, 10μM. AMP, ●; ADP, ○; ATP, △.

not be stabilized. Overgaard-Hansen (1965) has observed a transient depletion of the adenine nucleotide pool following the addition of glucose to Ehrlich ascites tumor cells incubated under aerobic conditions. When dilute (less than 10% by volume) cell suspensions were utilized, after 20 minutes of the incubation period had elapsed, ATP levels were restored to only about 60% of the levels which were present before addition of glucose and at this time in the incubation period, the rate of ATP resynthesis was maximal. For these reasons, one cannot designate which of the two reactions for the conversion of AMP to ATP limits the overall rate of this process.

2. Guanine-^{14}C as Precursor

In comparison to the complex situation which exists for the formation of ATP from AMP, the conversion of GMP to GTP is a relatively simple process. This latter conversion is catalyzed, in a two step sequence, by the enzymes guanylate kinase and nucleoside diphosphate kinase. The guanylate kinase reaction does not utilize GTP as the phosphate donor thereby eliminating a possible equilibrium reaction which could serve to complicate an analysis of experimental data such as that which occurs in the formation of ATP.

The formation of GMP, GDP and GTP from guanine-^{14}C are given in Fig. 24 for three (20, 50 and 100 μM) concentrations of precursor. Although the rate of GTP synthesis appears to be high throughout the incubation period, those of GDP and GMP synthesis remain comparatively low. In addition, whereas GMP does not accumulate significantly during the incubation period, GDP does appear to accumulate to a small extent. Apparently then, the rate-limiting step for the formation of GTP from GMP is that catalyzed by nucleoside diphosphate kinase.

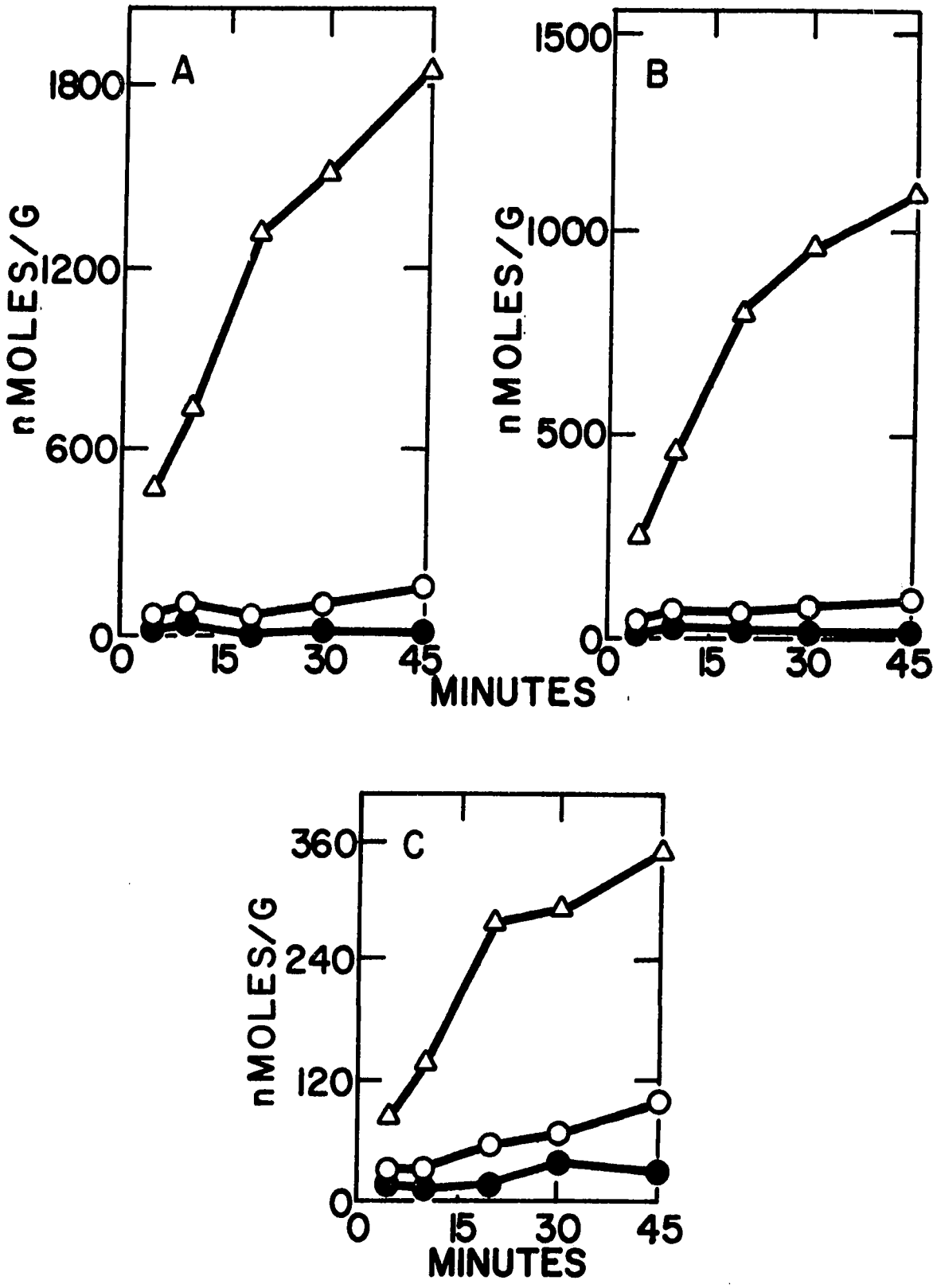


Figure 24. Incorporation of guanine-^{14}C into GMP, GDP, and GTP. Guanine-^{14}C concentration: Frame A, 100μM; Frame B, 50μM; Frame C, 20μM. GMP, ●; GDP, ○; GTP, △.

The above observations are also reflected when the formations of GTP, GDP and GMP are represented as percentages of the total acid-soluble nucleotide fraction (Fig. 25). When the 50 and 100 μM concentrations of guanine-^{14}C are used, the curves for both GDP and GTP reach plateau levels at about 7 and 81% of the total nucleotide fraction, respectively, after 20 minutes of incubation. GMP, at all times examined, appears to be present at very low levels (about 1% of total).

The decreased rates of GTP synthesis with time which are evident in Fig. 24, may reflect a modulation of the activity of nucleoside diphosphate kinase. Since guanylate kinase apparently is not limiting for GTP synthesis, this modulation of nucleoside diphosphate kinase activity probably is due to inhibition by reaction products rather than due to a paucity of substrate. It should be emphasized however, that a decrease in the rate of GDP synthesis might be very difficult to detect because of the slow rate at which GDP is formed.

The observation (see above) that the rates of ATP synthesis are very high even at early times in the incubation period and remain high throughout this period appears to indicate that the levels of ATP in these cells are high when the precursor is added. This occurrence was not unexpected however, since the cells are incubated with glucose for 20 minutes before addition of the precursor. High levels of ATP present when precursor is added may have some effect on the rates of conversion of guanine nucleotides to adenine nucleotides which appear to be quite low (Chapter V) in Ehrlich cells incubated under conditions identical to those used here. The rate-limiting step in the formation of adenine nucleotides from guanine nucleotides appears to be that catalyzed by GMP reductase, an enzyme which is believed to be inhibited

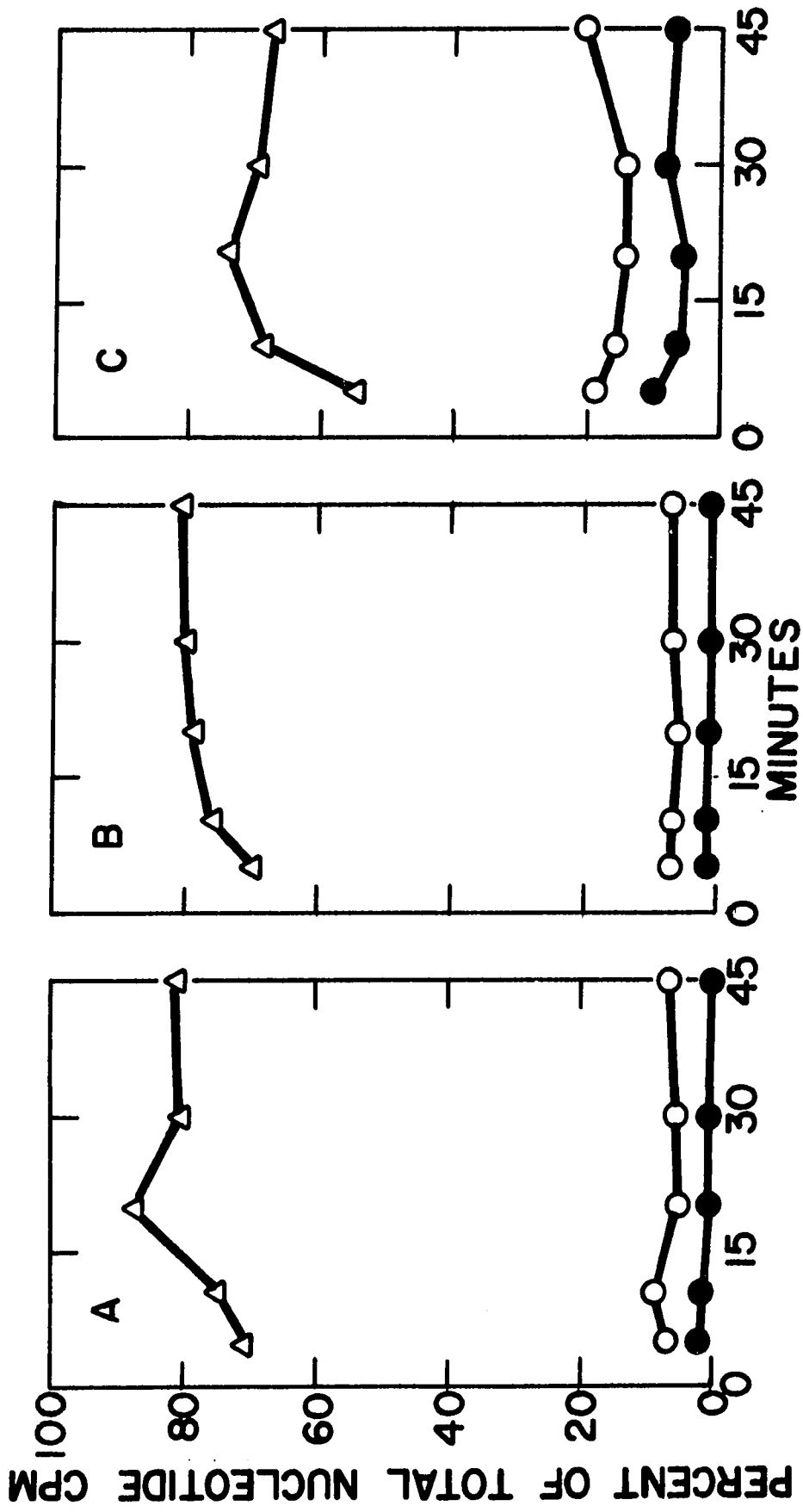


Figure 25. Synthesis of GMP, GDP, and GTP from guanine-^{14}C as percent of total nucleotide synthesis. Guanine-^{14}C concentration: Frame A, 100 μM; Frame B, 50 μM; Frame C, 20 μM. GMP, ●; GDP, ○; GTP, △.

by ATP. Although inhibition by ATP may be overcome, at least in isolated systems, by increasing the concentration of GMP, it is evident from the results presented above that when Ehrlich ascites tumor cells are incubated with guanine-^{14}C, the accumulation of GMP is negligible.

C. Summary

From the data outlined in the preceding sections, no firm conclusions can be drawn at the present time regarding the rate-limiting step for the formation of ATP from AMP. The rate of the conversion of GMP to GTP appears to be governed by the rate of the reaction catalyzed by nucleoside diphosphate kinase. The factors which regulate the rate of the nucleoside diphosphate kinase reaction are not presently evident.

V CONTROL OF PURINE RIBONUCLEOTIDE INTERCONVERSIONS

A. Introduction

Although the individual reactions of purine nucleotide interconversion have been examined in some detail in cell extracts and with partially purified enzymes, relatively little work has been done to elucidate the controls of these reactions when they are operative as an integrated system in the whole cell. In this chapter, these reactions are examined in Ehrlich ascites tumor cells *in vitro* and some attempt is made to delineate the rate-controlling steps in this complex series of reactions.

Studies with enteric bacteria carried out by Magasanik and coworkers (cf. Magasanik, 1962) have elucidated many of the reactions of these pathways and many potential control mechanisms. Their studies have shown that ATP is both an activator and a substrate for the formation of guanine nucleotides from adenine nucleotides as well as an inhibitor of GMP deamination. GTP is both an inhibitor of IMP formation from AMP (Setlow *et al.*, 1966) and a substrate for the formation of adenine nucleotides from IMP. Therefore, the levels of adenine nucleotides appear to regulate the levels of guanine nucleotides, and vice versa.

In rabbit erythrocytes *in vitro* (Hershko *et al.*, 1967), the rates of interconversion of adenine and guanine nucleotides appear to be quite low, suggesting that the activities of AMP deaminase and GMP reductase are low in these cells. Hershko *et al.* also postulate that the availability of glutamine may limit the conversion of XMP to GMP.

The most important factors expected to contribute to the regulation of the reactions of purine nucleotide interconversion in Ehrlich ascites tumor cells in vitro are (a) the relative activities of the enzymes which catalyze these reactions, (b) the control of these activities by nucleotides and (c) the availability of substrates of these enzymes. These aspects are briefly discussed below.

1. Relative Enzyme Activities

The relative activities of the enzymes of purine nucleotide interconversion in several mammalian cell types were measured by McFall and Magasanik (1960). These workers found that in L-cell extracts, the specific activities of the enzymes of these pathways were: IMP dehydrogenase, 0.003; XMP aminase, 0.178; AMPS synthetase, 3.1; AMPS lyase, 0.40. In extracts of Ehrlich ascites cells, these activities were: IMP dehydrogenase, 0.01; XMP aminase, 0.225; AMPS lyase, 0.84. The activities of AMP deaminase and GMP reductase were not determined in the latter case.

If one assumes that other factors were not limiting in these cells in vitro, it would appear, on the basis of the above data, that the synthesis of adenine nucleotides from IMP should take place much more readily than the synthesis of guanine nucleotides. In addition, the rate-limiting step for the conversion of IMP to AMP in L-cells would be that catalyzed by AMPS lyase, whereas the slow step in the formation of GMP from IMP would be that catalyzed by IMP dehydrogenase.

Santos et al. (1968) showed that in rat brain extracts, the specific activity of AMP deaminase was about 27-fold that of IMP dehydrogenase. Accordingly, if the above assumption is valid, IMP

dehydrogenase may limit the conversion of adenine nucleotides to guanine nucleotides. XMP aminase activity was not measured in these extracts, however.

However, it is uncertain whether substrate and cofactor concentrations for these enzymes are saturating in Ehrlich ascites tumor cells *in vitro*. In addition, as will be seen below, many of these enzymes are subject to allosteric control by nucleotides. Therefore, measurements of enzyme activities such as those made by McFall and Magasanik and Santos *et al.* tend to give an unrealistic picture of the situation which actually exists in the intact cell.

2. Control of Enzyme Activities by Nucleotides

Although most of the studies on control of enzyme activity by nucleotides have been done with partially purified enzyme preparations, they are of importance to the present study in that they indicate potential controlling factors. Accordingly, some of these studies are reviewed below.

a. AMPS Synthetase

AMPS synthetase is inhibited by a number of naturally-occurring purine nucleotides and purine nucleotide analogs. Probably the most important of these is GDP, a product of the reaction, which is competitive with respect to GTP (Wyngaarden and Greenland, 1963). In fact, the enzyme has a greater affinity for GDP than for GTP, and it was suggested that the relative intracellular concentrations of these compounds may regulate its activity. AMP, GMP and dGMP are competitive inhibitors with respect to IMP. A number of IMP analogs (Hampton, 1962a; Nichol *et al.*, 1967) are also inhibitory but by far

the most potent of these is 6-mercaptopurine ribonucleoside 5'-monophosphate.

In addition to the inhibition by purine nucleotides, this enzyme is strongly inhibited by an analog of L-aspartate, hadacidin (N-formylhydroxyaminoacetic acid) (Shigeura and Gordon, 1962). This inhibition, in which hadacidin competes with L-aspartate for the active site of the enzyme, is readily reversed by excess aspartate.

b. AMPS Lyase

Although AMP is a product inhibitor of this reaction (Cohen and Bridger, 1964), no other naturally-occurring purine nucleotide is known to have any effect on the activity of AMPS lyase. However, this enzyme is inhibited by several purine analog nucleotides. Hampton et al. (1962) noted that the enzyme from yeast was strongly inhibited by 6-mercaptopurine nucleotide but was not affected by 6-chloropurine nucleotide or by purine nucleotide. In addition, Hampton (1962b) noted that the L-succino form of the 6-thio analog of AMPS inhibited the cleavage of AMPS to AMP and fumarate. This analog itself was slightly active as a substrate and was cleaved to 6-mercaptopurine nucleotide plus either fumaric or malic acid.

c. AMP Deaminase

AMP deaminase has been postulated to be an important regulatory enzyme, and the effect of nucleotides on this enzyme has been postulated to be of premier importance in regulating purine nucleotide interconversions (Setlow et al., 1966); the enzyme from calf brain has been extensively studied in this regard. As is the case with AMP deaminases from other sources, the calf brain enzyme is strongly inhibited by GTP as well as being activated by ATP. The inhibition by

GTP is reversed by increasing the concentration of ATP. Conversely, activation by ATP is reversed by increasing the concentration of GTP (Setlow and Lowenstein, 1968). Both ATP and GTP are very effective at protecting the enzyme against heat inactivation. The kinetic properties of this enzyme suggest that the activity of the brain enzyme *in vivo* is regulated by the ratio of the concentrations of ATP and GTP. Opinions to the contrary have been expressed, however (Blakley and Vitols, 1968).

d. IMP Dehydrogenase

Various purine nucleotides are known to inhibit IMP dehydrogenase, and this inhibition is believed to be involved in the control of purine nucleotide interconversion (Mager and Magasanik, 1960). In the case of the enzyme from *A. aerogenes*, GMP and GDP inhibit competitively with respect to IMP, whereas ATP and GTP have little inhibitory effect (Mager and Magasanik, 1960; Hampton and Nomura, 1967). The enzyme from Sarcoma 180 cells is inhibited by XMP, AMP and GMP (Anderson and Sartorelli, 1966). Whereas XMP inhibits the Sarcoma 180 enzyme in a manner which is competitive with IMP, NADH, the other product of the reaction, is not competitive with respect to either IMP or NAD (Anderson and Sartorelli, 1968). The Ehrlich cell enzyme is inhibited by GMP only if the ratio of GMP to IMP is 10 or greater (McFall and Magasanik, 1960).

In addition to inhibition by naturally-occurring purine nucleotides, various analog nucleotides are potent inhibitors of this enzyme. IMP dehydrogenases from bacterial (Hampton, 1963; Hampton and Nomura, 1967) and mammalian sources (Salser *et al.*, 1960; Atkinson *et al.*, 1963; Anderson and Sartorelli, 1968) are inhibited by the

5'-nucleotides of 6-chloro-, 6-mercapto-, and 2-amino-6-mercaptopurine. These inhibitions are progressive, which suggests that the inhibitors react covalently with the enzyme (Hampton and Nomura, 1967; Hampton, 1963). Since IMP protects the enzyme against inhibition by these analog nucleotides, it is postulated that the analogs bind at the active site of IMP dehydrogenase (Hampton and Nomura, 1967; Anderson and Sartorelli, 1967).

e. XMP Aminase

Although the purine analog psicofuranine (9-D-psciofuranosyl-6-aminopurine) is known to inhibit the enzyme from bacteria (Schlecta, 1960), the XMP aminase from animal sources is not markedly affected by this compound. No information appears to be available concerning the effects of naturally-occurring or analog purine nucleotides on the activity of XMP aminase from mammalian sources.

f. GMP Reductase

GMP reductase from bacteria is strongly inhibited by ATP and to a much lesser extent by AMP and IMP. The effect of these inhibitors can be overcome completely by increasing the concentration of GMP. Reduced NADP has no effect on the inhibition by ATP, however. Pyrimidine nucleotides have no effect on the enzyme (Mager and Magasanik, 1960; Brox and Hampton, 1968). Partially purified GMP reductase is progressively inactivated by 6-chloro-, 2-amino-6-mercapto- and 6-mercaptopurine ribonucleotides. The rate of inhibition by these compounds is reduced by GMP but not by reduced NADP.

3. Availability of Substrates

Two enzymes of the purine nucleotide interconversion cycles

require amino acid substrates. L-aspartate is required for the AMPS synthetase reaction whereas L-glutamine is required for the amination of XMP to form GMP.

a. L-Glutamine

The concentration of glutamine in Ehrlich ascites tumor cells incubated in vitro is limiting for purine biosynthesis de novo (Henderson, 1962) and for protein synthesis (Rabinovitz et al., 1956). Although evidence has been obtained which suggests that glutamine is synthesized in these cells (Fontenelle and Henderson, 1969), the intracellular concentration of this compound is very low, presumably because it is rapidly deamidated (Coles and Johnstone, 1962; Henderson, 1963). Accordingly, the availability of an adequate supply of glutamine for the XMP aminase reaction may limit the rate of this reaction, and may limit the overall rate of the conversion of IMP to GMP.

b. L-Aspartate

When Ehrlich ascites tumor cells are exposed to high extracellular concentrations of aspartate, the synthesis of adenine nucleotides from glycine-^{14}C is increased (Fontenelle and Henderson, 1969). In addition, aspartate causes an increase in the specific activity of adenine synthesized from hypoxanthine via IMP. Normal intracellular levels of this compound may therefore be limiting for both de novo purine synthesis and the synthesis of AMP from IMP.

The possibility that some of these factors actually regulate the reactions of the cycles of purine nucleotide interconversion in Ehrlich ascites tumor cells in vitro is explored in this chapter. The

general method used in these experiments was to incubate Ehrlich cells with various concentrations of radioactive purine bases for varying time periods, after which the distribution of radioactivity among purine nucleotides was examined. All three purine bases whose rates of conversion to nucleotides were studied in the previous chapters were utilized. However, considerable emphasis has been placed on the metabolism of hypoxanthine in these experiments since inosinate serves a dual role as the end-point of the de novo route of purine nucleotide biosynthesis as well as the branch point for the cycles of purine nucleotide interconversion.

In addition, the reactions catalyzed by AMPS synthetase and XMP aminase are perturbed by supplementing the incubation medium with aspartate and glutamine, respectively, and by adding the inhibitors, hadacidin and DON. The effect of using incubation media other than the simple salts medium usually used is also examined.

B. Results and Discussion

1. Hypoxanthine-^{14}C as Precursor

Previous workers in this and other laboratories have observed that Ehrlich ascites tumor cells favor the synthesis of adenine nucleotides from IMP over the synthesis of guanine nucleotides from IMP. The enzyme that has been suspected to be responsible for the slow rate of GMP synthesis is IMP dehydrogenase.

When the incorporation of hypoxanthine-^{14}C into adenine nucleotides plus NAD, and into guanine nucleotides plus XMP are examined (Fig. 26), it is apparent that the distribution of radioactivity among these two groups of nucleotides is unequal, thus confirming the earlier

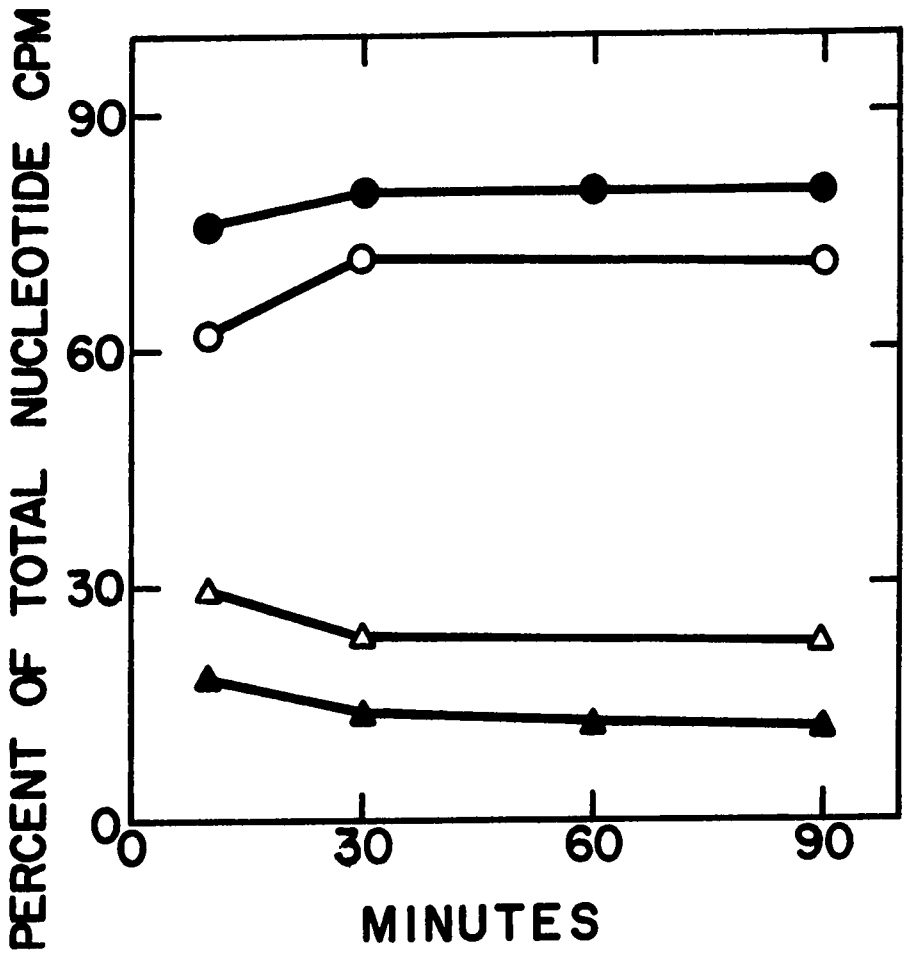


Figure 26. Relative incorporation of hypoxanthine-^{14}C into "adenine" and "guanine" nucleotides.
AMP + ADP + ATP + NAD: ● ○
GMP + GDP + GTP + XMP: ▲ △
Hypoxanthine-^{14}C- 5μM: ○ △
100μM: ● ▲

observations. With a low concentration of precursor (5 μM), the ratio of incorporation of hypoxanthine-^{14}C into adenine nucleotides relative to that into guanine nucleotides is approximately 3 after 30 minutes of incubation. When the hypoxanthine concentration is 100 μM however, the incorporation of base into adenine nucleotides is almost 6-fold that into guanine nucleotides. Therefore, the rate of one (or both) of the steps for the synthesis of GMP from IMP appears to be low in Ehrlich ascites tumor cells in vitro.

Since purine nucleotides are catabolized rapidly in Ehrlich cells, the incorporation of hypoxanthine-^{14}C into adenine nucleotides plus NAD plus adenosine (called "adenine compounds" below) and into guanine nucleotides plus XMP plus guanosine, xanthosine, guanine and xanthine (called "guanine compounds" below) was studied. Fig. 27 shows that at a precursor concentration of 5 μM, the incorporation of base into the guanine compounds is greater at early times than the incorporation into adenine compounds. The rate of incorporation into guanine compounds decreases more rapidly than does the rate of incorporation into adenine compounds, however. After 90 minutes of incubation, the ratio of the incorporation of precursor into the adenine compounds relative to that into the guanine compounds is about 1.3 (compare with 3 obtained above) indicating that the actual conversion of IMP into guanine nucleotides plus their degradation products is almost equivalent to the conversion of IMP into adenine nucleotides plus degradation products. When hypoxanthine-^{14}C is present at 100 μM, the rates of incorporation into adenine and guanine compounds are similar for about 30 minutes. At later times, the rate of incorporation of base into adenine compounds decreases whereas that of incorporation

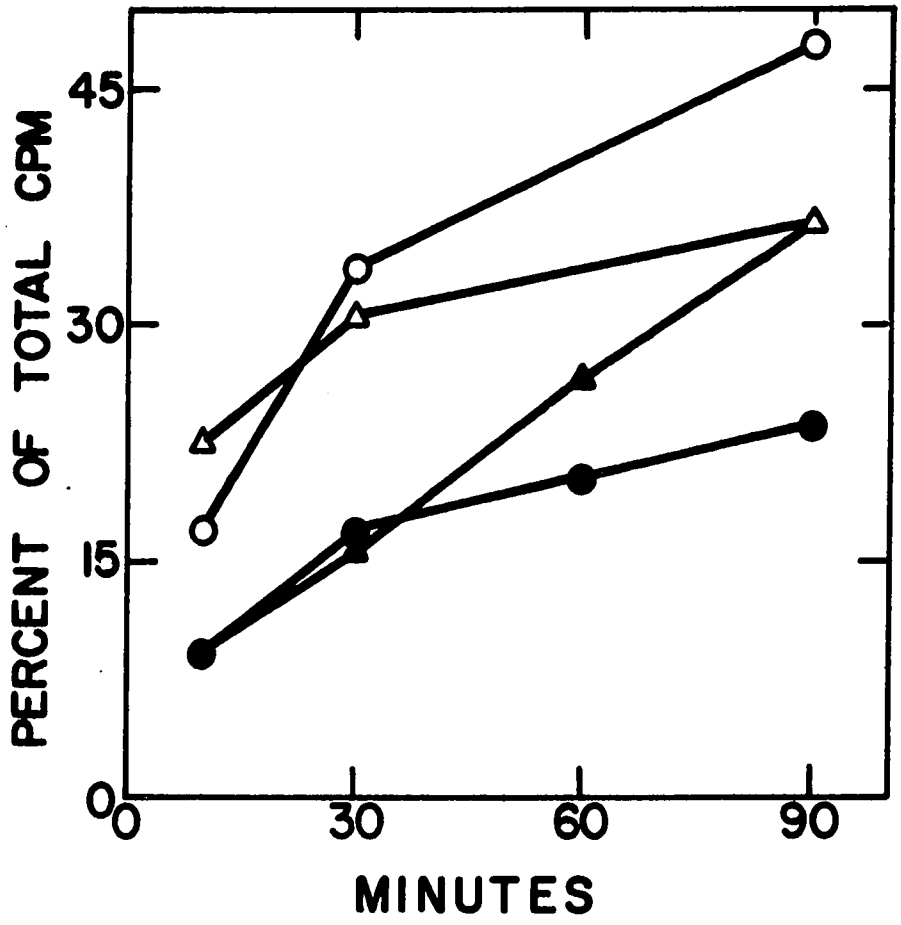


Figure 27. Relative conversion of hypoxanthine-^{14}C into "adenine" and "guanine" nucleotides, nucleosides and bases.

AMP + ADP + ATP + NAD + AR + A: ● ○

GMP + GDP + GTP + XMP + XR + X + GR + G: ▲ △

Hypoxanthine-^{14}C- 5μM: ○ △

100μM: ● ▲

into guanine compounds does not. In fact, after 90 minutes of incubation, the incorporation ratio is about 0.6. The large decrease in incorporation ratio observed when the precursor concentration is raised from 5 to 100 μM presumably is due to greater accumulation of degradation products of the guanine nucleotides (including XMP) as compared to those of the adenine nucleotides, and this may, in turn, reflect the slowness of one of the steps for the formation of GMP from IMP.

The data indicate that, whereas the conversion of inosinate into adenine and guanine nucleotides appears to be extremely biased towards the adenine nucleotides when only nucleotide metabolites are measured, when total metabolites are examined these conversions are almost equal. In these and other studies of metabolic pathways therefore, the investigator must examine all possible metabolites of a particular compound rather than just a certain segment of these metabolites in order not to obtain misleading conclusions. This procedure is extremely important in cases where the metabolites in question are rapidly and extensively converted to other compounds which remain undetected by the methods of assay used.

If IMP dehydrogenase were the rate-limiting enzyme for the conversion of IMP to GMP, IMP might be expected to accumulate. However, Fig. 28 shows that the concentration of IMP remains low throughout the incubation period. Similarly, if the XMP aminase reaction were the slow step in this conversion XMP should accumulate, but again this is not the case (Fig. 28). From these data, one cannot delineate the rate-controlling step for the formation of GMP from IMP. This apparent anomaly might be resolved by examining the accumulation of catabolites of nucleotides.

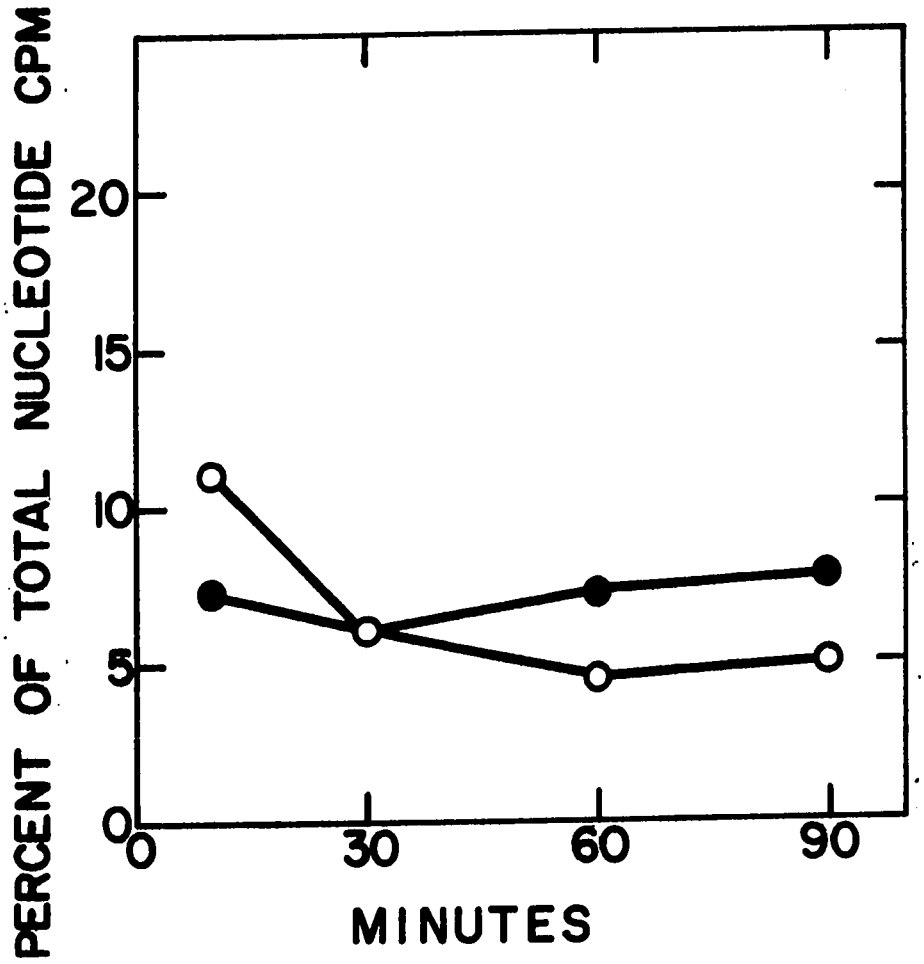


Figure 28. Formation of IMP and XMP from hypoxanthine-^{14}C.
IMP: ●
XMP: ○
Hypoxanthine-^{14}C: 100μM.

Xanthosine is a degradation product of XMP but not of the guanine nucleotides (see Chapter VI). When Ehrlich cells are incubated with hypoxanthine-^{14}C, xanthosine accumulates significantly (Fig. 29). In fact, after 90 minutes of incubation, almost 20% of the total radioactivity present in the sample is found as xanthosine. This observation implies that a significant amount of XMP is formed from IMP and the XMP so formed is not aminated to GMP but is dephosphorylated to xanthosine. Therefore, XMP aminase appears to limit the conversion of IMP to GMP more than does IMP dehydrogenase.

The possibility that the availability of glutamine is responsible for the slow rate of the XMP aminase reaction is examined below.

Delineation of the rate-controlling step in the conversion of IMP to AMP is quite difficult since (a) this process takes place rapidly in these cells, (b) we are unable to analyze for AMPS, the intermediate in this conversion and (c) as noted above, IMP does not accumulate when cells are incubated with hypoxanthine-^{14}C. Nevertheless, it is interesting to speculate that the availability of aspartate may be an important factor in establishing the slow step in this process. This aspect is studied below.

2. Adenine-^{14}C as Precursor

When adenine-^{14}C was used as precursor for nucleotide synthesis in Ehrlich ascites tumor cells <u>in vitro</u>, almost 95% of the total nucleotide fraction was composed of AMP + ADP + ATP. However, as noted above, measurements of nucleotide accumulations only do not take into account the actual total formation of nucleotides from the precursor since some of the nucleotides may be degraded during the incubation period. Accordingly, we also examined the conversion of adenine-^{14}C

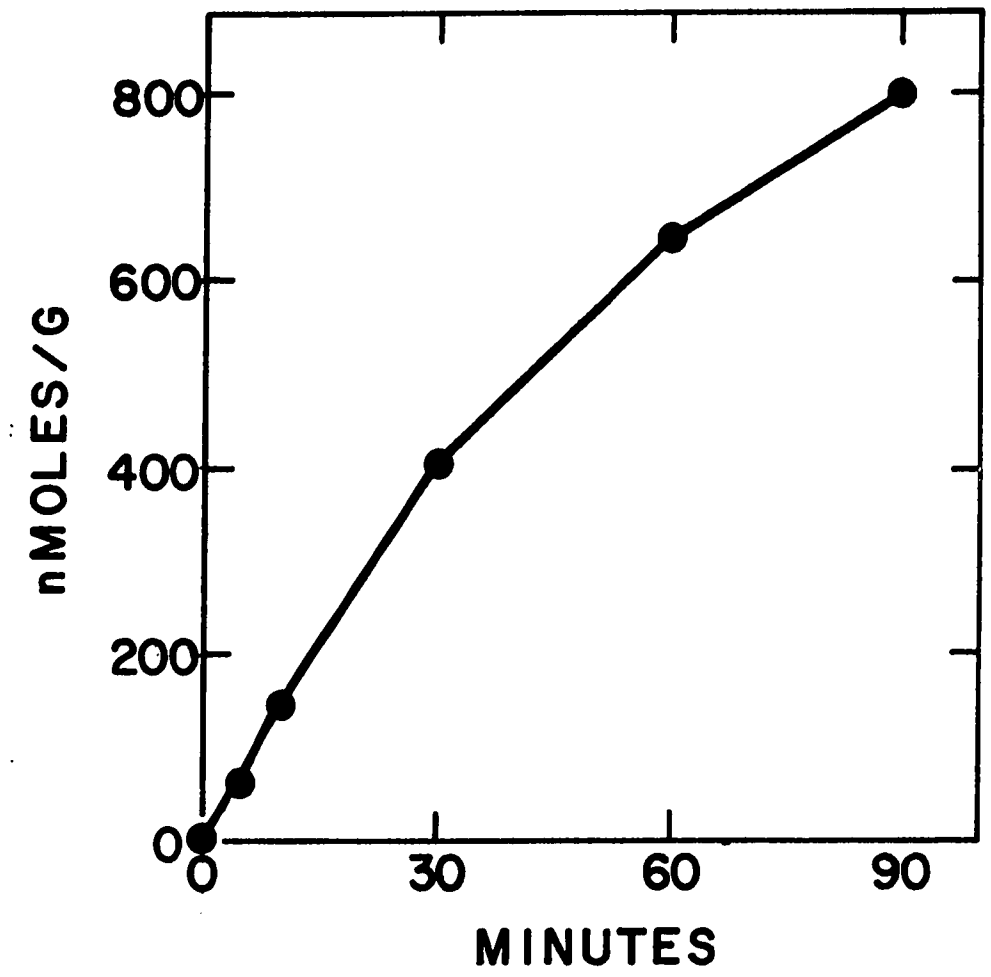


Figure 29. Formation of xanthosine from hypoxanthine-^{14}C. Hypoxanthine-^{14}C: 100μM.

into metabolites which do not contain adenine per se. It is apparent from Table II, that less than 15% of the adenine-^{14}C was converted to compounds which do not contain adenine even after 30 minutes of incubation. Therefore, the conversion of adenine nucleotides into other purine nucleotides takes place at low rates in these cells.

For the conversion of adenine nucleotides to guanine nucleotides at the monophosphate level, three reactions are required, AMP deaminase, IMP dehydrogenase and XMP aminase. Since neither IMP nor XMP accumulate when Ehrlich cells are incubated with adenine-^{14}C (Table III), the reaction catalyzed by AMP deaminase is suspect as the rate-limiting step in this conversion.

However, as noted above, XMP aminase appears to limit the formation of GMP from IMP. By analogy with the data obtained with hypoxanthine-^{14}C, if the reaction catalyzed by XMP aminase limits the conversion of AMP to GMP, xanthosine should accumulate when adenine-^{14}C is used as precursor. Fig. 30 shows that such is indeed the case. Therefore, XMP aminase appears to limit the conversion of AMP to GMP more than does AMP deaminase.

3. Guanine-^{14}C as Precursor

When Ehrlich cells are incubated in vitro with guanine-^{14}C, less than 10% of the precursor is converted to compounds which do not contain the guanine moiety per se (NGCM; Table IV). Xanthine, a possible catabolite of XMP, was not included in the NGCM totals since this compound may also be formed by the action of guanine deaminase on the precursor guanine-^{14}C as well as by the catabolism of guanine nucleotides.

Table II Conversion of Adenine-^{14}C Into Non-Adenine-Containing Metabolites* (NACM)

Adenine-^{14}C Concentration (μM)	Incubation Time (min)	% of Total CPM in NACM
20	10	7.2
	30	14.2
50	10	3.4
	30	6.7
100	10	2.5
	30	5.5

* NACM consists of IMP, XMP, GMP, GDP, GTP, H, HR, X, XR, G, GR.

Table III Conversion of Adenine-^{14}C Into IMP and XMP

Adenine-^{14}C Concentration (μM)	Incubation Time (min)	% of Total Nucleotide CPM in	
		IMP	XMP
20	10	0.8	0.3
	30	0.5	0.1
50	10	0.5	0.1
	30	0.3	0.2
100	10	0.5	0.2
	30	0.4	0.3

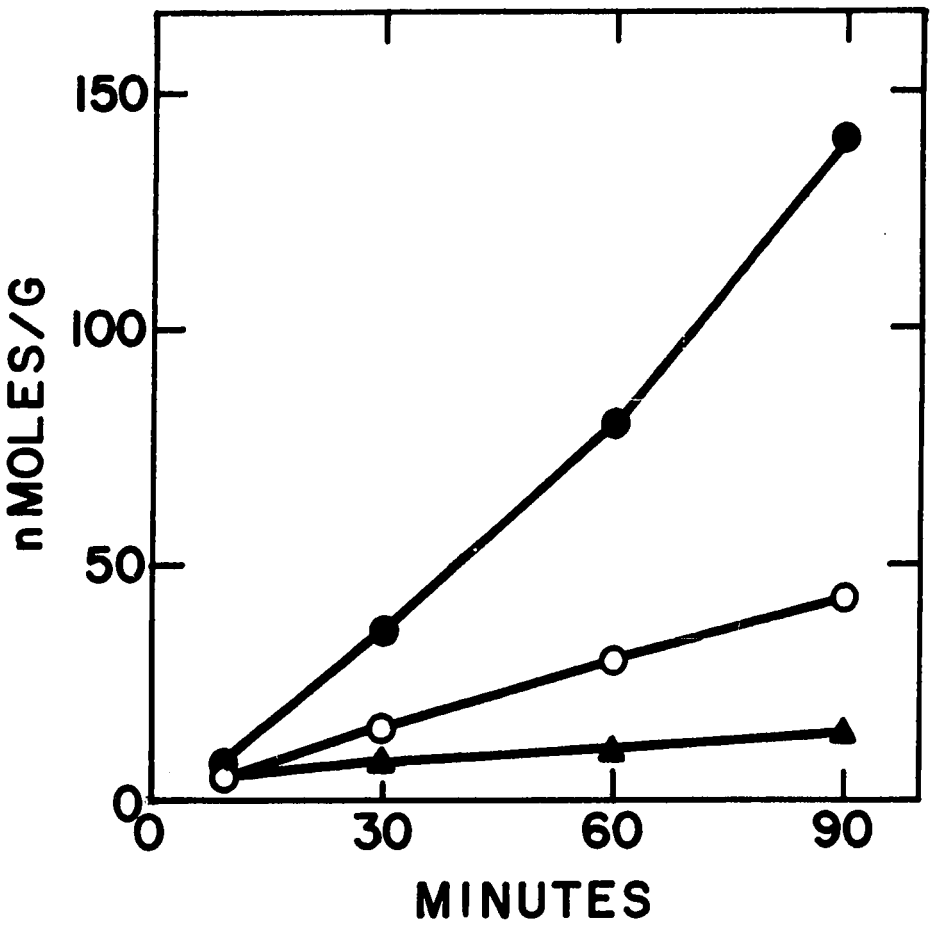


Figure 30. Xanthosine synthesis from adenine-^{14}C.
Adenine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

Table IV Conversion of Guanine-^{14}C Into Non-Guanine-Containing Metabolites* (NGCM)

Guanine-^{14}C Concentration (μM)	Incubation Time (min)	% of Total CPM in NGCM
20	10	5.6
	30	6.8
50	10	2.6
	30	2.7
100	10	2.2
	30	2.4

* NGCM consists of IMP, AMP, ADP, ATP, NAD, XMP, H, HR, A, AR, XR.

Table V Conversion of Guanine-^{14}C Into IMP

Guanine-^{14}C Concentration (μM)	Incubation Time (min)	% of Total Nucleotide CPM as IMP
20	10	0.7
	30	0.6
50	10	0.3
	30	0.1
100	10	0.1
	30	0.1

Since the acid-soluble nucleotide pool is composed to a large extent of adenine nucleotides, it is apparent that the conversion of GMP to AMP must take place very slowly in these cells. The possible rate-controlling reactions in this process are those catalyzed by GMP reductase, adenylosuccinate synthetase, and adenylosuccinate lyase. Since IMP does not accumulate when guanine-^{14}C is precursor (Table V), either GMP reductase or adenylosuccinate lyase appear to be limiting for the synthesis of AMP from GMP. However, evidence is presented below which implies that the reaction catalyzed by adenylosuccinate synthetase is the slow step in the conversion of IMP to AMP. The lack of IMP accumulation may therefore reflect the rapid degradation of this compound to inosine and hypoxanthine when IMP is not utilized for AMPS synthesis. Under no conditions did either inosine or hypoxanthine accumulate when guanine-^{14}C was used as precursor, however. Therefore, the formation of IMP from GMP takes place very slowly and GMP reductase appears to limit the conversion of GMP to AMP.

4. Perturbation of Reactions

The formations, both of guanylate and of adenylate, from inosinate consist of two reaction steps. In addition, in both cases, one of the steps utilizes an amino acid substrate. For guanylate formation, an amino donor is required in the xanthylate aminase reaction. In mammalian cells, this requirement is met by the amino acid glutamine. For adenylate formation, aspartate is required in the reaction catalyzed by adenylosuccinate synthetase.

To obtain greater insight into the rate-controlling steps for the synthesis of adenylate and of guanylate from inosinate, the

intracellular concentrations of aspartate and glutamine have been increased by adding these compounds to the incubation medium. In addition, the effects of metabolic inhibitors on the reactions in which aspartate and glutamine are utilized have been studied. The inhibitors used, hadacidin and DON, are believed to be antagonists of aspartate and glutamine, respectively,

The general method used in the other studies reported here previously was used in these experiments with one addition - the inhibitors or substrates were added at the start of the incubation period. Therefore, the cells were incubated with the substrate or inhibitor for 20 minutes before the radioactive precursor (hypoxanthine-^{14}C) was added. Two concentrations of hypoxanthine-^{14}C were used, 5 and 100 μM. Most emphasis is placed on the results obtained with 100 μM since the low precursor concentration results in very low counts in some of the metabolites. In addition, the rate-limiting steps are more apparent with the high concentration of precursor and hence effects of perturbation are more pronounced.

a. Control of the XMP Aminase Reaction

i. Glutamine

Since the availability of glutamine limits a number of metabolic processes requiring this compound as substrate, the XMP aminase reaction may also be limited by its availability. The effects of adding 2 mM glutamine on various aspects of nucleotide synthesis are outlined below.

Synthesis of acid-soluble purine nucleotides from hypoxanthine-^{14}C is increased significantly in the presence of glutamine (Fig. 31). Since glutamine is required for the formation of GMP from

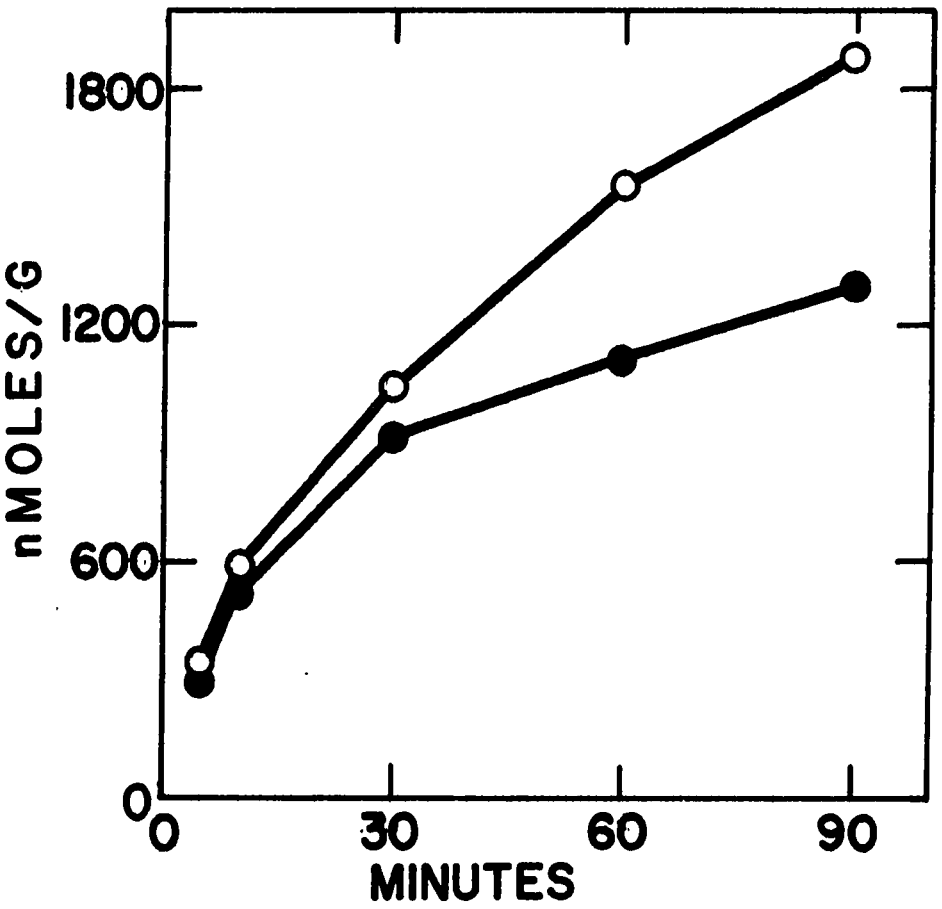


Figure 31. Effect of glutamine on total nucleotide synthesis from hypoxanthine-^{14}C.
Glutamine- 0: ●
2mM: ○
Hypoxanthine-^{14}C: 100μM.

IMP, the effects of glutamine on guanine nucleotide synthesis should be marked. Fig. 32 shows that in the presence of glutamine, the formation of guanine nucleotides from hypoxanthine-^{14}C is more than doubled over control values. Therefore, the intracellular concentration of glutamine appears to be limiting for the synthesis of GMP from IMP by limiting the rate of the XMP aminase reaction.

As proof of this hypothesis, Fig. 33 illustrates that the large accumulation of xanthosine observed in the absence of glutamine is reduced to a negligible level when glutamine is present in the incubation medium. Presumably, sufficient glutamine accumulates inside the cells, when it is added to the incubation medium, to react with the XMP formed from IMP and in this way, little XMP can accumulate and be dephosphorylated.

Although the incorporation of hypoxanthine-^{14}C into guanine nucleotides is increased in the presence of glutamine, the incorporation is still much lower than that into adenine nucleotides under the same conditions (compare Fig. 32 and 34). The most obvious explanation for this observation is that in the presence of glutamine, although the reaction catalyzed by XMP aminase is not limiting for the synthesis of GMP from hypoxanthine-^{14}C, that catalyzed by IMP dehydrogenase does limit this process. Presumably, the rate of the IMP dehydrogenase reaction is much slower than the rates of either of the reactions responsible for the formation of AMP from IMP.

When Fig. 32 and 33 are compared, it is obvious that the increased synthesis of guanine nucleotides observed in the presence of glutamine is not sufficient to account for the large decrease in xanthosine accumulation observed under the same conditions. In addition, in

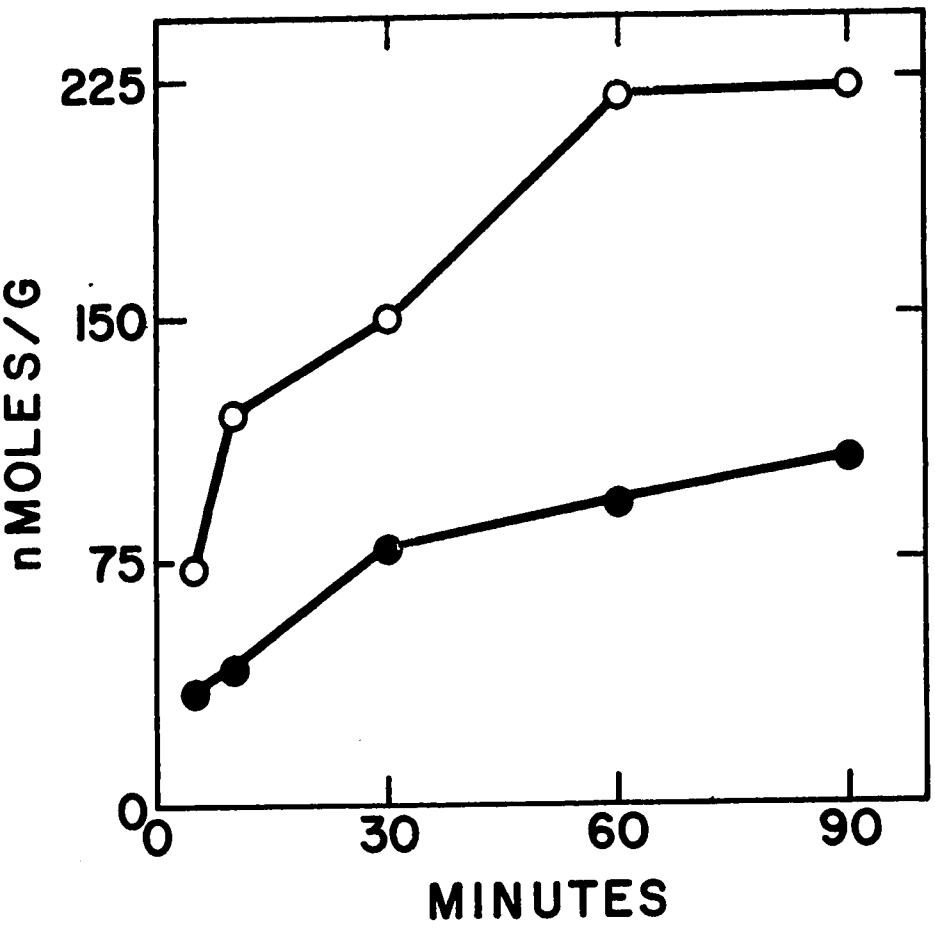


Figure 32. Effect of glutamine on guanine nucleotide synthesis from hypoxanthine-^{14}C.
Glutamine- 0: ●
2mM: ○
Hypoxanthine-^{14}C: 100μM.

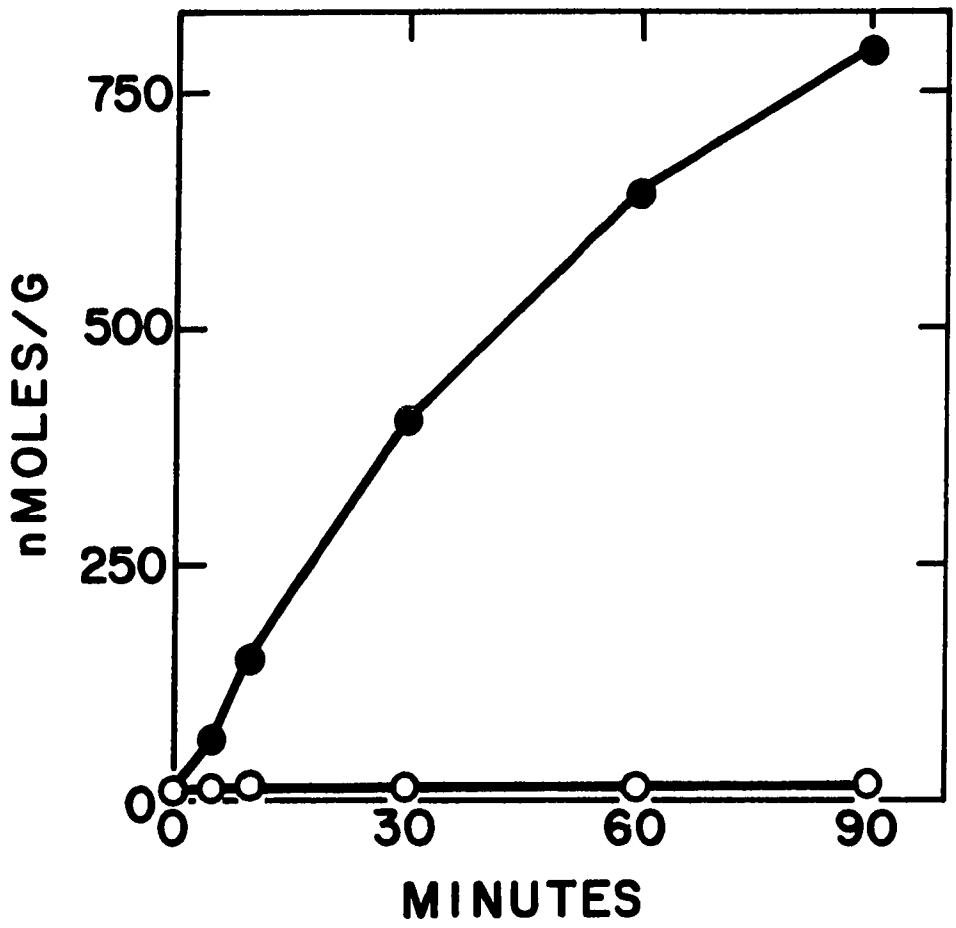


Figure 33. Effect of glutamine on xanthosine synthesis from hypoxanthine-^{14}C.
Glutamine- 0: ●
2mM: ○
Hypoxanthine-^{14}C: 100μM.

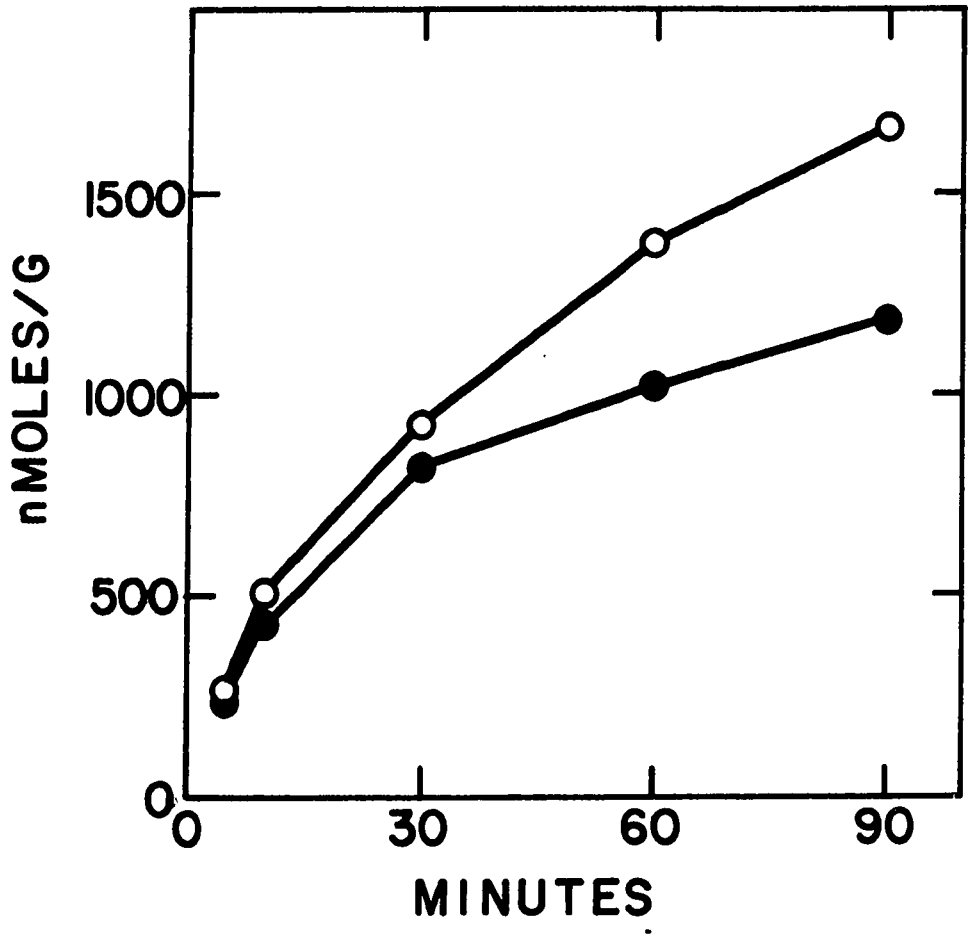


Figure 34. Effect of glutamine on adenine nucleotide synthesis from hypoxanthine-^{14}C.
Glutamine- 0: ●
2mM: ○
Hypoxanthine-^{14}C: 100μM.

the presence of glutamine, the total utilization of hypoxanthine-^{14}C for various metabolic processes is reduced over control values. The possibility exists that utilization of glutamine for metabolic reactions results in the release of ammonia which may alter the intracellular pH of the Ehrlich cells or actually inhibit some of the enzyme reactions.

In addition to its effects on guanine nucleotide synthesis from inosinate, the presence of glutamine in the incubation mixture has rather unexpected, but marked, effects on the incorporation of hypoxanthine-^{14}C into the adenine nucleotides. As Fig. 34 illustrates, addition of glutamine increases the synthesis of adenine nucleotides from inosinate in Ehrlich ascites tumor cells in vitro. Although the effects of glutamine on adenine nucleotide synthesis are not as pronounced as those on guanine nucleotide synthesis, they are quite substantial since the accumulation of adenine nucleotides is increased about 40% over control values. A possible explanation for this phenomenon is that aspartate, a required substrate for the synthesis of AMP from IMP is being formed in these cells from the glutamine present in the incubation medium. Although evidence has been found which supports this belief (Henderson, J. F. and Fontenelle, L. J., personal communication), this hypothesis assumes that the normal intracellular concentrations of aspartate are limiting for the synthesis of AMP from IMP.

ii. DON

DON is an analog of L-glutamine (Fig. 35) which inhibits amination reactions requiring glutamine as the amino donor. Notably susceptible to inhibition are the amination reactions of purine biosynthesis de novo. Since glutamine is also the amino donor for the XMP aminase reaction in mammalian cells, DON might be expected to

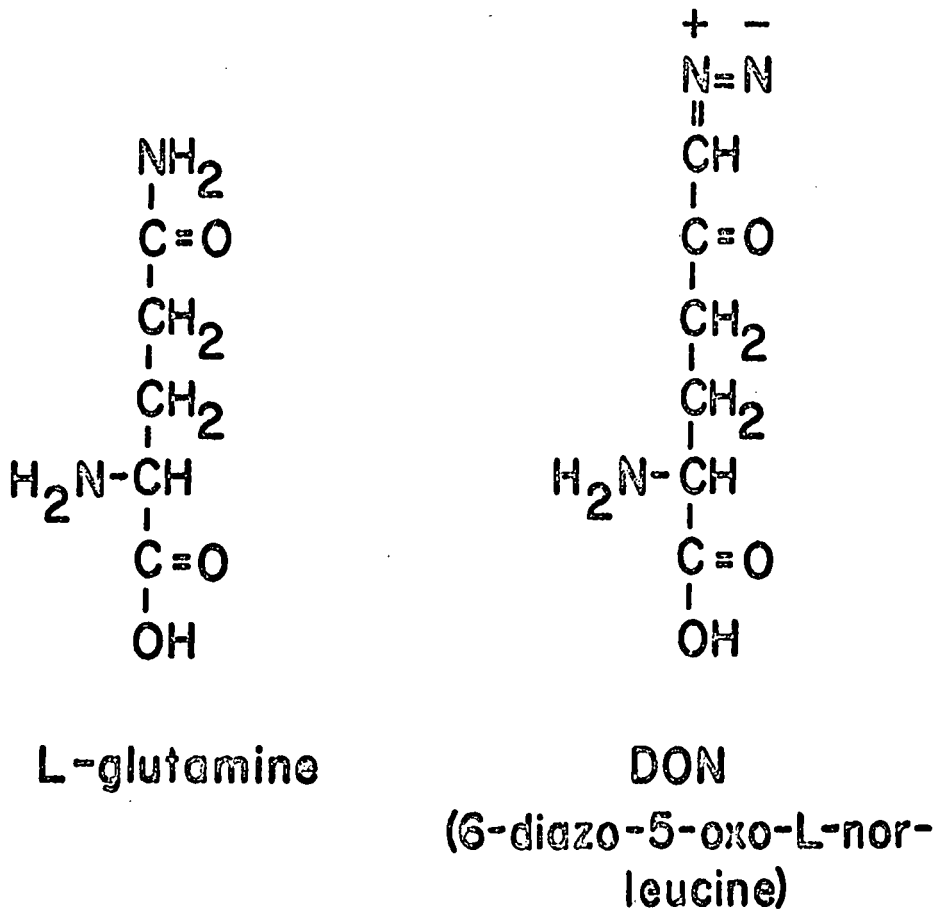


Figure 35. Structural formulae of L-glutamine and DON.

inhibit this reaction too. The effects of adding DON (35 μM) to the incubation medium when hypoxanthine-^{14}C is precursor are presented below.

DON inhibits the synthesis of acid-soluble purine nucleotides from hypoxanthine by about 43% after 90 minutes of incubation (Fig. 36) when the precursor is present at 100 μM.

If DON exerts its effect by inhibiting the XMP aminase reaction, the decreased incorporation of hypoxanthine-^{14}C into total acid-soluble purine nucleotides when DON is present should be reflected in decreased guanine nucleotide synthesis and, at the same time, increased formation of xanthosine. Fig. 37 shows that the formation of guanine nucleotides from hypoxanthine is reduced by more than 50% of control values when DON is added to the medium. At the same time, the accumulation of xanthosine is increased in the presence of DON (Fig. 38). Although the increased accumulation of xanthosine in the presence of DON appears to be small, it is sufficient to account for the decreased synthesis of guanine nucleotides from hypoxanthine observed under the same conditions. These observations are, of course, consistent with inhibition of XMP aminase by DON.

DON also inhibits the synthesis of adenine nucleotides from hypoxanthine-^{14}C by more than 50% after 90 minutes of incubation (Fig. 39). This unexpected, but significant, effect of DON might be explained as follows. If the formation of AMP from IMP is limited by the intracellular concentration of aspartate and if aspartate is synthesized from glutamine in Ehrlich cells <u>in vitro</u>, DON may inhibit AMP synthesis from IMP by blocking the formation of aspartate from glutamine.

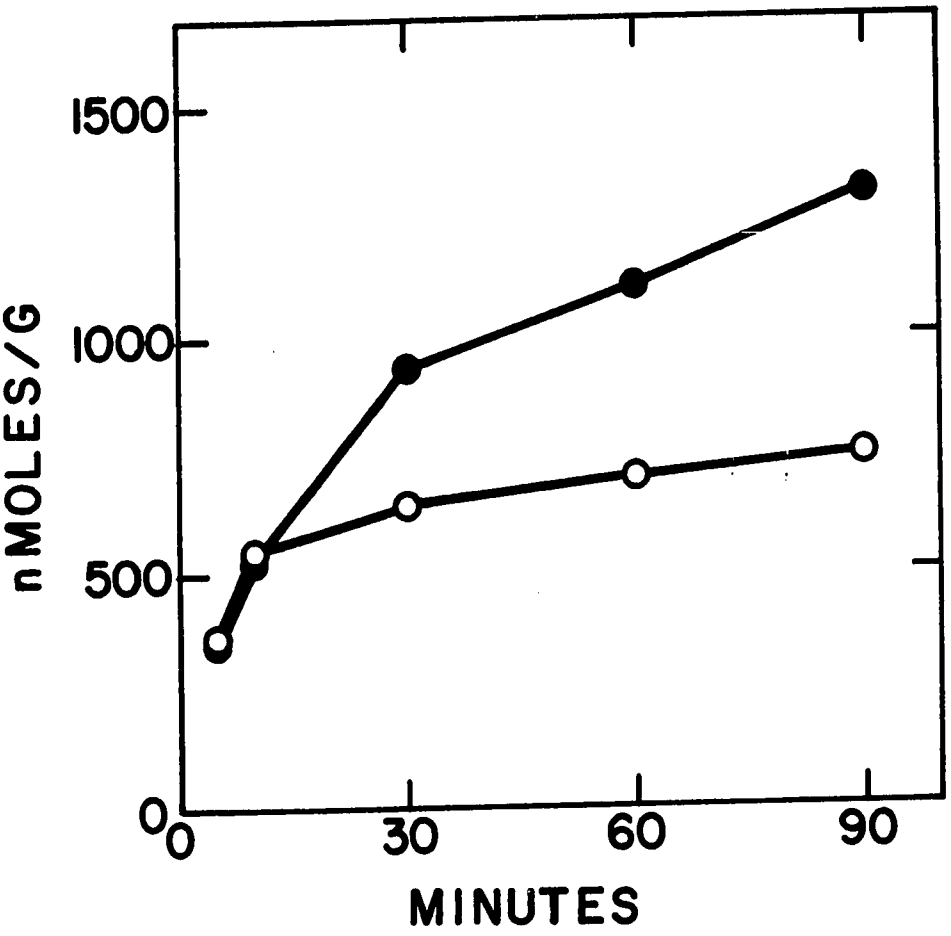


Figure 36. Effect of DON on total nucleotide synthesis from hypoxanthine-^{14}C.
DON: 0: ●
35μM: ○
Hypoxanthine-^{14}C: 100μM.

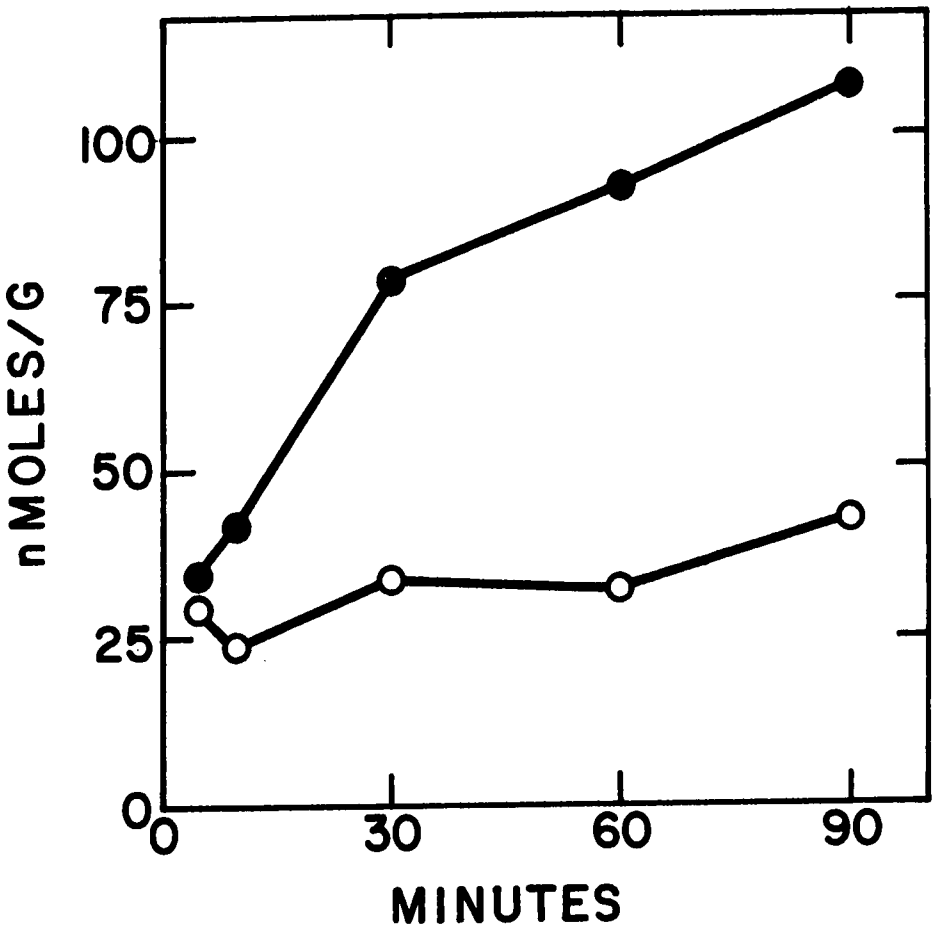


Figure 37. Effect of DON on guanine nucleotide synthesis from hypoxanthine-^{14}C.
DON- 0: ●
35μM: ○
Hypoxanthine-^{14}C: 100μM.

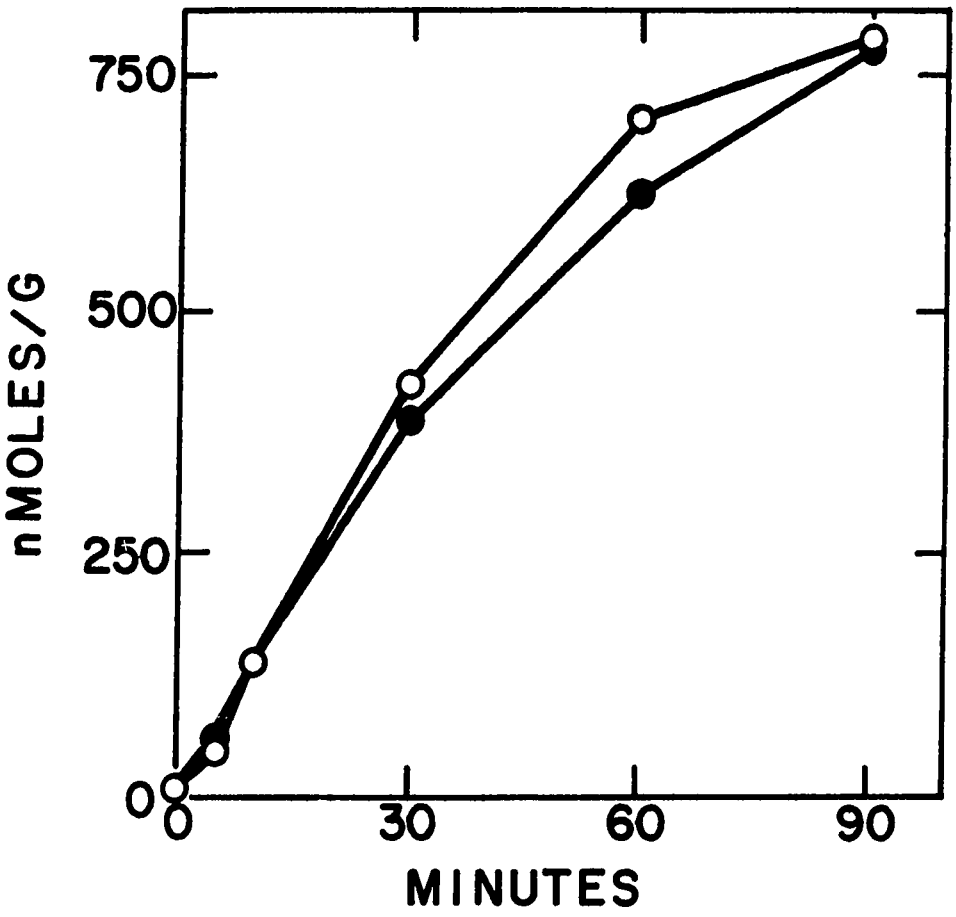


Figure 38. Effect of DON on xanthosine synthesis from hypoxanthine-^{14}C.
DON- 0: ●
35μM: ○
Hypoxanthine-^{14}C: 100μM.

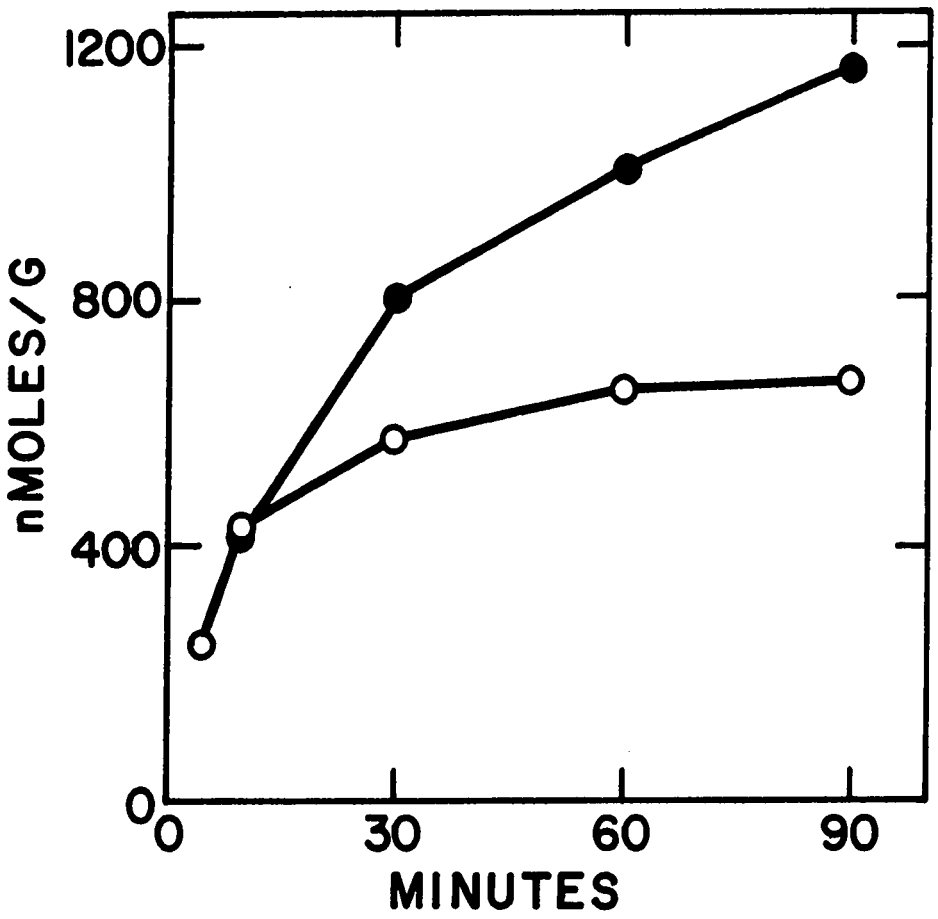


Figure 39. Effect of DON on adenine nucleotide synthesis from hypoxanthine-^{14}C.
DON- 0: ●
35μM: ○
Hypoxanthine-^{14}C: 100μM.

iii. Aspartate

Under control conditions, the reaction catalyzed by XMP aminase is the slow step in the conversion of IMP to GMP and consequently, xanthosine, the dephosphorylation product of XMP accumulates. When aspartate is present in the medium, the accumulation of xanthosine is reduced (Fig. 40). The possibility exists that some of the added aspartate is utilized to form glutamine, a required substrate for GMP formation from XMP. In this case, the formation of guanine nucleotides would be expected to be increased over control values when aspartate is added. As noted below, guanine nucleotide synthesis is scarcely affected, however. These observations may also reflect the greater utilization of inosinate for AMP synthesis in the presence of aspartate as compared to controls, so that less of the IMP formed from hypoxanthine-^{14}C is available for the synthesis of guanine nucleotides when aspartate is present. Accordingly, less XMP would be formed when aspartate is present and consequently less xanthosine would accumulate. Nevertheless, the synthesis of XMP from IMP in the presence of aspartate appears to be sufficient to give maximum rates of GMP synthesis since some xanthosine still accumulates and presumably the availability of glutamine still limits the conversion of IMP to GMP.

b. Control of the Adenylosuccinate Synthetase Reaction

i. Aspartate

The effects of aspartate on purine nucleotide synthesis from hypoxanthine-^{14}C are examined in this section. Although the concentration of aspartate in the incubation medium appears to be quite high (20 mM), this extracellular concentration is necessary to obtain significant intracellular levels of this compound since the uptake of this

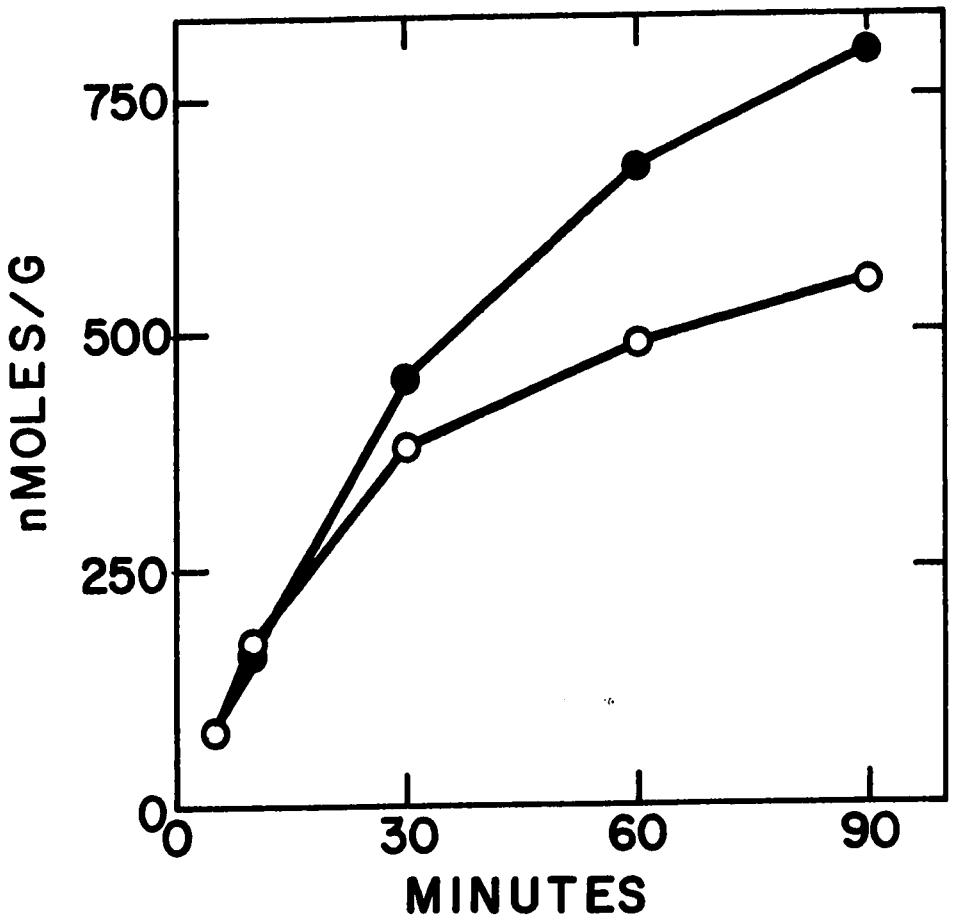


Figure 40. Effect of aspartate on xanthosine synthesis from hypoxanthine-^{14}C.
Aspartate- 0: ●
20mM: ○
Hypoxanthine-^{14}C: 100μM.

amino acid by Ehrlich cells is very slow (Johnstone and Scholefield, 1965).

When Ehrlich ascites tumor cells are exposed to high extracellular concentrations of aspartate, the formation of acid-soluble purine ribonucleotides from hypoxanthine-^{14}C (100 μM) is increased almost two-fold (Fig. 41) indicating that the intracellular concentrations of aspartate may be limiting for adenine nucleotide synthesis from preformed hypoxanthine. Presumably, the two-fold increase in total nucleotides is due to increased adenine nucleotide synthesis in the presence of aspartate. Guanine nucleotide synthesis, on the other hand should not be affected. As Fig. 42 indicates, after 90 minutes of incubation, the accumulation of adenine nucleotides is almost doubled, compared to controls, when aspartate is added. Guanine nucleotide synthesis is scarcely affected by aspartate, however. Apparently then, under normal conditions, the intracellular concentrations of aspartate are limiting for the synthesis of adenylate from inosinate; therefore, the reaction catalyzed by adenylosuccinate synthetase is rate-limiting for this process under normal <u>in vitro</u> incubation conditions.

ii. Hadacidin

Hadacidin (N-formylhydroxyaminoacetic acid) is a structural analog of L-aspartate (Fig. 43) which presumably exerts its effects by blocking the formation of AMPS from IMP (Shigeura and Gordon, 1962). The effects reported below of adding hadacidin to the incubation medium on purine nucleotide synthesis from hypoxanthine-^{14}C are consistent with this belief.

The incorporation of hypoxanthine-^{14}C (100 μM) into the total

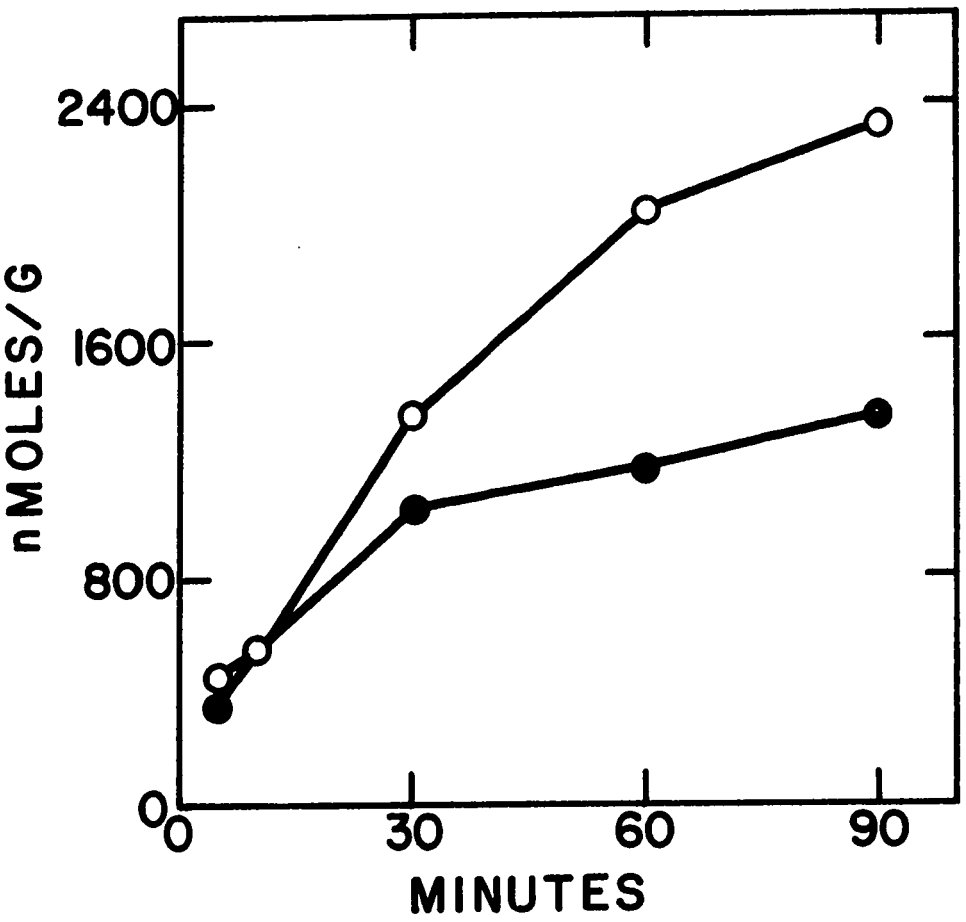


Figure 41. Effect of aspartate on total nucleotide synthesis from hypoxanthine-^{14}C.
Aspartate- 0: ●
20mM: ○
Hypoxanthine-^{14}C: 100μM.

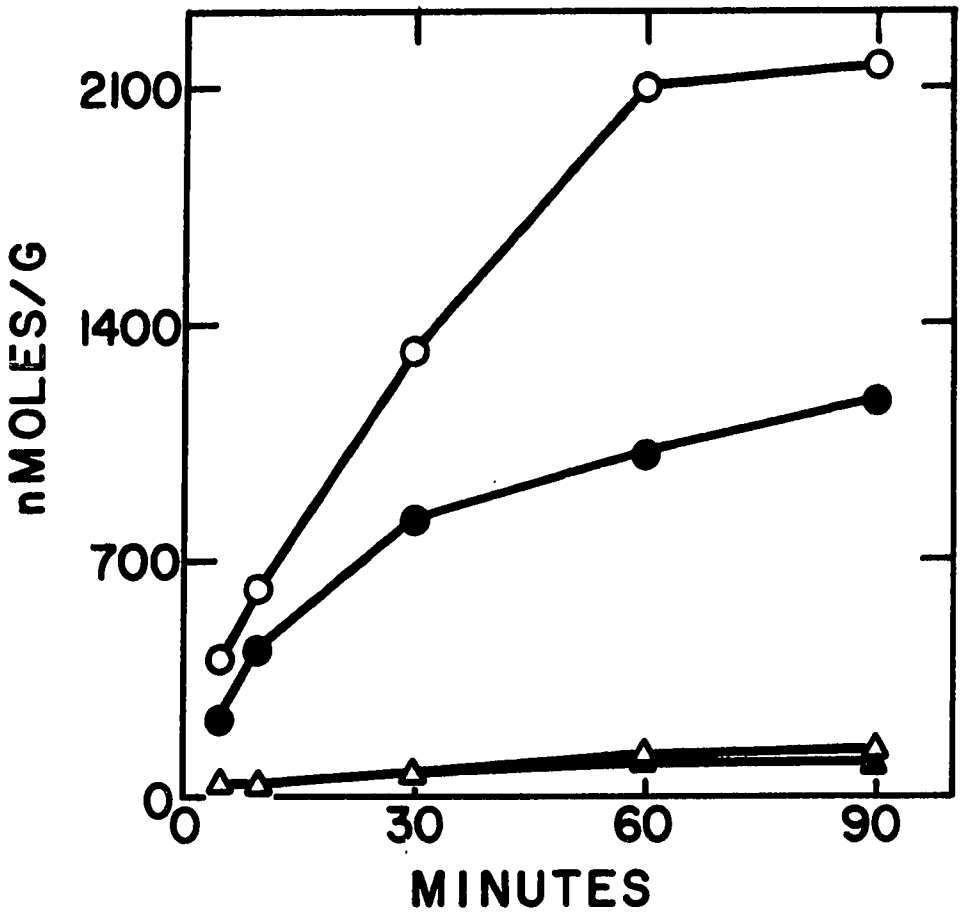
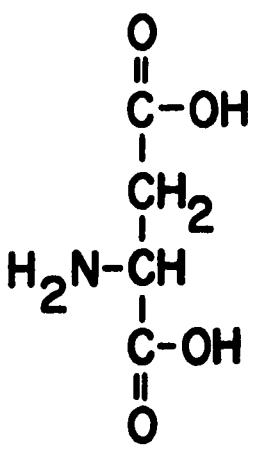
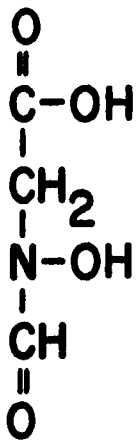


Figure 42. Effect of aspartate on adenine and guanine nucleotide synthesis from hypoxanthine-^{14}C.
Adenine nucleotides: ● ○
Guanine nucleotides: ▲ △
Aspartate- 0: ● ▲
20mM: ○ △
Hypoxanthine-^{14}C: 100μM.



L-aspartic acid



Hadacidin

(N-formylhydroxyamino-acetic acid)

Figure 43. Structural formulae of L-aspartate and hadacidin.

acid-soluble nucleotide fraction is markedly inhibited (almost 70% after 90 min) in Ehrlich ascites tumor cells incubated in vitro in the presence of hadacidin (Fig. 44). Since hadacidin inhibits adenylosuccinate synthetase, most of this reduction in total nucleotide synthesis should be due to inhibition of adenine nucleotide synthesis. Fig. 45 shows that, in the presence of hadacidin, the synthesis of adenine nucleotides is reduced to less than 20% of control values after 90 minutes of incubation. As expected, guanine nucleotide synthesis does not appear to be affected by the presence of this inhibitor.

5. Effects of Other Media on Nucleotide Synthesis from Hypoxanthine-^{14}C

At the time that the work reported in this dissertation was in progress, other workers in this laboratory were studying the effects of different incubation media on tumor cell metabolism in vitro. These workers were able to show that "Fischer's medium for leukemic cells of mice" (Fischer and Sartorelli, 1964) afforded a number of advantages over the salts medium used throughout the studies reported in this dissertation. In addition, still other workers in this laboratory found that supplementation of this culture medium with 10% horse serum permitted the growth of Ehrlich ascites tumor cells in tissue culture. Therefore, it was of interest to examine the effects of such media on the metabolism of hypoxanthine-^{14}C by Ehrlich ascites tumor cells in vitro and to compare the results obtained with those reported previously when Krebs-Ringer solution was used as the incubation medium.

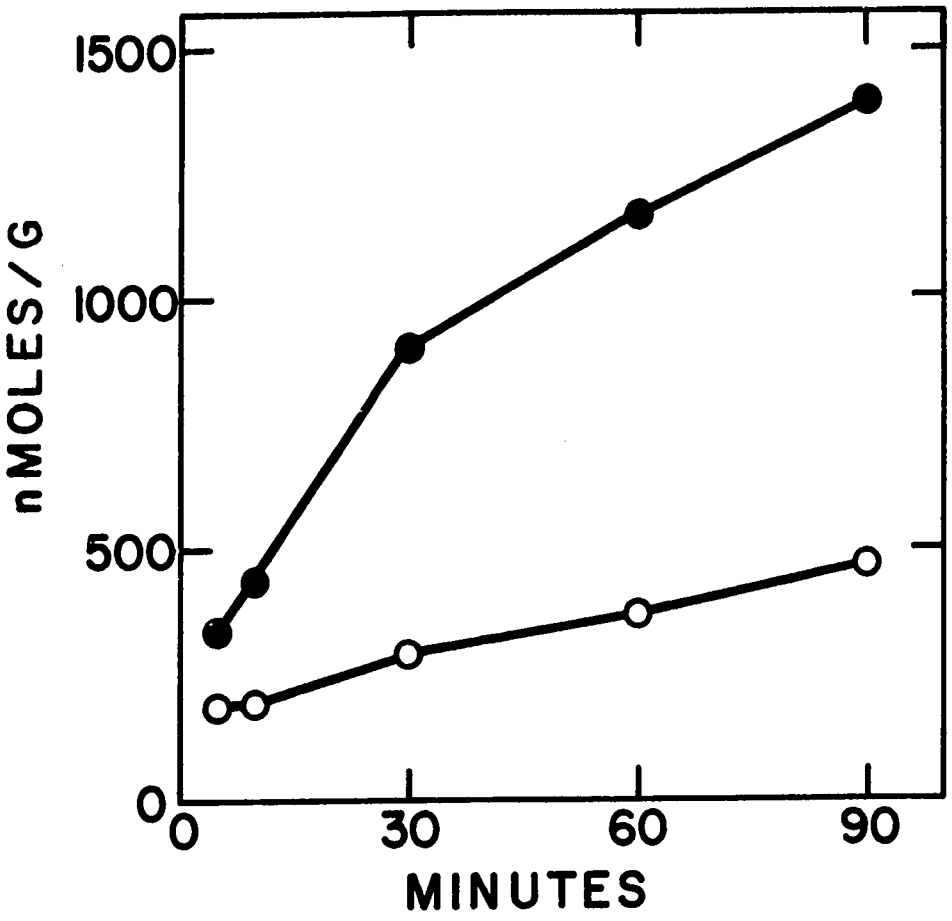


Figure 44. Effect of hadacidin on total nucleotide synthesis from hypoxanthine-^{14}C.
Hadacidin- 0: ●
100µg/ml: ○
Hypoxanthine-^{14}C: 100µM.

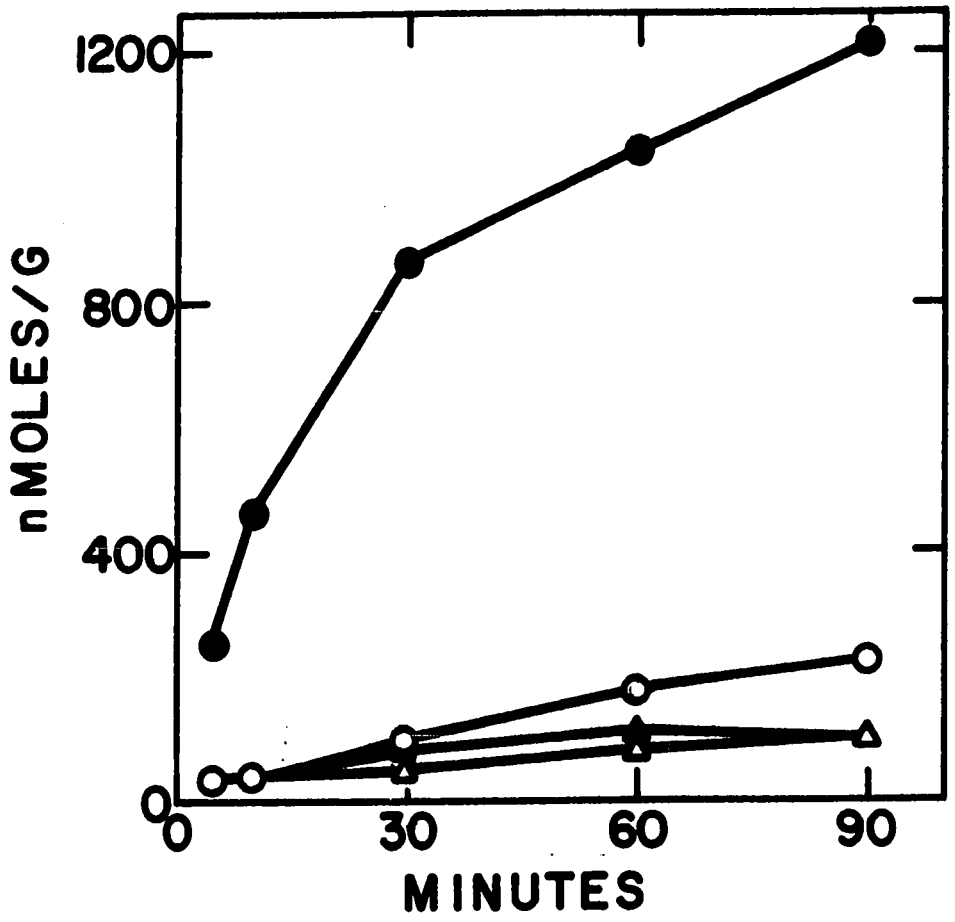


Figure 45. Effect of hadacidin on adenine and guanine nucleotide synthesis from hypoxanthine-^{14}C.
Adenine nucleotides: ● ○
Guanine nucleotides: ▲ △
Hadacidin- 0: ● ▲
100ug/ml: ○ △
Hypoxanthine-^{14}C: 100μM.

a. Fischer's Medium

"Fischer's medium for leukemic cells of mice" is a chemically defined medium which contains simple salts, glucose, amino acids (including asparagine and glutamine), and vitamins and which is "bicarbonate-buffered". Because of the nature of the sampling procedure used in these experiments, the "gassing" procedure (with 95% O_2/5% CO_2) needed for bicarbonate-buffered media would have been difficult to accomplish. Since Fischer's medium is obtained from the supplier as a dry powder to which $NaHCO_3$ is to be added, for the experiments reported in this section, $NaHCO_3$ is replaced by sodium phosphate to give a final phosphate concentration of 25 mM. Under these conditions, the buffering capacity of the medium is sufficient to maintain the pH at or near physiological levels for the duration of the incubation period.

The effect on the synthesis of total acid-soluble purine ribonucleotides of incubating cells in the presence of Fischer's medium as compared to Krebs-Ringer solution is given in Fig. 46. In Fischer's medium, almost twice as much of the hypoxanthine-^{14}C is incorporated into total nucleotides than when Krebs-Ringer solution is used.

When Fischer's medium is used (Fig. 47), the synthesis of adenine nucleotides is increased by about 70% over that found with Krebs-Ringer solution. This effect may be due to the presence of L-asparagine in the Fischer's medium, from which aspartate may be formed. Since aspartate appears to be limiting for adenine nucleotide synthesis in these cells in vitro (see previous data), the formation of aspartate in this manner would be expected to increase the amounts of adenine

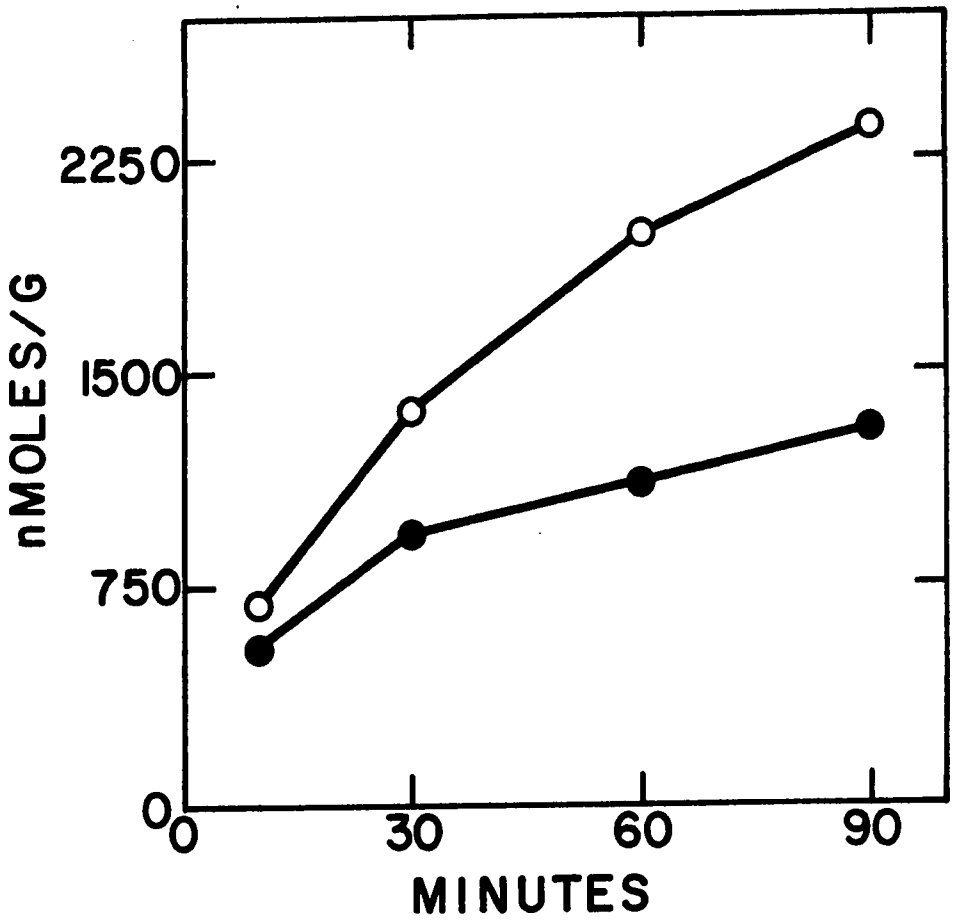


Figure 46. Comparison of total nucleotide synthesis from hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

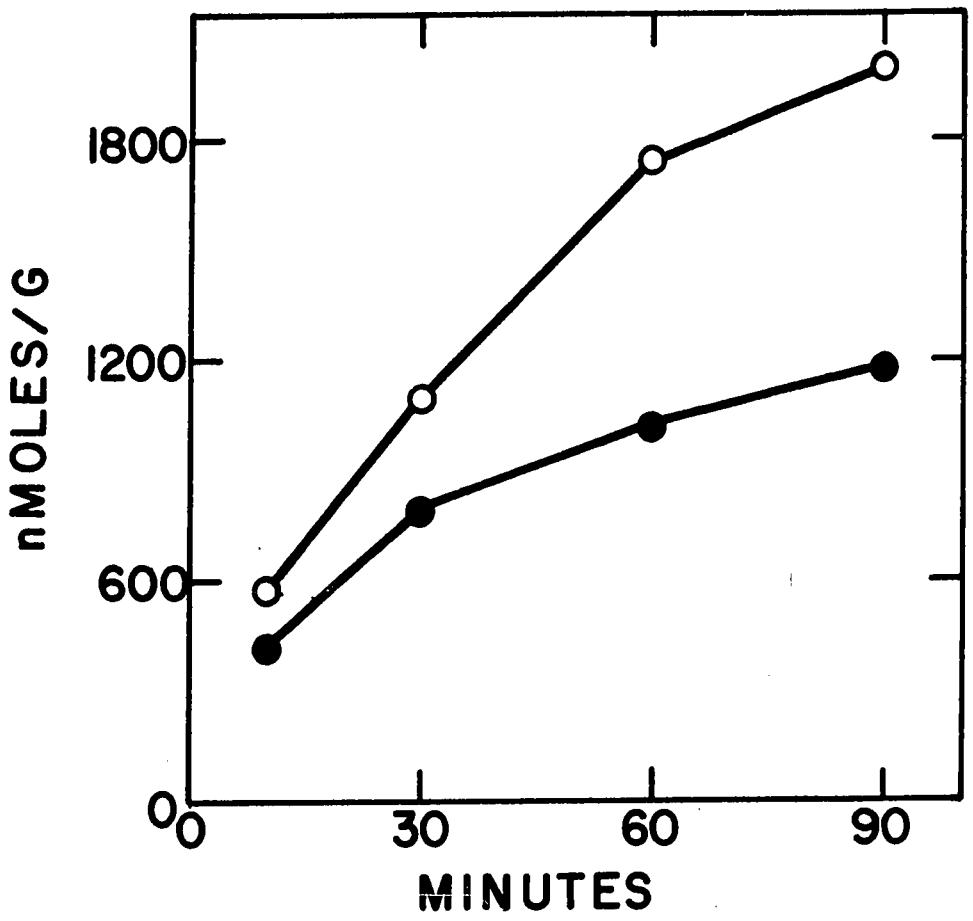


Figure 47. Comparison of adenine nucleotide synthesis from hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

nucleotides synthesized from hypoxanthine-^{14}C when Fischer's medium is used.

Fig. 48 illustrates that guanine nucleotide synthesis is increased more than four-fold when Fischer's medium is used instead of Krebs-Ringer solution. Since glutamine is rate-limiting for guanylate synthesis from inosinate, the glutamine present in Fischer's medium would be expected to cause the large increases in the synthesis of guanine nucleotides observed in Fig. 48. The fact that the synthesis of guanine nucleotides is much lower than that of adenine nucleotides even in Fischer's medium may be explained by assuming that the IMP dehydrogenase reaction becomes rate-limiting for the formation of GMP from IMP when glutamine is supplied to the cells (see previous data).

b. Horse Serum

Since the addition of horse serum is known to have beneficial effects on the propagation of Ehrlich ascites tumor cells by tissue culture techniques, we have examined its effects on the synthesis of purine nucleotides from hypoxanthine-^{14}C in Ehrlich ascites tumor cells <u>in vitro</u>. For these experiments, we have used Krebs-Ringer solution and Fischer's medium, both supplemented with 10% horse serum and unsupplemented.

The supplementation of the incubation medium results in small, but significant, increases in the synthesis of purine nucleotides from hypoxanthine-^{14}C (Fig. 49). Both adenine and guanine nucleotide syntheses from hypoxanthine-^{14}C (5 μM) appear to be enhanced by the addition of horse serum, regardless of the incubation medium used (Fig. 50 and 51).

It is impossible at this time, to rationalize these effects

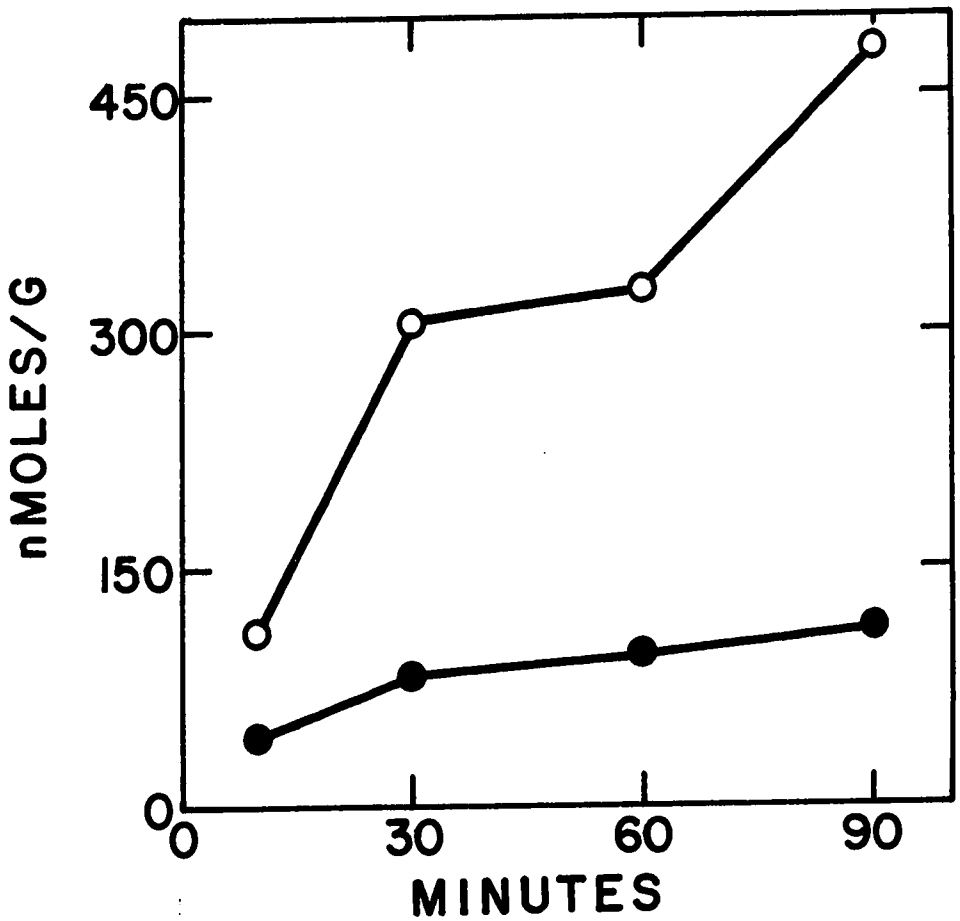


Figure 48. Comparison of guanine nucleotide synthesis from hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

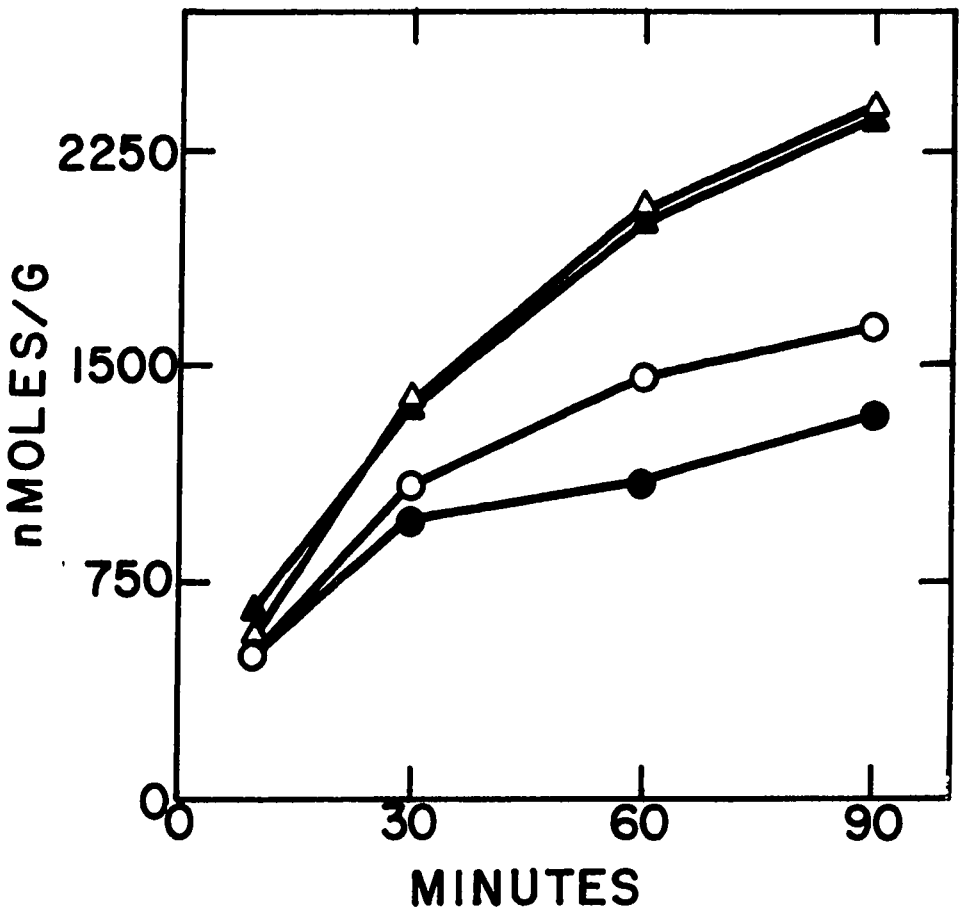


Figure 49. Effect of horse serum on total nucleotide synthesis from hypoxanthine-^{14}C in different media.
Horse serum- 0: ● ▲
10%: ○ △
Krebs-Ringer medium: ● ○
Fischer's medium: ▲ △
Hypoxanthine-^{14}C: 100μM.

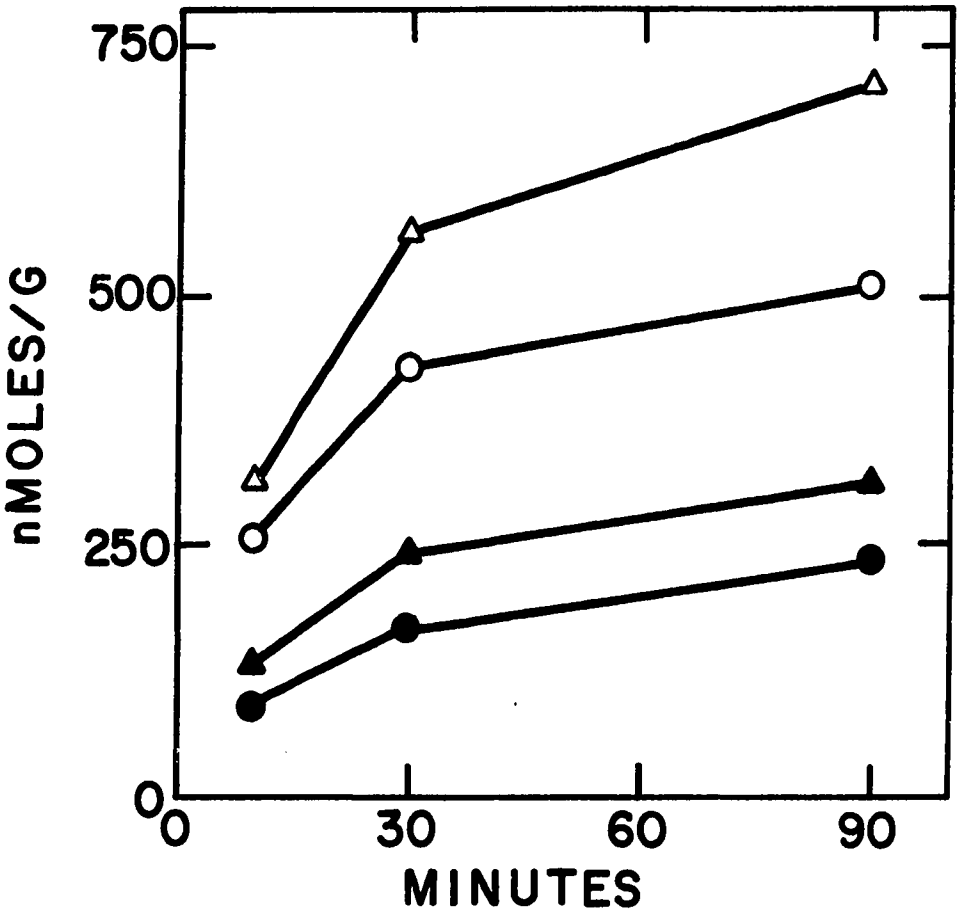


Figure 50. Effect of horse serum on adenine nucleotide synthesis from hypoxanthine-^{14}C in different media.

Horse serum- 0: ● ▲
10%: ○ △
Krebs-Ringer medium: ● ○
Fischer's medium: ▲ △
Hypoxanthine-^{14}C: 5μM.

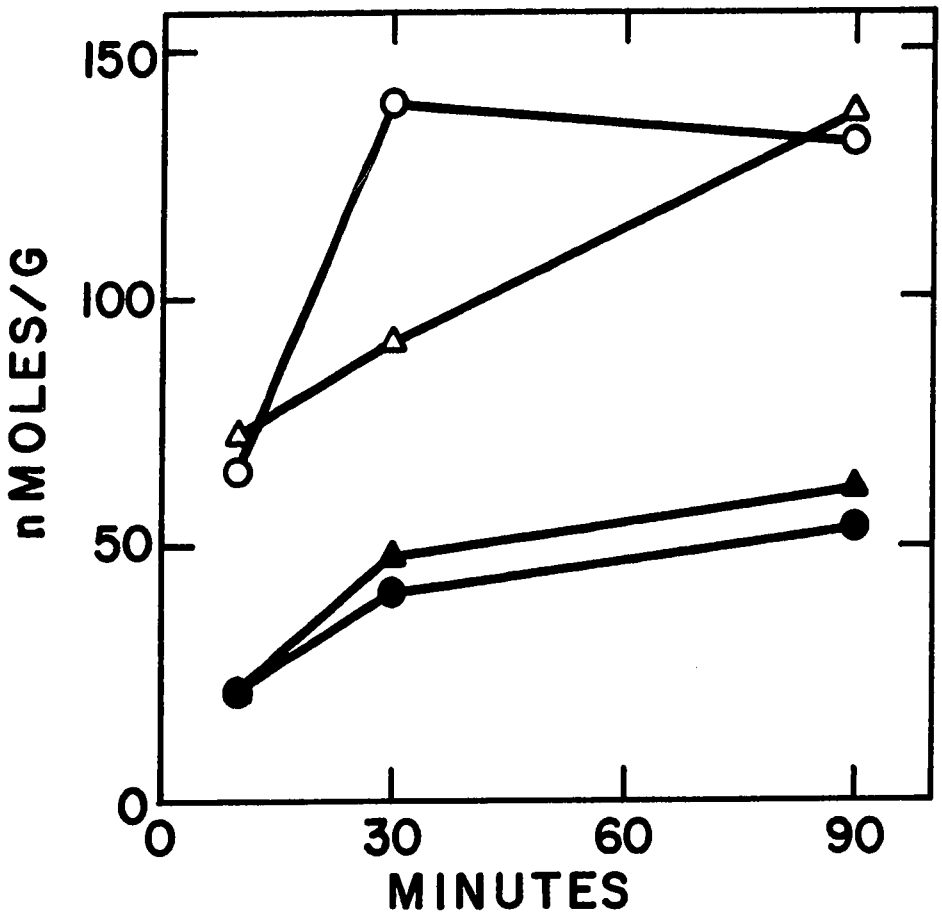


Figure 51. Effect of horse serum on guanine nucleotide synthesis from hypoxanthine-^{14}C in different media.

Horse serum- 0: ● ▲
10%: ○
Krebs-Ringer medium: ● ○
Fischer's medium: ▲ △
Hypoxanthine-^{14}C: 5μM.

of horse serum on purine nucleotide metabolism, simply because the composition of horse serum is unknown. Presumably, active enzymes and other nutrients in the horse serum are responsible for the observed effects.

C. Summary

It is apparent, from the data presented in this chapter, that the rates of interconversion of adenine and guanine nucleotides in Ehrlich ascites tumor cells *in vitro* are quite low. The rates of conversion of AMP into GMP and of GMP into AMP appear to be limited by the reactions catalyzed by XMP aminase and GMP reductase, respectively.

The concentrations of non-purine substrates such as L-aspartate and L-glutamine appear to be limiting, under normal conditions, for the formation of AMP and GMP, respectively from IMP. Structural analogs of these non-purine substrates, namely hadacidin and DON, markedly inhibit the reactions catalyzed by adenylosuccinate synthetase and XMP aminase, respectively.

"Fischer's medium for leukemic cells of mice" supports increased synthesis of both adenine and guanine nucleotides from hypoxanthine-^{14}C as compared to that observed with Krebs-Ringer solution as incubation medium. These increases are presumably due to the presence of non-purine substrates in the Fischer's medium; these substrates are limiting for nucleotide synthesis when Krebs-Ringer solution is used.

Addition of 10% horse serum to either Fischer's medium or Krebs-Ringer solution results in increased synthesis of both adenine and guanine nucleotides from hypoxanthine-^{14}C as compared to controls. The explanation for these effects is not apparent, however.

VI CONTROL OF PURINE RIBONUCLEOTIDE CATABOLISM

A. Introduction

Purine nucleotides are catabolized by various types of animal, plant and bacterial cells to compounds which may be excreted or reutilized in order to maintain an equilibrium between their synthesis and degradation. Many pathways potentially exist for purine catabolism in cells, but a particular cell utilizes only certain of them. Also, when alternative pathways do exist in a cell, one cannot always identify their relative contributions.

Although the pathways of purine nucleotide catabolism have been established for some time, only recently has work been undertaken to examine the factors which control these reactions both from the standpoint of the alternative pathways which are operative and from the standpoint of the steps which are rate-controlling in these reaction sequences. In the present study therefore, we have attempted to define the catabolic pathways of purine nucleotide metabolism and the rate-limiting reactions in these pathways as they exist in Ehrlich ascites tumor cells in vitro.

As with the other reactions of purine nucleotide metabolism, most of the work so far reported on possible control mechanisms has been done on individual enzymes in isolated systems. Some of this work is reviewed below. However, several investigations have appeared which are concerned with regulation of catabolic pathways in tissue extracts and in intact cells. Some of these studies are also reviewed below.

In general terms, the reactions of purine nucleotide catabolism are those of dephosphorylation, deamination, glycosidic bond

cleavage and oxidation. For this dissertation, the catabolic reactions present in mammalian cells will be emphasized; the reactions catalyzed by purine nucleoside kinases will also be examined here.

1. Studies of Individual Enzymes

a. Dephosphorylation

Nucleoside di- and triphosphates are dephosphorylated by enzymes of different specificities than those which dephosphorylate nucleoside monophosphates. In addition to the dephosphorylation of triphosphates by enzymes which utilize them as substrates (see Chapter IV), these compounds are dephosphorylated by inorganic pyrophosphatase, nucleoside triphosphatases and nucleoside diphosphatases. However, little information is available regarding the control of these enzymes.

Nucleoside monophosphates may be dephosphorylated to nucleosides by non-specific acid or alkaline phosphatases and by specific 5'-nucleotidases. Although little is known about the control of the non-specific phosphatases, 5'-nucleotidases from most mammalian tissues are subject to inhibition by purine and pyrimidine nucleosides. In addition, cardiac muscle 5'-nucleotidase is known to be inhibited competitively by ATP, GTP and ITP (Baer et al., 1966). Although the enzyme from sheep brain is strongly inhibited by ATP, UTP and CTP, GTP has no effect (Ipata, 1968). In addition, this preparation is not inhibited by purine or pyrimidine bases, nucleosides or 2'- or 3'-nucleotides.

b. Deamination

Enzymes exist in some tissues for the deamination of adenine and guanine, their nucleosides and their nucleotides. Most of these

deaminases are fairly specific and catalyze irreversible reactions. The enzymes responsible for the deamination of AMP and GMP have been examined in the previous chapter and that discussion will not be reiterated here.

Adenine deaminase, although present in plants and microorganisms, has not, to this reviewer's knowledge, been detected in mammalian cells. In contrast, guanine deaminase is widely distributed in nature. GTP activates the guanine deaminase from rat liver by reducing the K_m for guanine (Josan and Krishnan, 1968). Other than this effect, little is known about the metabolic control of this enzyme in cells.

Until recently, only indirect evidence for the existence of guanosine deaminase was available. This enzyme has been purified from Pseudomonas convexa and been shown to be inhibited by aminoimidazole-carboxamide ribonucleoside (Ishida et al., 1969). No reports of the regulation of the activity of this enzyme in mammalian cells are available at the present time, however.

Adenosine deaminase is widely distributed in plants, bacteria and animal cells. In spite of the large amount of research undertaken on this enzyme since its discovery, its actual role in cellular metabolism is unclear. In addition, very few studies on effectors of the activity of adenosine deaminase are in the literature. Inosine is known to be a product inhibitor of the reaction catalyzed by this enzyme and numerous nucleoside analogs are known to be inhibitory (cf Baker, 1967). Neither ATP nor GTP has any effect on this enzyme, however (Burger and Lowenstein, 1967).

c. Glycosidic Bond Cleavage

Cleavage of the glycosidic bond of purine nucleosides may theoretically take place either phosphorolytically or hydrolytically. Although purine ribonucleoside hydrolases have been detected in yeast and in bacteria, by far the most important reaction in mammalian tissues is the phosphorolytic cleavage of nucleosides.

Purine nucleoside phosphorylase catalyzes the reversible cleavage of purine nucleosides to purine bases and ribose-1-phosphate in the presence of P_i. Since, in partially purified preparations of this enzyme, nucleoside synthesis is favored over nucleoside cleavage (Friedkin and Kalckar, 1961), the coupling of nucleoside phosphorylase with nucleoside kinases (see below) was once thought to be an important route of nucleotide synthesis. However, evidence has accumulated against this belief and it is now believed that the major role of phosphorylase is catabolic.

Studies on the highly purified purine nucleoside phosphorylase from human erythrocytes have shown that hypoxanthine, guanine and xanthine, but not adenine, are substrates for nucleoside synthesis in the presence of either ribose-1-phosphate or deoxyribose-1-phosphate. In addition to these naturally occurring purine bases, certain purine analogs can act as substrates or inhibitors, and of these, 8-azaguanine, 6-mercaptopurine and allopurinol (4-hydroxypyrazolo(3,4-d)-pyrimidine) bind most tightly to the enzyme (Krenitsky et al., 1968). The enzyme from human erythrocytes is subject to substrate activation by inosine whereas that from bovine spleen is not (Agarwal and Parks, 1969).

d. Oxidation

Although the presence of xanthine oxidase in mammalian tissue has been known for more than sixty years, the mechanisms for the regulation of its activity are very complex and relatively obscure at the present time (cf. Knox and Greengard, 1963). This enzyme is inhibited by a wide range of purines, pteridines and other heterocyclic compounds which are oxidized very slowly or not at all by the enzyme. An interesting example of such compounds is the potent inhibitor allopurinol. Xanthine oxidase is also subject to inhibition by excess substrate.

That xanthine oxidase is important *in vivo* for the excretion of excess purines is well documented. In fact, Bergel *et al.* (1957) promote the view that xanthine oxidase may be one of the most important enzymes of purine metabolism and believe that it controls the general pool of biological purines by catabolizing hypoxanthine and xanthine to uric acid.

e. Nucleoside Kinases

Adenosine kinase, purified from mammalian tissues in 1967, has a wide specificity with respect to both the base and sugar moieties as well as a wide specificity with respect to the nucleoside triphosphate utilized as the phosphate donor (Lindberg *et al.*, 1967; Schnebli *et al.*, 1967; Murray, 1968). Although 6-mercaptopurine ribonucleoside and 2-fluoro-6-mercaptopurine ribonucleoside are not phosphorylated by this enzyme, they do inhibit adenosine phosphorylation. With the adenosine kinase from Ehrlich ascites tumor cells, AMP, GMP, IMP, ADP, dADP, IDP and 6-mercaptopurine ribonucleotide compete with ATP for binding to the enzyme (Murray, 1968). Apparently, the activity of this enzyme is regulated by the relative concentrations of AMP, ADP and ATP.

Until very recently, no direct evidence was found for the existence, in mammalian tissues, of kinases capable of phosphorylating inosine or guanosine. However, in 1967, Pierre et al. (1967) observed the phosphorylation of both inosine and guanosine in cell-free extracts of a subline of the Ehrlich ascites carcinoma which lacks hypoxanthine-guanine phosphoribosyltransferase activity. Recently, a partial purification of inosine kinase from these cells was reported (Pierre and LePage, 1968). No information is available as yet concerning the metabolic regulation of either of these kinases, however.

2. Studies of Integrated Systems

Overgaard-Hansen (1965), in a study of the transient exhaustion of the adenine nucleotide pool following addition of glucose to Ehrlich ascites tumor cells in vitro, obtained evidence that this depletion took place via the reaction sequence AMP → IMP → inosine → hypoxanthine rather than by the sequence AMP → adenosine → inosine → hypoxanthine. In rabbit erythrocytes in vitro, both adenine and guanine nucleotides appear to be catabolized via pathways which involve IMP (Hershko et al., 1967). These pathways involve deamination of AMP and GMP to IMP, reactions catalyzed by AMP deaminase and GMP reductase, respectively. The IMP so formed may be degraded to inosine and hypoxanthine, or converted to XMP which is subsequently degraded to xanthosine and xanthine.

The activities of various enzymes of nucleotide metabolism have been studied in extracts of rat brain by Santos et al. (1968). One aspect of this study showed that the activity of adenosine deaminase was more than 6-fold that of AMP deaminase. On the basis of these

observations, if one assumes that the dephosphorylation of nucleotides is not limiting for nucleotide catabolism and that no factors other than enzyme activities are responsible for controlling nucleotide catabolism, it may be postulated that AMP is degraded via the pathway AMP → adenosine → inosine → hypoxanthine in preference to a pathway involving AMP deaminase. That such assumptions are valid in intact cells is far from certain, however.

The complexities involved in defining the pathways of nucleotide catabolism and the regulation of these pathways are aptly demonstrated by the work of Burger and Lowenstein (1967). Apparently extracts of rat heart, like many other tissues, catabolize AMP by two pathways. The first involves deamination to yield IMP followed by dephosphorylation to yield inosine, while the second involves dephosphorylation to adenosine, followed by deamination to yield inosine. ATP activates the first and inhibits the second pathway since it activates AMP deaminase and inhibits AMP phosphatase. GTP however, reverses the action of ATP on AMP deaminase and also inhibits AMP phosphatase. Neither triphosphate inhibits or activates adenosine deaminase. In the absence of any triphosphate therefore, AMP is degraded mainly by the second pathway. When ATP is present, the first pathway is preferred. In the presence of GTP, AMP hydrolysis is inhibited regardless of the route followed. These authors feel that most other animal tissues possess both pathways of AMP degradation and postulate that any differences observed in the different tissues are probably due to the presence of the two pathways in different proportions. Although other aspects of these pathways appear to have been overlooked by Burger and Lowenstein, and although extracts rather than intact tissues are

used in their studies, this work illustrates the problems which must be considered in attempting to evaluate the diverse pathways of nucleotide catabolism.

In the present study, therefore, we have attempted to elucidate some of the pathways which exist in Ehrlich ascites tumor cells in vitro by which purine nucleotides are catabolized, and have attempted to define some of the rate-controlling reactions of these pathways. We have utilized studies similar to those outlined in the previous chapter of this dissertation in that we have followed the incorporation of radioactivity from exogenously-supplied ^{14}C-labelled purine bases into acid-soluble purine nucleotides, nucleosides and bases. The findings obtained in such experiments are outlined and discussed in the following sections.

B. Results and Discussion

1. Adenine-^{14}C as Precursor

The total conversion of adenine-^{14}C into nucleotides, nucleosides and bases is given in Fig. 52. Greater than 90% of the base is converted after 90 minutes of incubation even when the precursor concentration is 100 μM.

From previous results, it is apparent that adenine must be converted to nucleotides as the first step in its metabolism in Ehrlich ascites tumor cells in vitro. That very little adenine is degraded to hypoxanthine or utilized for adenosine synthesis may be observed from Fig. 53. In this figure, it is apparent that the synthesis of other purine nucleosides and bases is quite low during early times of incubation. At later times of incubation, however, these compounds are

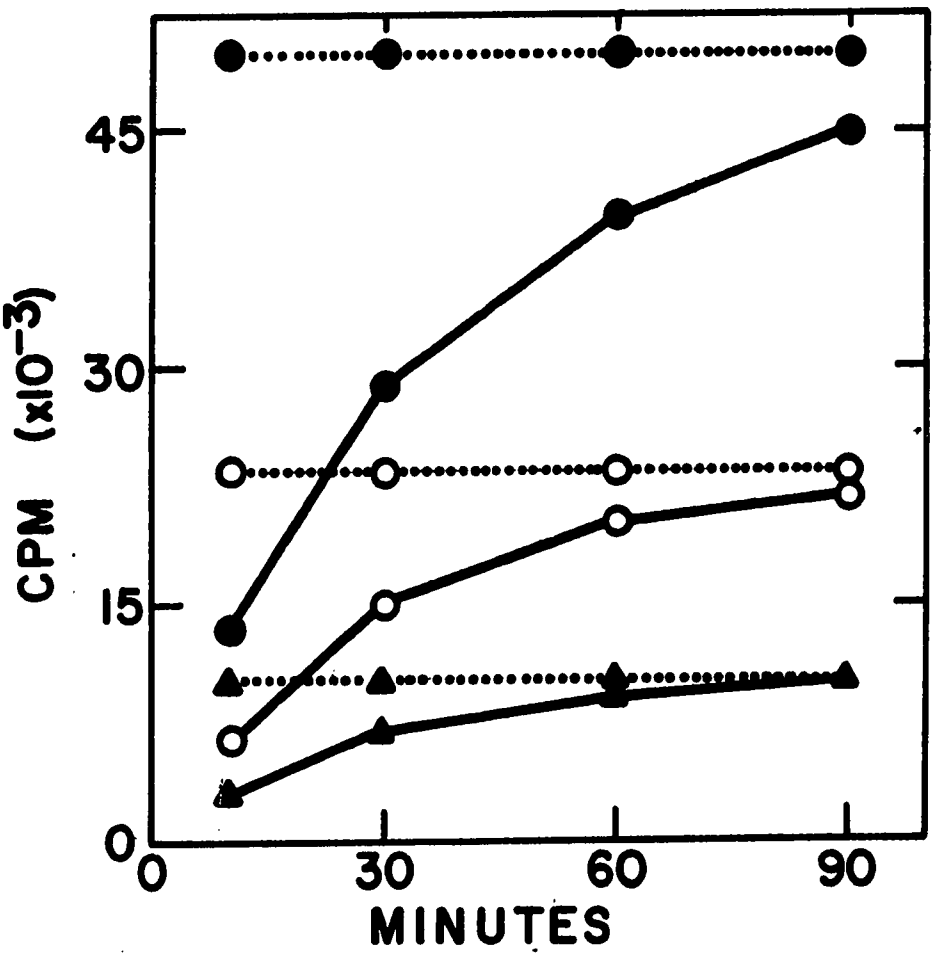


Figure 52. Metabolism of adenine-^{14}C.
Adenine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●
Total nucleotides plus nucleosides plus bases: ——
Total radioactivity: •••••••

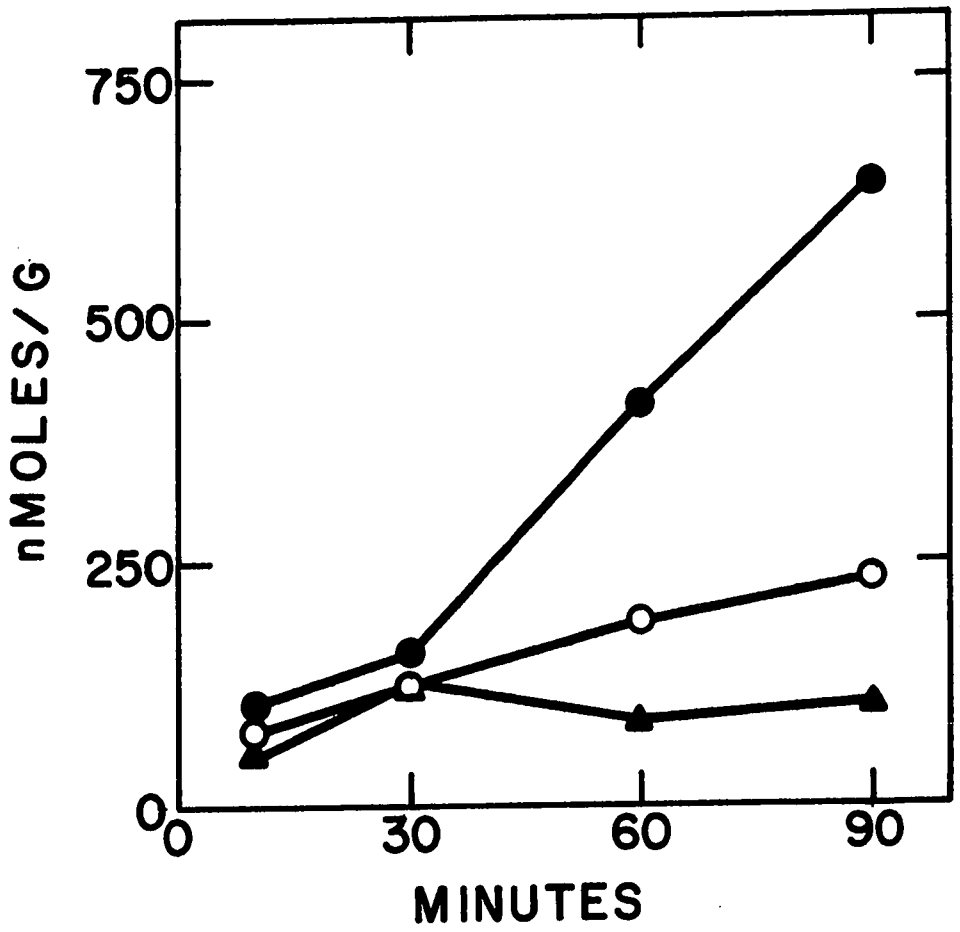


Figure 53. Conversion of adenine-^{14}C into nucleosides plus bases.
Adenine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

synthesized to a much greater extent. Presumably then, such compounds arise via breakdown of nucleotides formed from adenine-^{14}C rather than via metabolism of the adenine per se. These results are in accord with the observations of other investigators that (a) Ehrlich cells do not contain active adenine deaminase and (b) the purine nucleoside phosphorylase of Ehrlich cells cannot utilize adenine as a substrate for adenosine synthesis.

It should be noted however, that since Ehrlich cells do contain an active adenosine kinase, one might not observe the formation of adenosine via the phosphorylase reaction simply because the adenosine would be utilized for nucleotide synthesis immediately upon formation. In contrast, the action of adenosine deaminase on any adenosine so formed would be observed in the results diagrammed in Fig. 53 since this figure includes all purine nucleosides and bases (other than adenine) which accumulate.

The greatest amount of radioactivity found in the purine nucleoside plus base fraction is after 90 minutes when the initial adenine-^{14}C concentration is 100 μM. Even then, less than 20% of the total radioactivity present in the sample is found in these compounds. This fairly low level may reflect the low rates of degradation of adenine nucleotides caused by the rapid incorporation of the precursor into ATP, since only nucleoside monophosphates are good substrates for 5'-nucleotidase.

As noted above, AMP may be catabolized by two routes as follows:

Pathway 1 AMP → adenosine → inosine → hypoxanthine

Pathway 2 AMP → IMP → inosine → hypoxanthine

Since neither IMP nor adenosine accumulate when Ehrlich ascites tumor cells are incubated with adenine-^{14}C, the relative contributions made by each of these pathways for AMP degradation are difficult to determine.

Regardless of the preferred pathway for AMP catabolism in Ehrlich cells, inosine and hypoxanthine should be produced. Fig. 54 shows the accumulation of inosine plus hypoxanthine when cells are incubated with adenine-^{14}C (20, 50 and 100 μM). When the two lowest precursor concentrations are used, the formation of inosine plus hypoxanthine is quite low and does not appreciably increase with time. Although the accumulation of these compounds is also low at early incubation times with 100 μM precursor, this accumulation increases markedly during the incubation period.

If one assumes that AMP is degraded via Pathway 1, the accumulation of inosine plus hypoxanthine might be expected to be directly related to the precursor concentration. That is, as the adenine-^{14}C concentration increases from 20 to 50 to 100 μM, increases of 2.5 and 2.0-fold, respectively, the accumulation of inosine plus hypoxanthine should likewise increase 2.5 and 2.0-fold unless, of course, 5'-nucleotidase and adenosine deaminase become saturated with substrate or inhibited in some way, in which case the increases in inosine plus hypoxanthine accumulation would be lower than the increases in precursor concentration. However, it is apparent from Fig. 54, that as the adenine-^{14}C concentration increases 2.5 and 2.0-fold the corresponding increases in inosine plus hypoxanthine formation are about 2.8 and 3.8-fold after 60 minutes of incubation and 1.7 and 6.1-fold after 90 minutes of incubation.

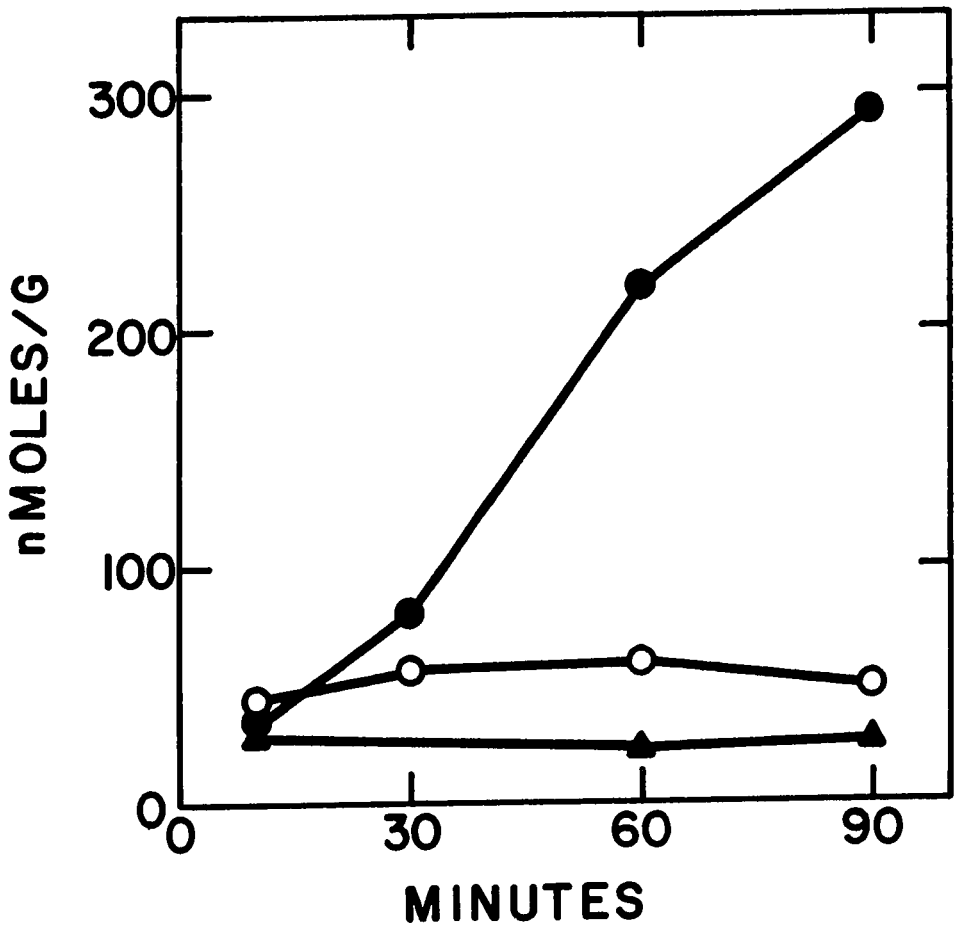


Figure 54. Formation of inosine plus hypoxanthine from adenine-^{14}C.
Adenine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

The unexpected accumulations of inosine plus hypoxanthine observed when adenine-^{14}C concentration is increased from 50 to 100 μM may be explained if the AMP is catabolized via the reaction sequence of Pathway 2. As precursor concentration increases, AMP formation increases and consequently more IMP is formed. The IMP so produced may be degraded to inosine and hypoxanthine or utilized for GMP synthesis. As noted in the previous chapter, the conversion of IMP to GMP takes place at low rates in Ehrlich cells incubated under the conditions used in these experiments. Although the XMP aminase reaction is rate-limiting for this conversion, the reaction catalyzed by IMP dehydrogenase may become saturated with substrate if IMP is not being utilized for AMP synthesis. Therefore, as more IMP is formed by the deamination of AMP, the IMP dehydrogenase reaction might become saturated by IMP and consequently more inosine and hypoxanthine would be produced. These effects would, of course, be most marked at high substrate concentrations.

Since the XMP aminase reaction limits the formation of GMP from IMP, the accumulation of xanthosine (xanthylate does not accumulate under these conditions) should show similar marked effects with increased precursor concentration to those observed on inosine plus hypoxanthine accumulation. Fig. 55 shows, in fact, that when the adenine-^{14}C concentration is increased from 50 to 100 μM, a 2.0-fold increase, the accumulation of xanthosine is increased 2.7-fold after 60 minutes of incubation and 3.2-fold after 90 minutes.

These observations are consistent with the belief that the major route of AMP degradation in Ehrlich ascites tumor cells <u>in vitro</u> is via Pathway 2, i.e.:- AMP → IMP → inosine → hypoxanthine. However,

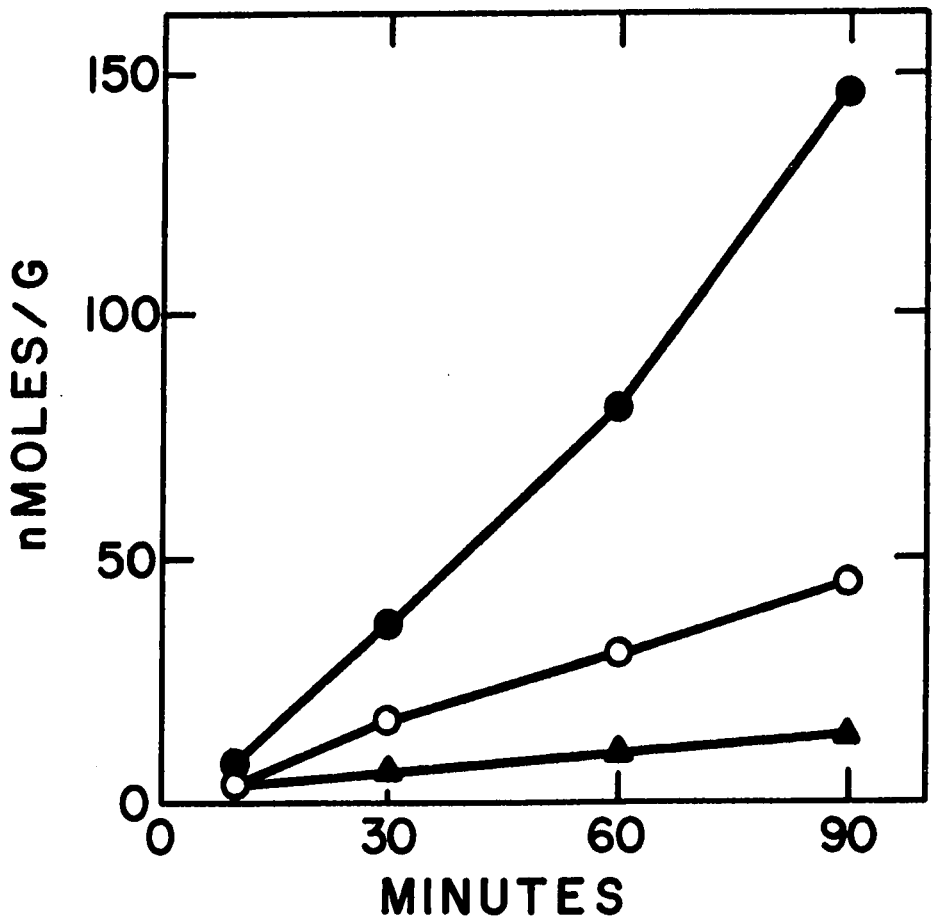


Figure 55. Formation of xanthosine from adenine-^{14}C.
Adenine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

one cannot state with certainty that the alternate pathway for AMP degradation, i.e.:- AMP → adenosine → inosine → hypoxanthine, is not operative to some extent in these cells.

If AMP is catabolized via Pathway 2, the fact that IMP does not accumulate in these experiments seems to indicate that the dephosphorylation of IMP is not the rate-limiting step in this reaction sequence. Although inosine does accumulate to a small extent when the adenine-^{14}C concentration is 100 μM, the accumulation of hypoxanthine is much greater (Fig. 56). Therefore, the rate of the purine nucleoside phosphorylase reaction does not appear to limit the degradation of AMP to hypoxanthine. Presumably then, the reaction catalyzed by AMP deaminase is rate-limiting for this sequence of reactions. It is interesting to note that some AMP does accumulate (Chapter IV) under the conditions used in these experiments.

The utilization of adenine for the formation of adenine nucleotides and other nucleotides and the relative amounts of catabolism of these compounds are difficult to define without more precise knowledge of the degradative pathways. The relatively small role played by the catabolic pathways in these experiments may be illustrated by the following relationship. If one assumes that all of the degradation of adenine nucleotides takes place through the formation of adenosine and if all of the IMP formed from AMP is used for XMP synthesis (i.e.:- none of the inosinate is degraded), when Ehrlich ascites tumor cells have been incubated with adenine-^{14}C (initial concentration 100 μM) for 30 minutes, the following relationship is apparent:

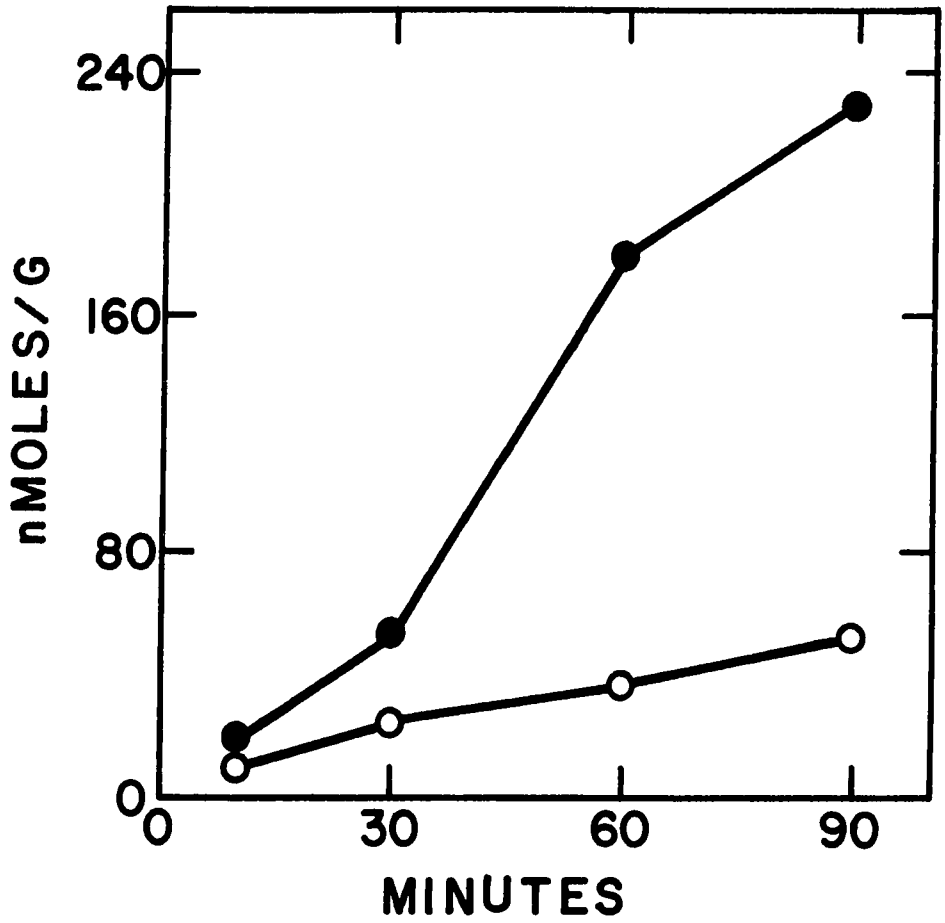
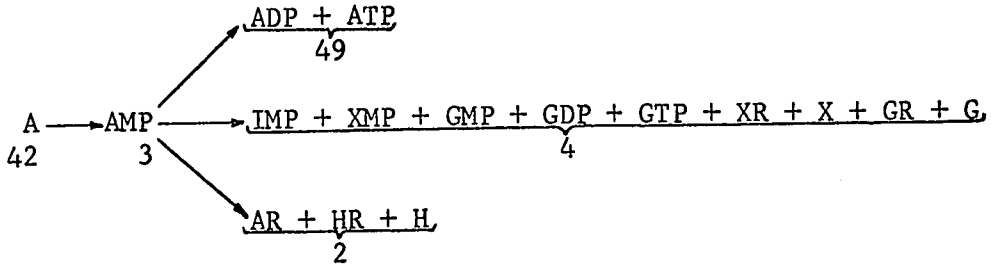


Figure 56. Relative formation of hypoxanthine and inosine from adenine-^{14}C.
Hypoxanthine: ●
Inosine: ○
Adenine-^{14}C: 100μM.



with the figures representing the percentage of total radioactivity contributed by the different compounds. The roles played by the catabolic reactions would become more evident at longer times of incubation.

2. Guanine-^{14}C Precursor

Fig. 57 illustrates the conversion of guanine-^{14}C into acid-soluble purine nucleotides, nucleosides and bases as a function of incubation time. It is apparent that the incorporation of radioactivity from the precursor is almost complete after 90 minutes of incubation when the guanine-^{14}C concentration is either 20 or 50 μM. At a level of 100 μM, 82% of the base is converted after 90 minutes.

Conversion of radioactivity from guanine-^{14}C into other purine bases and nucleosides may take place by (a) direct conversion of guanine into these compounds and (b) formation of nucleotides from guanine followed by degradation of the nucleotides into nucleosides and bases. The formation of purine bases and nucleosides from the precursor is shown in Fig. 58. Although by far the greatest amount of radioactivity is present in these compounds after 90 minutes of incubation, significant amounts are present at the early incubation times regardless of the initial guanine-^{14}C concentration. In fact, for all three concentrations of guanine-^{14}C used, about one-third of the amount of radioactivity present in the purine bases and nucleosides after 90 minutes

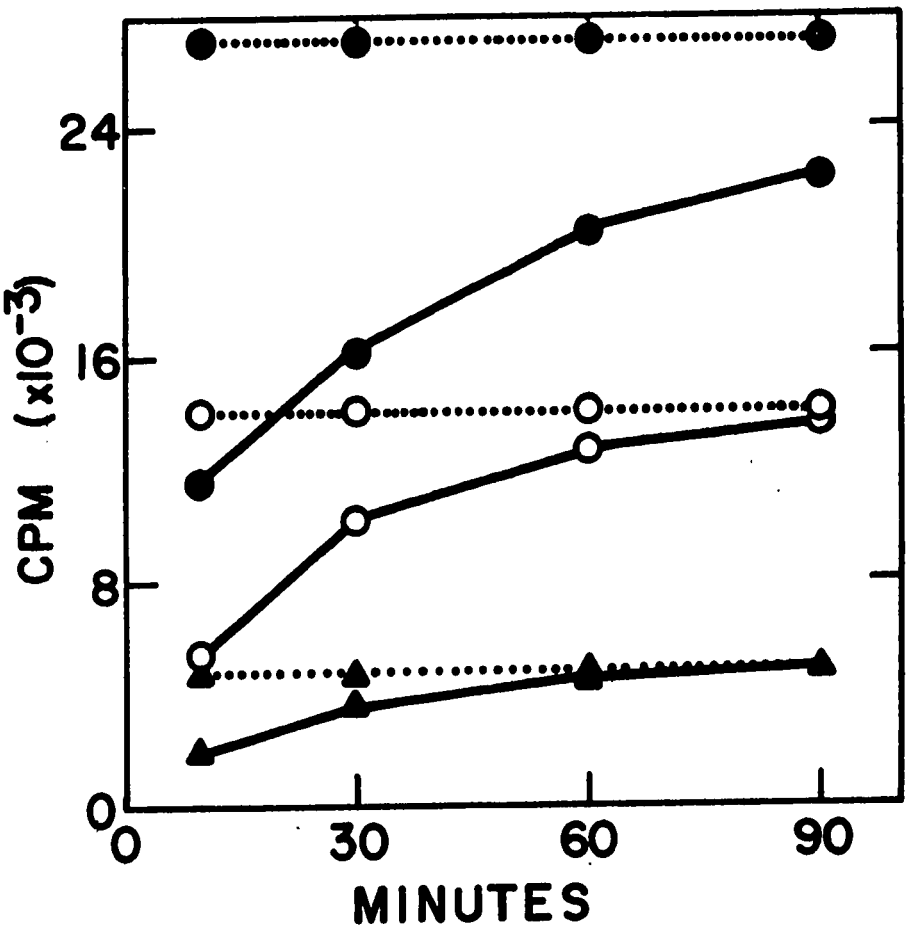


Figure 57. Metabolism of guanine-^{14}C.
Guanine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●
Total nucleotides plus nucleosides plus bases: ——
Total radioactivity: ••••••

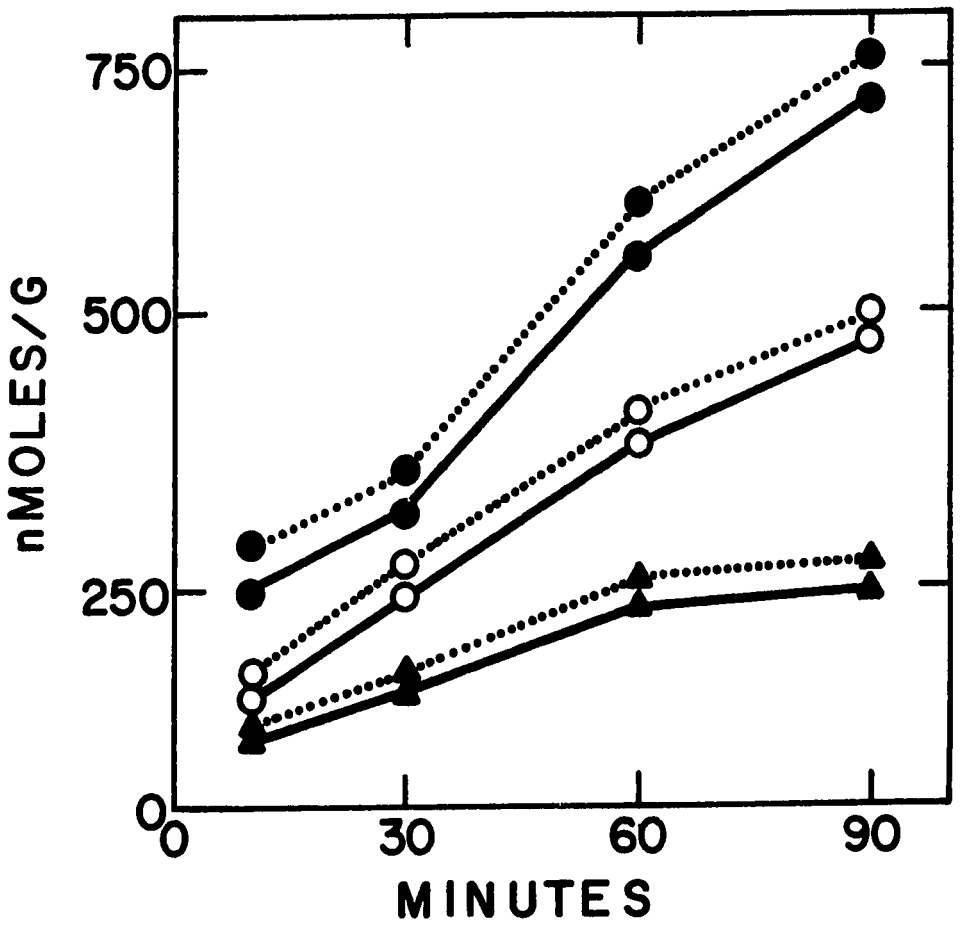


Figure 58. Formation of nucleosides plus bases from guanine-^{14}C.
Total nucleosides plus bases: ••••••••
X + XR + GR + UA: ——
Guanine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

of incubation is present after only 10 minutes. These fairly large amounts of purine bases and nucleosides present at early times in the incubation period presumably indicate the conversion of significant amounts of guanine-^{14}C into these compounds directly, without prior conversion to the nucleotide level.

Since the conversion of guanine nucleotides into IMP and adenine nucleotides appears to be very low in Ehrlich ascites tumor cells in vitro (Chapter V), almost all of the radioactivity which appears in purine nucleosides and bases during incubation with guanine-^{14}C should be found in catabolites of guanine nucleotides and metabolites of guanine itself. When one examines the formation of xanthosine, xanthine, guanosine and uric acid, it is apparent (Fig. 58) that such is the case.

On closer examination of the distribution of radioactivity from guanine-^{14}C, one finds that the amount of xanthosine which accumulates is negligible at all time periods examined, even when the precursor concentration is 100 μM (Fig. 59). This observation indicates that the formation of xanthosine from guanosine mediated by guanosine deaminase is very low or that the degradation of xanthosine to xanthine by purine nucleoside phosphorylase is so rapid that no xanthosine accumulation is detectable. However, as noted in Chapter V, xanthosine does accumulate when hypoxanthine-^{14}C is used as precursor (see also the following section). Therefore, the fact that no xanthosine accumulates when guanine-^{14}C is the precursor indicates the guanosine deaminase activity is negligible in Ehrlich ascites tumor cells in vitro.

Another possible reason for the lack of xanthosine formation during incubation with guanine-^{14}C does exist however. If the activity

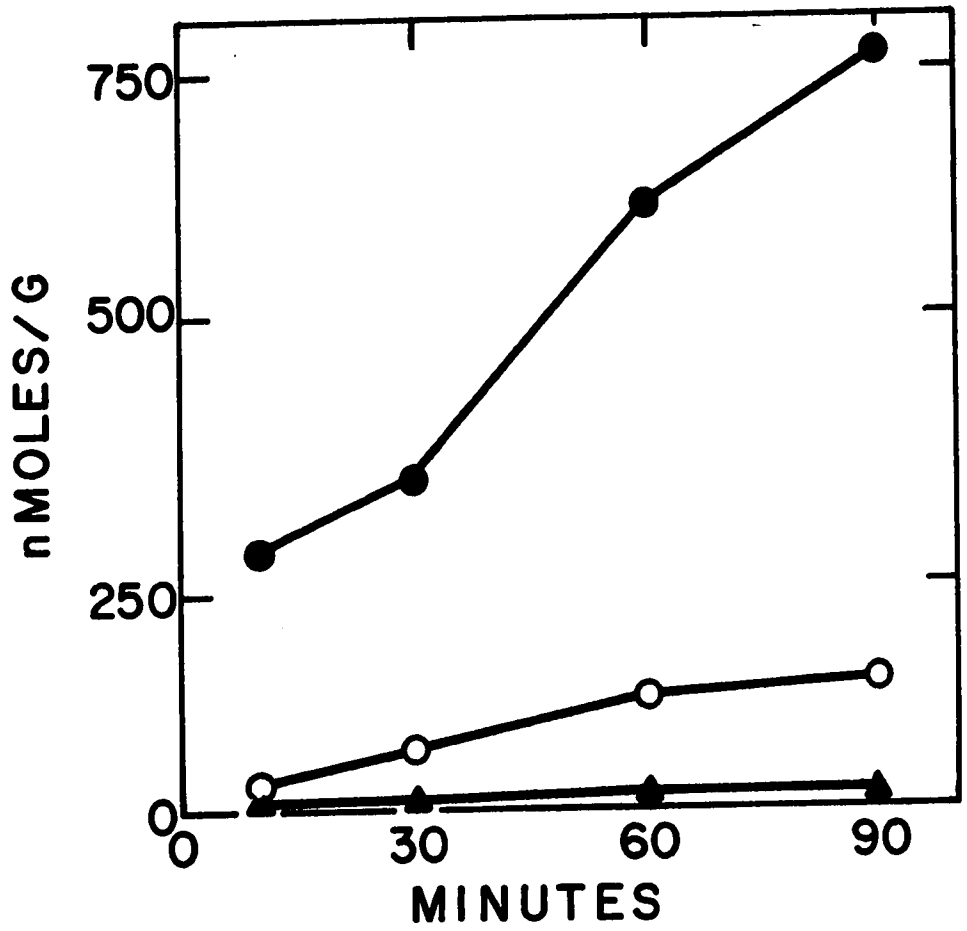


Figure 59. Formation of guanosine and xanthosine from guanine-^{14}C.
Total nucleosides plus bases: ●
Guanosine: ○
Xanthosine: ▲
Guanine-^{14}C: 100μM.

of purine nucleoside phosphorylase is much greater than that of guanosine deaminase, guanosine might be cleaved to guanine immediately upon formation. This reaction would result in very low amounts of guanosine present as a substrate for guanosine deaminase. However, Fig. 59 illustrates that a small amount of guanosine does accumulate, at least when the precursor concentration is initially 100 μM.

The small amounts of guanosine which accumulate in these experiments may arise from either degradation of guanine nucleotides or from guanine directly by the reaction catalyzed by nucleoside phosphorylase. However, as can be seen in Fig. 59, the formation of guanosine is very low at early times in the incubation period when the amount of precursor guanine is still very high (about 60% of the total radioactivity after 10 minutes incubation - Chapter III) and guanosine only accumulates at longer incubation times. Presumably then, the guanosine formed when Ehrlich ascites tumor cells are incubated with guanine-^{14}C arises from the degradation of guanine nucleotides formed with the precursor and not from the action of purine nucleoside phosphorylase on the precursor itself.

Xanthine is by far the greatest contributor to the purine base plus nucleoside fraction when guanine-^{14}C is the precursor. Fig. 60 illustrates that xanthine itself contains more than 50% of the radioactivity incorporated into this fraction. Xanthine may be formed directly from the precursor guanine by the action of guanine deaminase or it may be formed by the degradation of nucleotides initially synthesized from guanine. It seems fairly evident that xanthine is produced via both routes in these cells. At early times in the incubation procedure, most of the xanthine probably arises from the action of

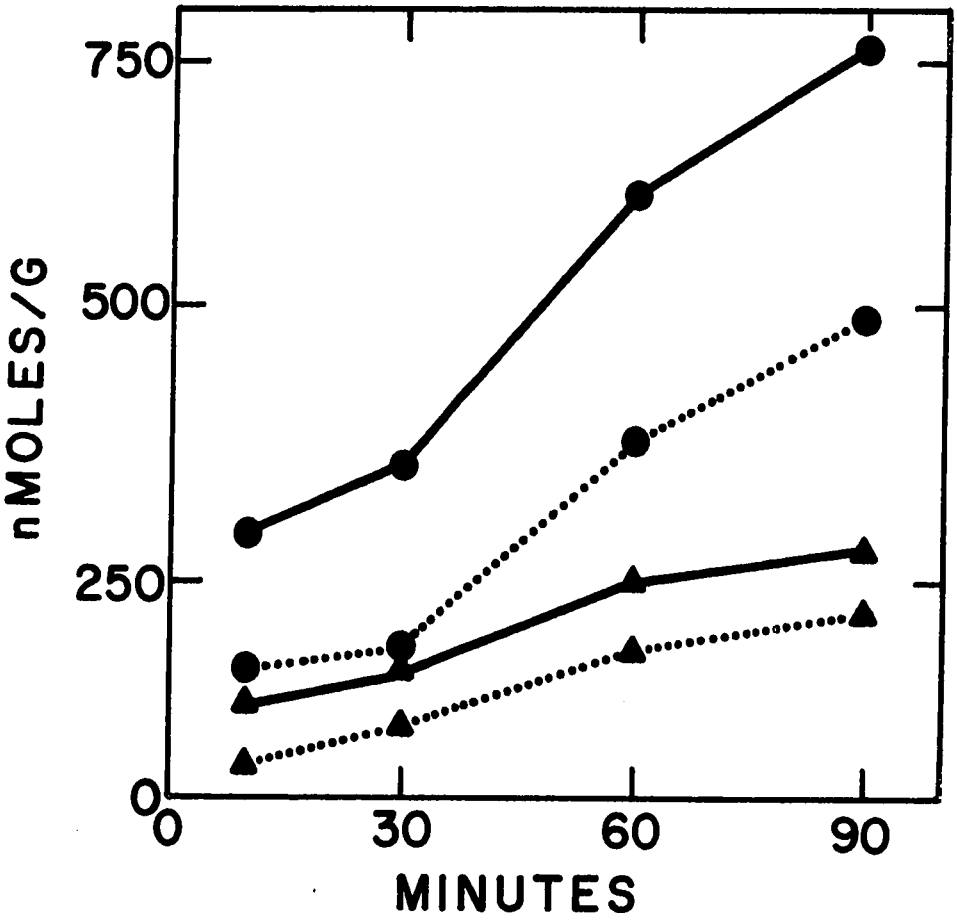


Figure 60. Formation of xanthine from guanine-^{14}C.
Total nucleosides plus bases: ——
Xanthine: ••••••••
Guanine-^{14}C- 20μM: ▲
100μM: ●

guanine deaminase on guanine-^{14}C whereas at later times in the incubation period, degradation of guanine nucleotides is responsible for most of the xanthine which accumulates.

Uric acid is synthesized at very low rates when guanine-^{14}C is precursor (Fig. 61) indicating that the reaction catalyzed by xanthine oxidase is slow in Ehrlich ascites tumor cells in vitro. Support for this belief is also evident in the observation that hypoxanthine accumulates when adenine-^{14}C is used as precursor (see above).

Since GMP does not accumulate under these incubation conditions (see Chapter IV), the dephosphorylation of GMP does not limit the catabolism of guanine nucleotides. The fact that some guanosine does accumulate seems to indicate that the reaction catalyzed by purine nucleoside phosphorylase is saturated by substrate under these conditions and therefore may be limiting for the degradation of guanine nucleotides to xanthine.

3. Hypoxanthine-^{14}C as Precursor

The conversion of hypoxanthine-^{14}C into other acid-soluble purine compounds is given in Fig. 62. When the precursor concentration is 20 μM, this conversion is almost complete after 90 minutes of incubation. In contrast, almost 30% of the hypoxanthine-^{14}C is unconverted after 90 minutes when the precursor concentration is 100 μM. Nevertheless, the amount of hypoxanthine converted to other purine compounds is much greater when the precursor is present at a concentration of 100 μM as compared to 50 μM. Since nucleotide synthesis from hypoxanthine-^{14}C is independent of precursor concentration above 50 μM (Chapter III), some of the hypoxanthine-^{14}C appears to be utilized as a substrate for

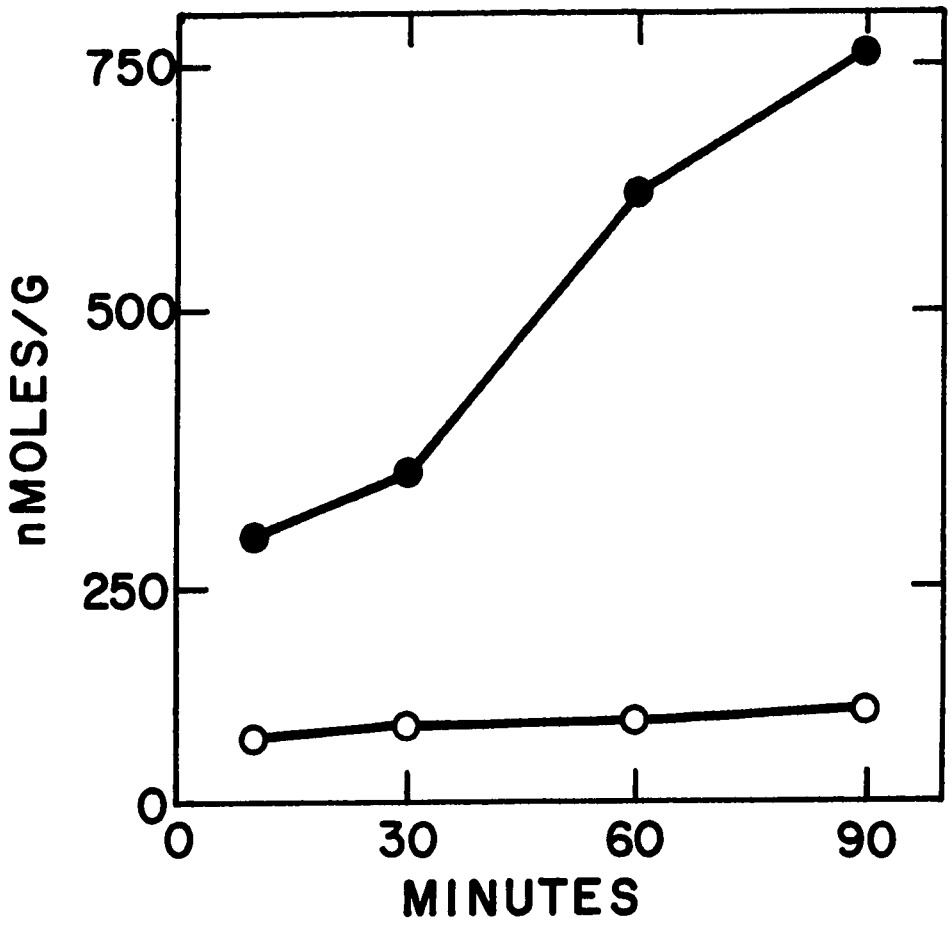


Figure 61. Formation of uric acid from guanine-^{14}C.
Total nucleosides plus bases: ●
Uric acid: ○
Guanine-^{14}C: 100μM.

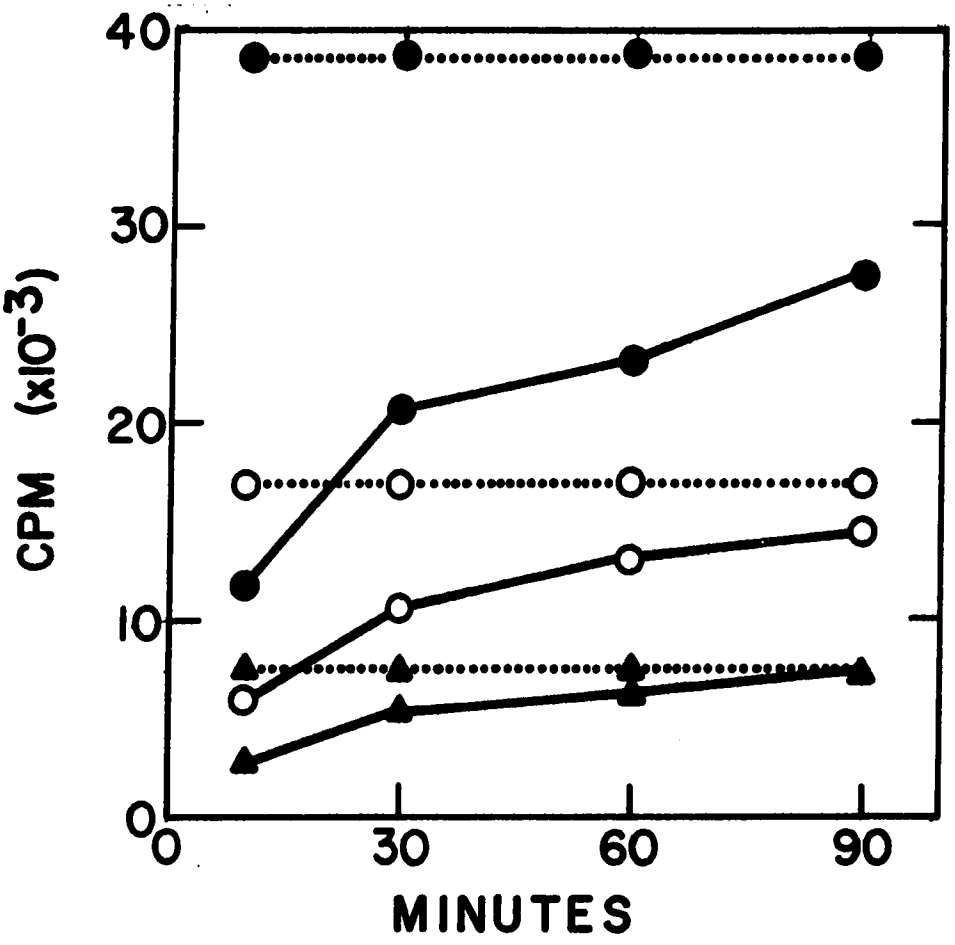


Figure 62. Metabolism of hypoxanthine-^{14}C.
Hypoxanthine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●
Total nucleotides plus nucleosides plus bases: ——
Total radioactivity: ••••••

reactions other than that catalyzed by hypoxanthine-guanine phosphoribosyltransferase.

Fig. 63 illustrates the formation of purine nucleosides and bases from hypoxanthine-^{14}C. Although the accumulations of these compounds are greatest after 90 minutes of incubation regardless of initial precursor concentration, about 50% of the amounts of these compounds found after 90 minutes are present after only 10 minutes of incubation. This observation indicates that significant amounts of purine nucleosides and bases are being formed directly from the precursor before it is incorporated into nucleotides. Of major interest in this regard are the compounds inosine and xanthine.

The accumulations of inosine during incubation of Ehrlich cells with three concentrations of hypoxanthine-^{14}C are shown in Fig. 64. The marked accumulations of inosine which occur at early times in the incubation period are presumably due to the action of purine nucleoside phosphorylase on the precursor base. This belief is supported by the fact that the increase in inosine formation when the precursor concentration is increased 2-fold (from 50 to 100 μM) is greater than can be accounted for by the increase in hypoxanthine-^{14}C concentration. Since the phosphoribosyltransferase is saturated with substrate when the hypoxanthine concentration is greater than 50 μM, the additional hypoxanthine-^{14}C present when the concentration is raised from 50 to 100 μM might then be available for the synthesis of inosine via the phosphorylase reaction.

It is also apparent from Fig. 64 that maximum accumulation of inosine takes place after about 30 minutes of the incubation period has elapsed. The most obvious explanation for this phenomenon is that

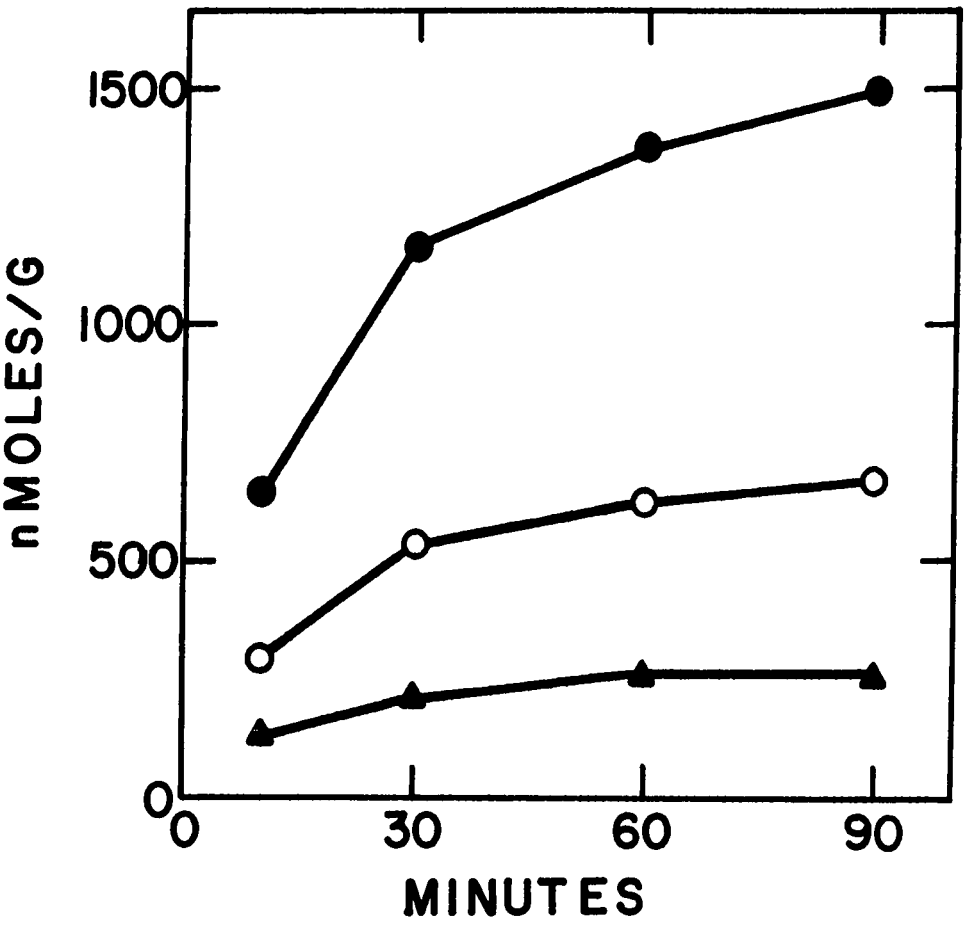


Figure 63. Formation of nucleosides plus bases from hypoxanthine-^{14}C.
Hypoxanthine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

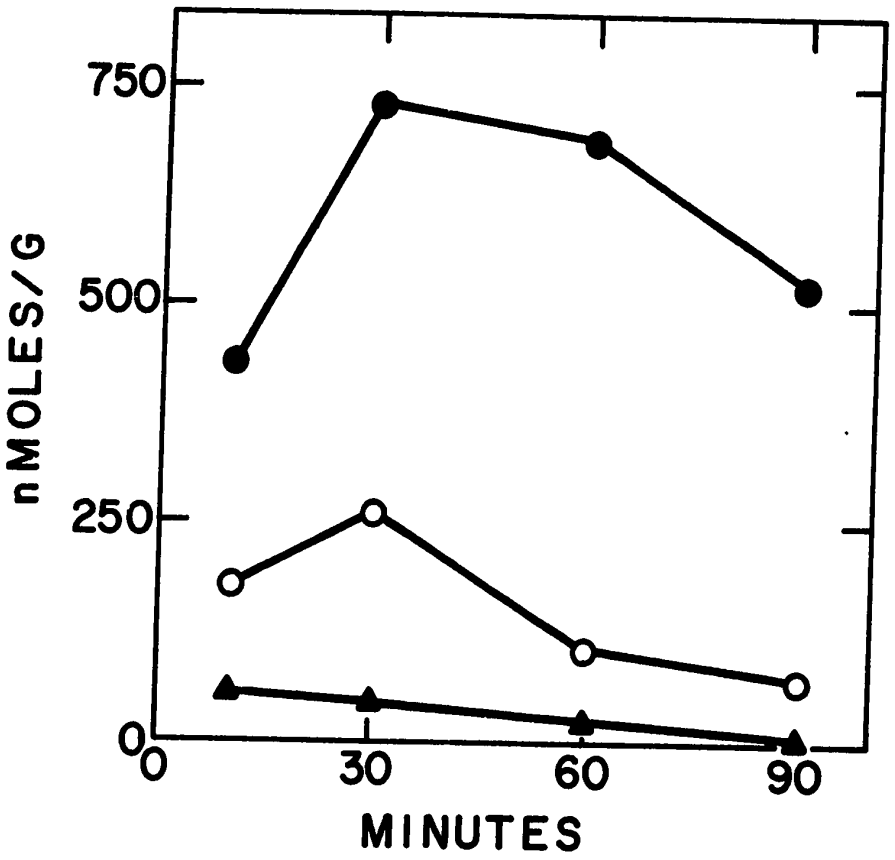


Figure 64. Formation of inosine from hypoxanthine-^{14}C.
Hypoxanthine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

since inosine may be synthesized both by the action of nucleoside phosphorylase on hypoxanthine and by the degradation of adenine nucleotides and IMP, perhaps after 30 minutes of the incubation period has elapsed, the synthesis of inosine by both pathways is sufficiently high to give maximum accumulation of inosine. Before 30 minutes, when the hypoxanthine-^{14}C concentrations are relatively high, most of the inosine is probably produced via the phosphorylase reaction. After 30 minutes, most of the inosine which accumulates is presumably due to the degradation of nucleotides; the phosphorylase reaction may then have a catabolic, instead of its earlier anabolic, role.

The activity of inosine kinase appears to be quite low in these cells since large accumulations of inosine can occur. If, by analogy to adenosine kinase, inosine kinase is subject to product inhibition, the activity of inosine kinase might be masked since IMP is being produced from the precursor hypoxanthine at the same time that inosine is being formed.

In addition to inosine, xanthine may also be produced from hypoxanthine-^{14}C directly, by the action of xanthine oxidase. The time courses of xanthine formation are given in Fig. 65. The fact that xanthine accumulation is very low at early times in the incubation period and the fact that xanthine accumulation after 10 minutes of incubation is independent of initial hypoxanthine-^{14}C concentration would seem to indicate that very little xanthine is produced from hypoxanthine-^{14}C via the xanthine oxidase reaction. Accordingly, xanthine must be formed by the action of purine nucleoside phosphorylase on xanthosine, the dephosphorylation product of XMP, and/or by the action of guanine deaminase on guanine, a catabolite of guanine nucleotides.

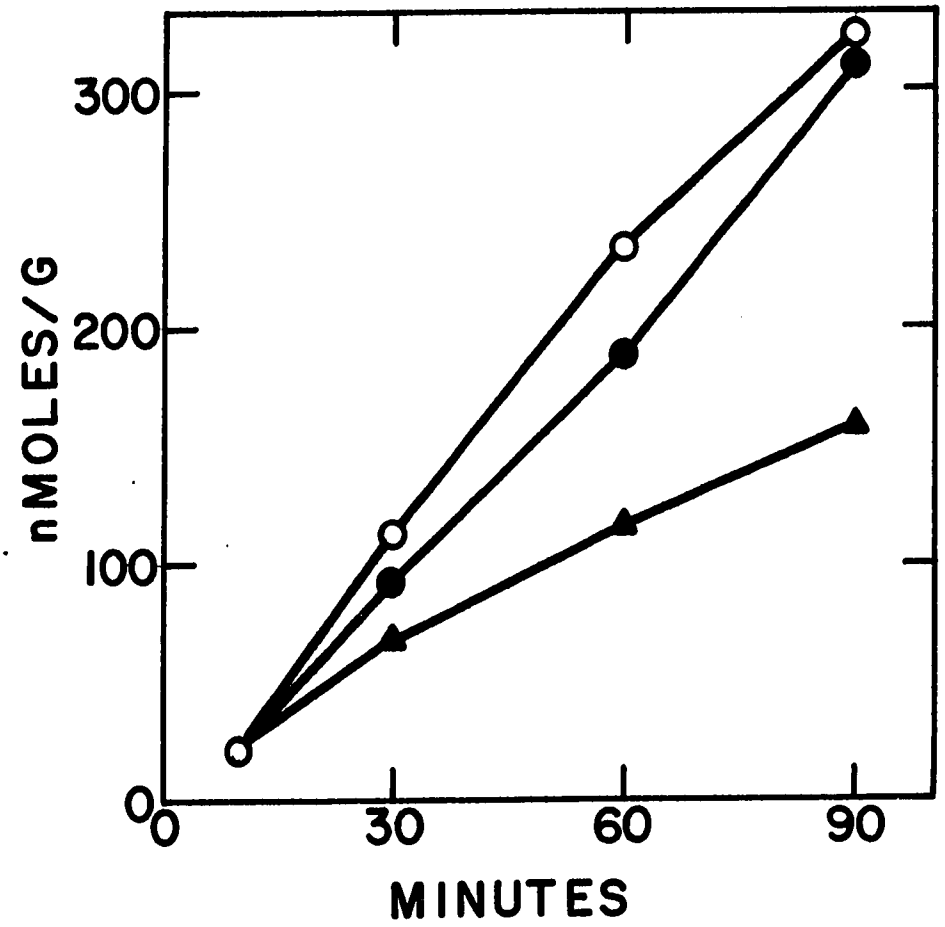


Figure 65. Formation of xanthine from hypoxanthine-^{14}C.
Hypoxanthine-^{14}C- 20μM: ▲
50μM: ○
100μM: ●

Fig. 66 shows the formation of xanthine from hypoxanthine-^{14}C when glutamine or DON is present in the incubation medium. If xanthine is being formed solely from the degradation of guanine nucleotides, when DON is present in the incubation medium, very little xanthine should be produced, and in the presence of glutamine, the formation of xanthine should exceed control values. However, it is apparent from Fig. 66 that this relationship is completely reversed since xanthine accumulation in the presence of DON equals control values whereas xanthine production in the presence of glutamine is less than 20% of controls. Therefore, the major portion of the xanthine which accumulates when Ehrlich ascites tumor cells are incubated under control conditions with hypoxanthine-^{14}C appears to be formed from the action of purine nucleoside phosphorylase on xanthosine.

Support for this hypothesis may be obtained from Fig. 67 which illustrates the formation of xanthosine under the same conditions as outlined above for xanthine. In general, the accumulation of xanthosine roughly parallels that of xanthine. Since no xanthosine accumulates in the presence of glutamine, the small amount of xanthine which accumulates under these conditions may arise from the degradation of guanine nucleotides. Alternatively, the nucleoside phosphorylase may be sufficiently active to cleave all of the xanthosine formed in the presence of glutamine. In the presence of DON, xanthosine accumulation exceeds that of controls whereas accumulation of xanthine does not exceed but rather equals control values. This observation may indicate that the purine nucleoside phosphorylase is cleaving xanthosine at maximum rates under control conditions.

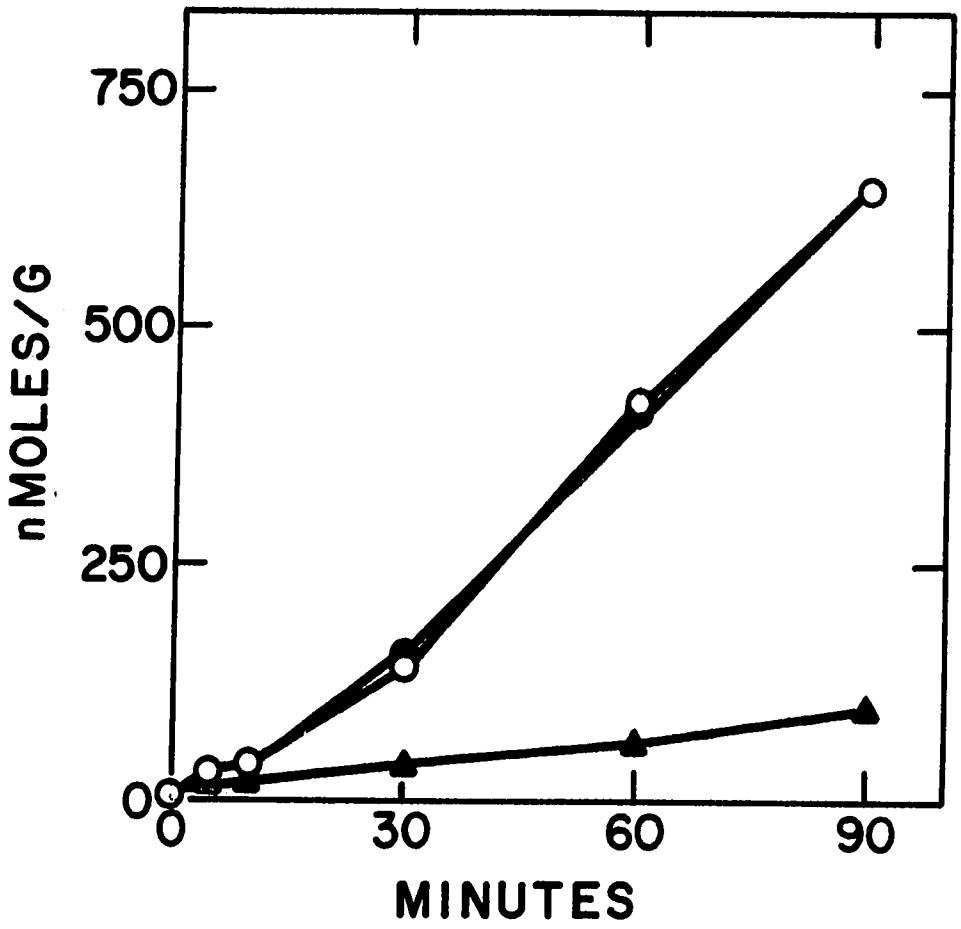


Figure 66. Effects of DON and glutamine on xanthine formation from hypoxanthine-^{14}C.

DON: 0: ●
35μM: ○
Glutamine: 0: ●
2mM: ▲
Hypoxanthine-^{14}C: 100μM.

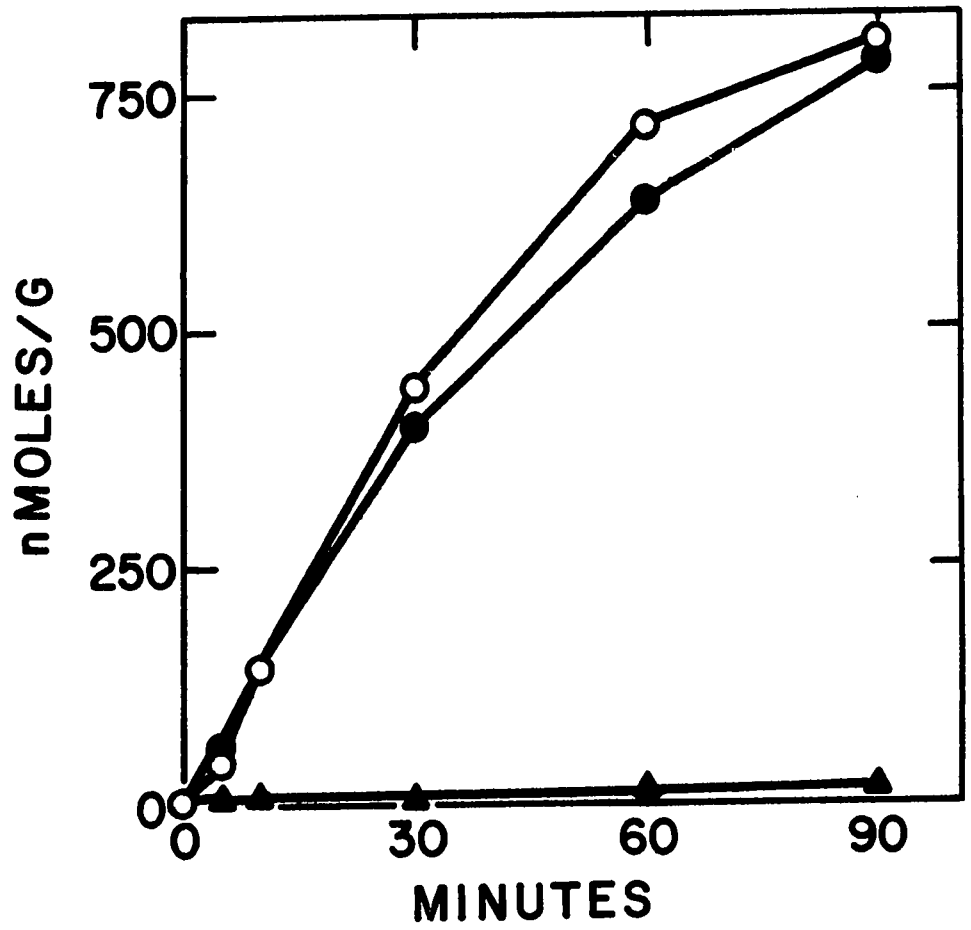


Figure 67. Effects of DON and glutamine on xanthosine formation from hypoxanthine-^{14}C.

DON: 0: ●
35μM: ○
Glutamine: 0: ●
2mM: ▲
Hypoxanthine-^{14}C: 100μM.

The dephosphorylation of nucleotides is not limiting for the degradation of IMP or XMP under control conditions since neither of these compounds accumulate. Since both inosine and xanthosine do accumulate however, the rates of the reactions catalyzed by purine nucleoside phosphorylase appear to limit the degradation of these nucleotides to their constituent bases. The formation of xanthine and uric acid from hypoxanthine derived from IMP and of uric acid from xanthine derived from XMP appear to take place quite slowly by virtue of the low rates of the xanthine oxidase reaction in these cells.

When the incubation medium (Krebs-Ringer) is replaced by Fischer's medium or supplemented by 10% horse serum, the effects on the accumulation of purine nucleosides and nucleotides are quite marked when Ehrlich cells are incubated with hypoxanthine-^{14}C. The effects of replacing the Krebs-Ringer solution with Fischer's medium are quite predictable on the basis of the results obtained when glutamine is added to Krebs-Ringer solution. The accumulation of xanthosine plus xanthine is reduced over 90% (Fig. 68). The small amount of these compounds which does accumulate when Fischer's medium is used, is probably contributed entirely by xanthine since xanthine, but not xanthosine accumulates in the presence of glutamine (compare Figs. 66, 67 and 68). The formation of inosine is also reduced in Fischer's medium as compared to control values (Fig. 69) as is the utilization of hypoxanthine-^{14}C (Fig. 70). Presumably, these effects can be ascribed to the presence of glutamine in Fischer's medium (see above).

Although addition of horse serum to Krebs-Ringer phosphate has little effect on the total accumulation of xanthosine plus xanthine

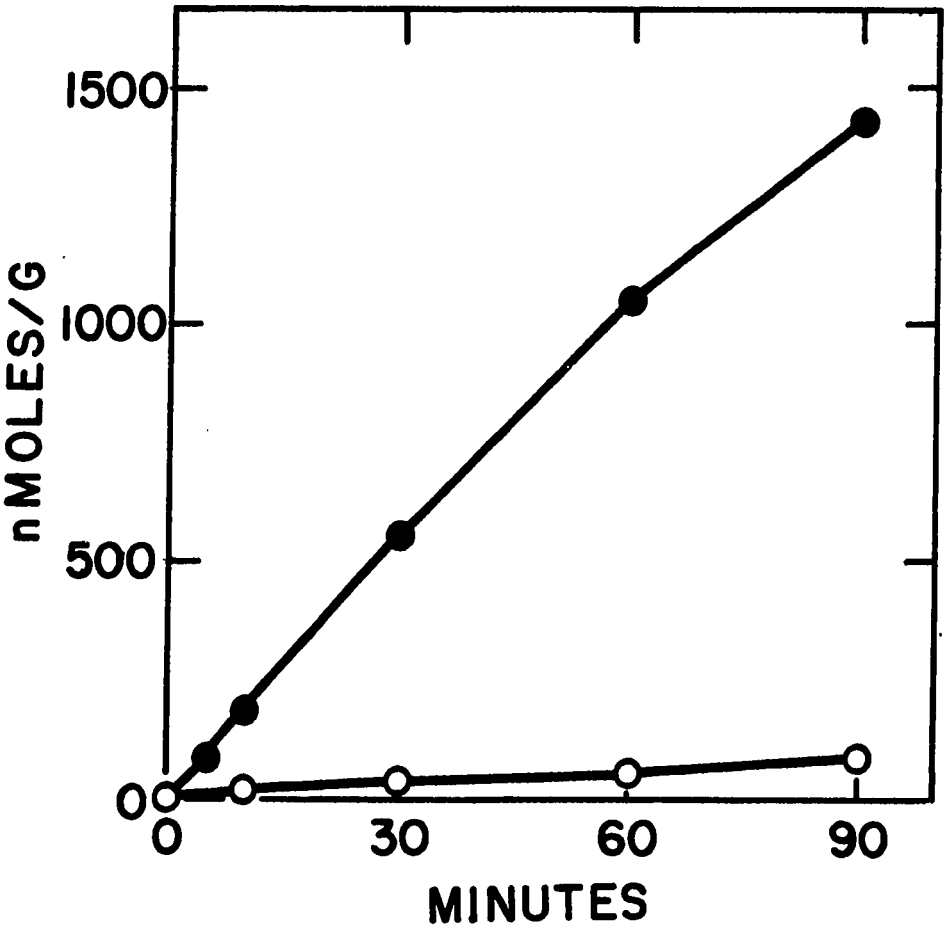


Figure 68. Comparison of xanthosine plus xanthine synthesis from hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

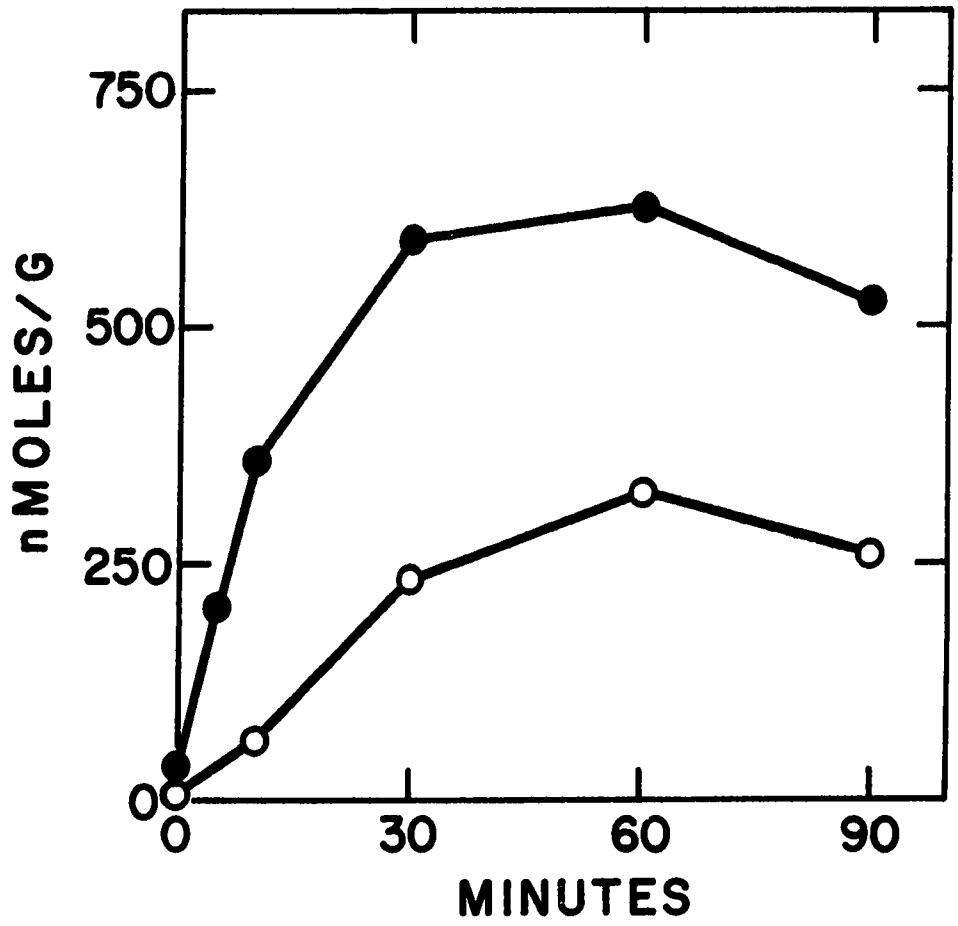


Figure 69. Comparison of inosine synthesis from hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

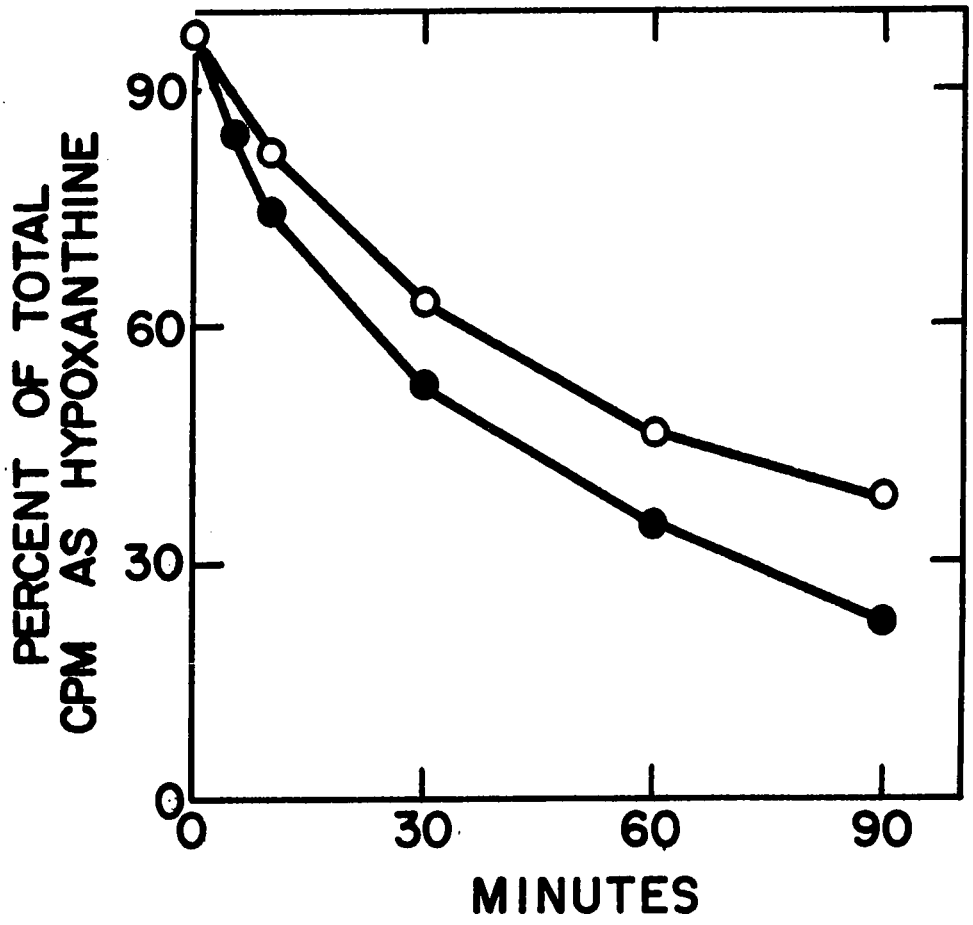


Figure 70. Total metabolism of hypoxanthine-^{14}C in different media.
Krebs-Ringer medium: ●
Fischer's medium: ○
Hypoxanthine-^{14}C: 100μM.

(Fig. 71), the relative formations of these two compounds are greatly altered (Fig. 72). In the absence of serum, the accumulation of xanthine is about 80% that of xanthosine. However, when horse serum is added, the accumulation of xanthosine is about twice that of xanthine after 90 minutes of incubation. These observations are difficult to explain at the present time.

C. Summary

The major route of adenine nucleotide catabolism in Ehrlich ascites tumor cells in vitro involves deamination of AMP to IMP, dephosphorylation of IMP to inosine and cleavage of inosine to hypoxanthine. The rate of the AMP deaminase reaction may limit this process although the reaction catalyzed by purine nucleoside phosphorylase also takes place at low rates.

The degradation of guanine nucleotides takes place via the sequence of reactions catalyzed by 5'-nucleotidase, purine nucleoside phosphorylase and guanine deaminase with the phosphorylase reaction being the slowest step. Guanosine deaminase activity appears to be low or absent in these cells.

With hypoxanthine-^{14}C as precursor, the synthesis of inosine reaches maximum values at about 30 minutes. The formation of inosine from the precursor base appears to take place readily. The dephosphorylation of nucleotides does not appear to limit the rates of their catabolism in Ehrlich ascites tumor cells, but the rates of the phosphorylase reactions may do so. Large amounts of xanthine accumulate with hypoxanthine as precursor; the majority of this accumulation takes place via the cleavage of xanthosine. The rate of the xanthine oxidase

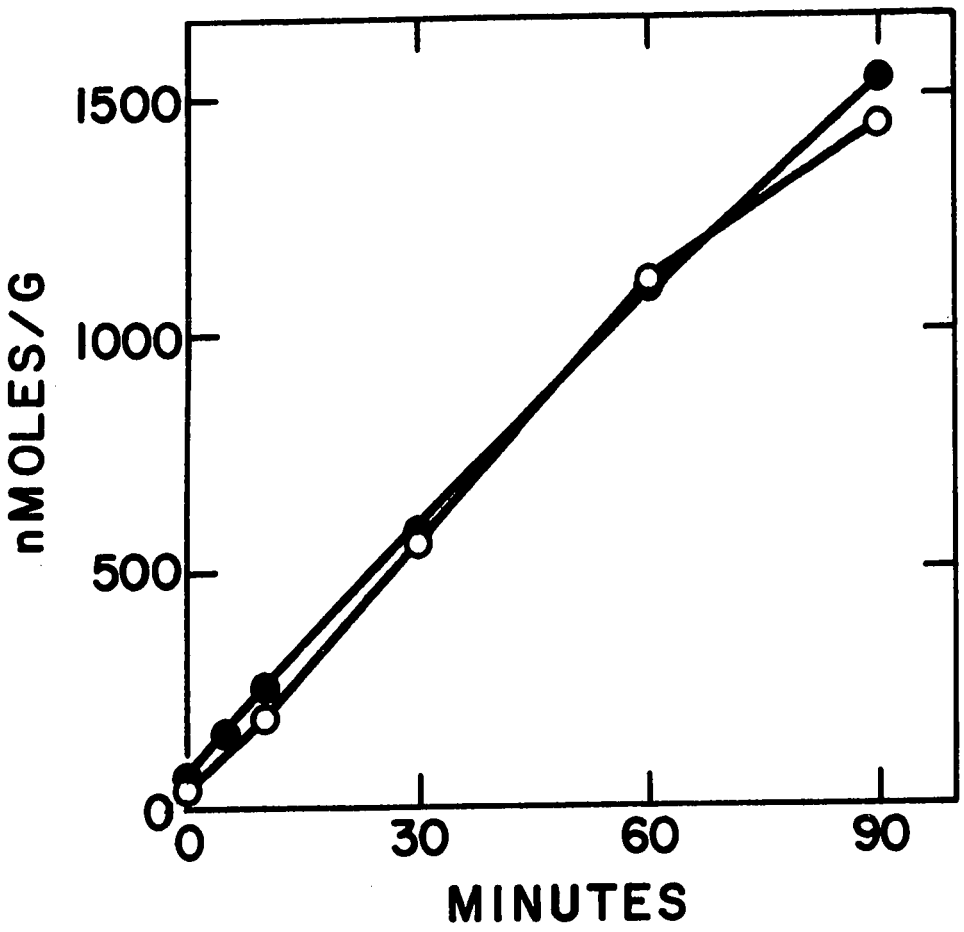


Figure 71. Effect of horse serum on xanthine plus xanthosine synthesis from hypoxanthine-^{14}C.
Horse serum: 0: ●
10%: ○
Hypoxanthine-^{14}C: 100μM.

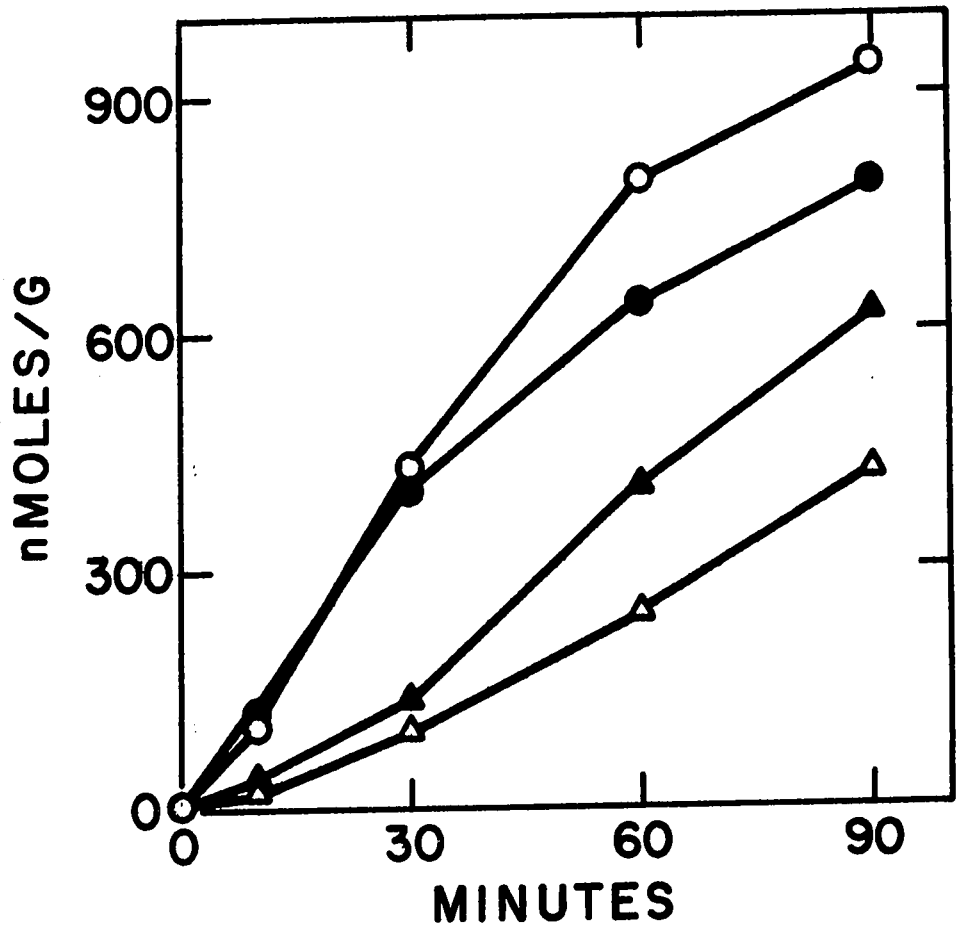


Figure 72. Effect of horse serum on xanthine and xanthosine synthesis from hypoxanthine-^{14}C.
Horse serum: 0: ● ▲
10%: ○ △
Xanthine: ▲ △
Xanthosine: ● ○
Hypoxanthine-^{14}C: 100μM.

reaction is quite slow in these cells.

When the incubation medium (Krebs-Ringer solution) is supplemented with glutamine or replaced by Fischer's medium, the catabolism of nucleotides is greatly reduced. Supplementation of the incubation medium with 10% horse serum does not appear to have significant effects on the total catabolism of nucleotides but may have specific effects on some of the reactions of nucleotide catabolism. The reactions catalyzed by nucleoside phosphorylase are markedly affected by horse serum. The reasons for these effects are not clear at the present time, however.

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