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INHIBITION OF PURINE METABOLISM—COMPUTER-ASSISTED ANALYSIS OF DRUG EFFECTS

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Abstract—Procedures are described by which the apparent rates of eight enzymes and three other parameters of purine ribonucleotide metabolism can be calculated from data obtained using intact cells, and the effects of drugs on each separately determined. An APL/360 computer program is used.

PURINE ribonucleotides are essential for cell growth and function, and the pathways of their synthesis and conversion have proven to be fruitful areas of chemotherapeutic attack. Although the effects of drugs on pathways or groups of enzymes of purine metabolism can be measured relatively easily in intact cells *in vitro* or *in vivo*, the determination of effects of drugs on individual enzymes in such complex systems is much more difficult. We report here an analytical procedure by which the effects of drugs on the apparent rates of several individual enzymes of purine metabolism may be determined. The analysis has been facilitated by the use of a suitable computer program written in the APL/360 language.

METHODS AND RESULTS

Experimental system

In order to investigate the effects of drugs on a wide range of enzymes of purine ribonucleotide synthesis and interconversion, hypoxanthine-8-^{14}C (52·0 mc/m-mole, Schwarz BioResearch) was employed as precursor and its metabolic transformations were examined. The routes of hypoxanthine metabolism which were studied are shown in Fig. 1.*

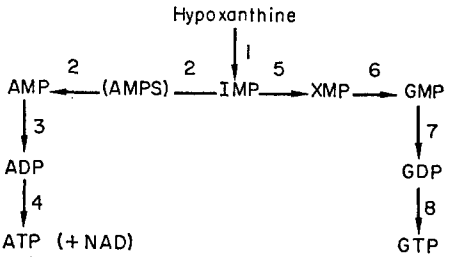

FIG. 1. Routes of hypoxanthine metabolism.

* Abbreviations: AMP, ADP, ATP, the mono, di and triphosphates of adenosine; GMP, GDP, GTP, the mono, di and triphosphates of guanosine; IMP, inosine monophosphate; XMP, xanthosine monophosphate; NAD, nicotinamide adenine dinucleotide; AMPS, adenylosuccinate.

Ehrlich ascites tumor cells were collected and diluted in Fischer's medium modified to contain 25 mM phosphate buffer, pH 7·4, without bicarbonate, to make a 2·5 per cent cell suspension by volume. The tumor cell suspension (80 μl), additional medium (5 μl) and test compounds (10 μl, 1 mM final concentration) were incubated in small plastic tubes (10 × 75 mm, Falcon Plastics) for 20 min at 37° with shaking. Hypoxanthine-^{14}C was then added (5 μl, 0·1 mM final concentration) and the incubation continued. After 60 min, 5 μl of 4·2 M cold perchloric acid was added to each tube and the cell extract was neutralized by the addition of 5 μl of 4·42 M KOH. Samples were chilled, centrifuged and 10 μl of the supernatant was spotted for chromatography.

Purine ribonucleotides were separated by thin-layer chromatography on polyethyleneimine–cellulose according to the method of Crabtree and Henderson.[1] The amount of radioactivity in the following purine nucleotides was measured: GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP and NAD. Results of duplicate measurements were averaged.

Adenylosuccinate (AMPS) was not isolated and hence the adenylosuccinate synthetase and adenylosuccinate lyase reactions are not distinguished. Previous studies[1] have suggested that the former is rate limiting for the conversion of IMP to AMP.

Ehrlich ascites tumor cells were incubated for 60 min in modified Fischer's medium with 100 μM hypoxanthine-^{14}C, in the presence and absence of drugs. Under these conditions 82·6 per cent of the radioactivity was converted to nucleotides, 1·6 per cent was oxidized directly to xanthine plus uric acid, 0·8 per cent was converted via nucleotides to other purine bases and ribonucleosides, 11·8 per cent was converted to acid-insoluble material and 3·2 per cent remained as hypoxanthine.

Both because the screening efforts have been directed primarily to reactions of ATP and GTP synthesis from hypoxanthine and because the amounts of radioactivity in bases and nucleosides were so small, the latter have been ignored in this analysis. As a simplification, the radioactivity in the acid-insoluble fraction (nucleic acids) derived from ATP and GTP has also not been included in this analysis.

Enzyme rates

The apparent rate of each of the enzymic reactions illustrated in Fig. 1 is taken to be represented by the sum of the radioactivity in all ribonucleotides which occur further along the pathway. Thus reactions 1–8 in Fig. 1 are represented by the arithmetic sums which are shown in Table 1. The enzyme names employed in Table 1 correspond

TABLE 1. SUMMATIONS REPRESENTING APPARENT RATES OF ENZYMATIC PROCESSES IN FIG. 1.

Process	Enzyme system	Summation
1	Hypoxanthine phosphoribosyltransferase	ATP + ADP + AMP + NAD + GTP + GDP + GMP + XMP + IMP
2	AMPS synthetase + lyase	ATP + ADP + AMP + NAD
3	AMP kinase	ATP + ADP + NAD
4	ADP kinase	ATP + NAD
5	IMP dehydrogenase	GTP + GDP + GMP + XMP
6	GMP synthetase	GTP + GDP + GMP
7	GMP kinase	GTP + GDP
8	GDP kinase	GTP

to the metabolic processes measured and are not necessarily intended to imply the involvement of only one enzyme. For example "ADP kinase" represents total ATP formation from ADP.

In addition, three other parameters of purine ribonucleotide metabolism were computed. The ratios of radioactive nucleoside triphosphates (ATP and GTP) to their respective mono and diphosphates gave a measure of what may be called the "energy status" of the radioactive adenine and guanine nucleotide pools. The relative rates of conversion of IMP to adenine and guanine nucleotides were also calculated. These functions are shown in Table 2.

TABLE 2. ADDITIONAL PARAMETERS OF PURINE RIBONUCLEOTIDE METABOLISM

Parameter	Function
Adenine nucleotide energy status	ATP ÷ (AMP + ADP)
Guanine nucleotide energy status	GTP ÷ (GMP + GDP)
Adenine/guanine nucleotides	(ATP + ADP + AMP + NAD) ÷ (XMP + GMP + GDP + GTP)

Computation of enzyme rates

While it is possible to compute the apparent rates of the various enzymes illustrated in Fig. 1 by use of manual techniques, the advantages of accuracy and speed which accompany a computer-assisted analysis suggested that the latter approach would be more valuable. The program was written in APL/360, a terminal-oriented language devised by Iverson;[2] the computer used was an IBM 360/67. The program first requests input of an identification number by which the drug being tested may be recorded on the print-out; control data are identified by the numeral 1. The values of the radioactivity in GTP, ATP, GDP, ADP, GMP, XMP, IMP, AMP and NAD are then entered for control and drug-treated cells. Any number of different drugs may be tested against a single set of control values in a single run. Any units may be employed for the quantitites of the different nucleotides (counts per minute, micromoles per gram of cells, etc.) provided that the method of expressing the results is consistent within any given run.

As an example of the use of these procedures, Table 3 gives the apparent rates of enzymes of hypoxanthine metabolism in cells incubated in the presence and absence of mycophenolic acid, a known inhibitor of IMP dehydrogenase.[3]

Computation of fractional inhibition

Fractional inhibition of each of the enzymes studied may be obtained by division of the apparent rates in the presence of the drug by the corresponding control values. This process is also performed by the program and Table 4 shows the results of such calculations applied to the data of Table 3.

Inspection of these data shows that the primary effect of mycophenolic acid was inhibition of IMP dehydrogenase, the rate of which was approximately 10 per cent that of hypoxanthine phosphoribosyltransferase, the preceding enzyme in the pathway (Fig. 1). The enzymes synthesizing ATP from IMP have increased rates, which may be due to an increased availability of IMP for AMPS synthetase and lyase due to

TABLE 3. APPARENT RATES OF ENZYMES AND PARAMETERS OF PURINE METABOLISM*

Parameter	Control (cpm)	Mycophenolic acid (cpm)
Hypoxanthine phosphoribosyl/transferase	11,473	12,174
AMPS synthetase + lyase	9271	10,507
AMP kinase	8867	10,242
ADP kinase	7998	9265
IMP dehydrogenase	1055	137
GMP synthetase	972	108
GMP kinase	835	86
GDP kinase	702	38
Adenine nucleotide energy status	6·027	7·387
Guanine nucleotide energy status	2·600	0·543
Adenine/guanine nucleotides	8·788	76·693

* Cells were incubated with 100 μM hypoxanthine-^{14}C for 60 min in the presence and absence of 1·0 mM mycophenolic acid.

TABLE 4. FRACTIONAL INHIBITION BY MYCOPHENOLIC ACID

Parameter	Mycophenolic acid (fraction of control)
Hypoxanthine phosphoribosyl/transferase	1·061
AMPS synthetase + lyase	1·133
AMP kinase	1·155
ADP kinase	1·158
IMP dehydrogenase	0·130
GMP synthetase	0·111
GMP kinase	0·103
GDP kinase	0·054
Adenine nucleotide energy status	1·226
Guanine nucleotide energy status	0·209
Adenine/guanine nucleotides	8·727

inhibition of the alternative pathway of IMP metabolism. The increased adenine/guanine nucleotide ratio also reflects inhibition of IMP dehydrogenase. These data also suggest that the conversion of GDP to GTP is significantly inhibited by mycophenolic acid, since the fractional rate of this process is about half that of the previous step.

Computation of per cent inhibition

The analysis applied above to the data of Table 4 remains only semiquantitative because the *apparent* fractional inhibition of any particular enzyme (except hypoxanthine phosphoribosyltransferase) is influenced by the rate of the enzyme(s) preceding it in Fig. 1. Thus 90 per cent inhibition of IMP dehydrogenase leads to an apparent 90 per cent inhibition of GMP synthetase, GMP kinase and GDP kinase simply because there is little radioactivity in the guanine nucleotides due to the block in IMP dehydrogenase. This drawback is corrected in the next step of the program, which calculates the per cent inhibition of each individual process and compensates for inhibition of preceding steps in the pathways of metabolism.

Per cent inhibition is given by:

$$P = \left(\frac{f^p - f}{f^p}\right) \times 100 = (1 - f/f^p) \times 100$$

where P is the per cent inhibition, f is the fractional inhibition of the process considered and f^p is the fractional inhibition of the preceding process. The value of f^p for conversion of hypoxanthine to IMP is 1. The sequence of processes illustrated in Fig. 1 defines the choice of other f^p values. The results of calculating per cent inhibition for the different enzymic processes are shown in Table 5. These data are obtained from Table 4 and represent inhibition by mycophenolic acid. Negative values for percentage inhibition represent augmented processes.

TABLE 5. PER CENT INHIBITION BY MYCOPHENOLIC ACID

Parameter	Computation	Inhibition (%)
Hypoxanthine phosphoribosyl/transferase	$\left(1 - \frac{1{\cdot}061}{1}\right) \times 100$	−6·1
AMPS synthetase + lyase	$\left(1 - \frac{1{\cdot}133}{1{\cdot}061}\right) \times 100$	−6·8
AMP kinase	$\left(1 - \frac{1{\cdot}155}{1{\cdot}133}\right) \times 100$	−1·9
ADP kinase	$\left(1 - \frac{1{\cdot}158}{1{\cdot}155}\right) \times 100$	−0·3
IMP dehydrogenase	$\left(1 - \frac{0{\cdot}130}{1{\cdot}061}\right) \times 100$	87·7
GMP synthetase	$\left(1 - \frac{0{\cdot}111}{0{\cdot}130}\right) \times 100$	14·6
GMP kinase	$\left(1 - \frac{0{\cdot}103}{0{\cdot}111}\right) \times 100$	7·2
GDP kinase	$\left(1 - \frac{0{\cdot}054}{0{\cdot}103}\right) \times 100$	47·6

It may be seen from Table 5 that the suggestions based on careful scrutiny in Table 4 are confirmed. IMP dehydrogenase is greatly inhibited by mycophenolic acid and there is, in addition, marked inhibition of GDP kinase.

DISCUSSION

Studies of effects of drugs on purine metabolism in intact cells and tissues have gradually come to involve the measurement of radioactivity in more and more ribonucleotides, and Hill *et al.*,[4,5] for example, have recently done such measurements on from six to eight compounds. In most cases, however, it still has been found difficult to assign a drug effect to a specific enzyme on the basis of such results and even more difficult to determine its effect quantitatively on a particular enzyme in the intact cell.

The methods presented here for computation of apparent rates of enzymes in intact cells and of drug effects, expressed in per cent inhibition of individual enzymes, appear to have overcome many of these difficulties. This procedure is sensitive to small changes in apparent rates of the enzymes involved, to weak effects of drugs and the use of a terminal-operated computer for analysis of the data lends the added advantages of speed and convenience. The data presented here for mycophenolic acid confirm previous reports[3] that it inhibits IMP dehydrogenase, but in addition it has also been shown to inhibit GDP kinase. This procedure has also been applied to 92 purine analogues and derivatives.[6]

The relationship between the apparent enzyme rates, measured here on the basis of total radioactivity measurements and the true rates depends on such considerations as mixing of radioactive and nonradioactive ribonucleotides and relative sizes of nucleotide pools and may vary for different reactions and in different cells. However, other studies with Ehrlich ascites tumor cells (reference 1 and unpublished data) suggest that radioisotope flow from IMP to ATP and GTP is virtually equivalent to the observed changes in chemical composition of the triphosphate pools.

Extension or modification of the experimental and computational methods could broaden the applicability of this approach. The consideration of radioactivity incorporated into the nucleic acid fraction, derived from ATP and GTP, has proved valuable in some situations where the contribution of this fraction cannot be ignored. The analysis of drug effects *in vivo* is an important extension of the study of drug effects *in vitro* and the methodology permitting such an analysis is being developed. Modification of the details of computation would also render this method applicable to screening programs using labeled purine nucleotide precursors other than hypoxanthine.

Program listings are shown in the Appendix.

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APPENDIX

Program HSCREEN

The program HSCREEN written in the APL language for the APL/360 Terminal System calculates apparent rates, fractional inhibition and percent inhibition of enzymes and parameters represented by the variable DATA. Input of each set of data for nucleotides is requested in a specified order and control data must be entered first and assigned the identification number (C.R.U. Number) 1.

DATA

C.R.U. NUMBER
HGPRTASE
AMPS SYNTH.+LYASE
AMP KINASE
ADP KINASE
IMP DEHYDROGENASE
GMP SYNTHETASE
GMP KINASE
GDP KINASE
TOTAL A NUCLEOTIDES
A ENERGY STATUS
TOTAL G NUCLEOTIDES
G ENERGY STATUS
A/G NUCL. RATIO

```
    ∇ HSCREEN;V;RV;I
[1]   'ENTER DATA IN THE FOLLOWING ORDER.  WHEN COMPLETE ENTER A ZERO.';RV←⍳I←0
[2]   'CRU   GTP   ATP   GDP   ADP   GMP   XMP   IMP   AMP   HAD'
[3]  LABEL1:→(1≠⍴,V←⍞)/LABEL2
[4]   RV←RV,V[1],(+/1↓V),(+/V[3 5 9 10]),(+/V[3 5 10]),(+/V[3 10]),(+/V[2 4 6 7]),(+/V[2 4 6]),(+/V[2 4]),V[2]
[5]   RV←RV,(+/V[3 5 9 10]),(V[3]÷+/V[5 9]),(+/V[2 4 6 7]),(V[2]÷+/V[4 6]),((+/V[3 5 9 10])÷(+/V[2 4 6 7]))
[6]   →LABEL1,I←I+1
[7]  LABEL2:(1 0 ,13⍴1)\[1] DATA,(10 3) DFT RV←⍉(I,14)⍴RV
[8]   ' '
[9]   'EXPRESSED AS FRACTION OF THE CONTROL VALUE:'
[10]  ' '
[11]  (1 0 ,13⍴1)\[1] DATA,(11 5) DFT RV←(0 1)↓RV÷⍉(⍴⍉RV)⍴RV[;1]
[12]  ' '
[13]  'PERCENTAGE INHIBITION:'
[14]  ' '
[15]  V←(9,I)⍴RV[1;],100×1-((,RV)[I+⍳8×I])÷((I←I-1)⍴1),(,(1 0)+(¯10 0)↓RV),RV[2;],,(5 0)+(¯6 0)↓RV
[16]  (1 0 ,8⍴1)\[1]((¯5 0)↓DATA),(11 1) DFT V
    ∇
```