Simultaneous Estimation of Rates of Pyrimidine and Purine Nucleotide Synthesis de novo in Cultured Human Cells*

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The requirement of the pathways of pyrimidine and purine nucleotide synthesis de novo for CO₂ was exploited in a method for simultaneous estimation of the rates of operation of these pathways in cultured human lymphoblasts and fibroblasts. Rates of incorporation of [¹⁴C]bicarbonate into pyrimidine and purine compounds were constant for up to 2 h and were proportional to cell number in the assay. Incorporation rates appeared to reflect rates of synthesis of pyrimidine and purine compounds in individual cell strains under conditions in which: 1) carbamyl phosphate concentrations were less than 0.1% of the hourly flux of labeled bicarbonate into the pyrimidine pathway; 2) specific activities of bicarbonate pools were apparently unchanged.

Alterations in rates of [¹⁴C]bicarbonate incorporation during incubation of normal and hypoxanthine-guanine phosphoribosyltransferase (EC 2.7.6.1)-deficient lymphoblasts with purine bases and purine and pyrimidine nucleosides were in accord with previous observations using alternative methods for the individual estimation of rates of purine or pyrimidine synthesis. In addition, comparable increases in [¹⁴C]bicarbonate incorporation into purines and pyrimidines were demonstrated in human lymphocytes during exposure to phytohemagglutinin, a stimulus previously shown to accelerate rates of pyrimidine and purine synthesis. These findings provided evidence for the validity of the present method in assessing rates of pyrimidine and purine synthesis.

High correlations between log phase growth rates of individual lymphoblast lines and their rates of incorporation of label were observed. Although specific and consistent differences were observed in rates of [¹⁴C]bicarbonate incorporation into pyrimidine and purine compounds in normal, hypoxanthine-guanine phosphoribosyltransferase-deficient, and 5-phosphoribosyl 1-pyrophosphate synthetase (EC 2.7.6.1) superactive strains, lack of information concerning the specific activities of intracellular bicarbonate pools in different cell strains restricts absolute comparisons of rates of nucleotide synthesis between cell strains.

Among methods described for the study of specific biosynthetic pathways in intact cells, measurement of radiolabeled precursor incorporation into pathway intermediates or end products, or both, has been commonly employed. The rates of operation of the pathways of pyrimidine and purine nucleotide synthesis de novo in mammalian cells in vitro have been studied individually utilizing [¹⁴C]bicarbonate (1, 2) and either [¹³C]formate (3-5) or [¹⁴C]glycine (6, 7), respectively. Short-term isotopic studies of the rates of these pathways have assumed a close relationship between rates of label incorporation and rates of nucleotide synthesis (1-4, 6, 7). The present study describes an isotopic method for the simultaneous estimation of rates of pyrimidine and purine synthesis in individual human lymphoblast lines and fibroblast strains in culture. In the course of applying this method, which depends upon measurement of the rates of incorporation of the common precursor, [¹⁴C]bicarbonate, into pyrimidine and purine compounds, close correlations between growth rates of individual lymphoblast cultures and their rates of synthesis of these compounds became apparent and are described. In addition, rates of incorporation of label into pyrimidines and purines in normal cells were compared with those in cells bearing specific genetic abnormalities in enzymes of purine metabolism, and these findings, as well as responses of certain of these cultures to chemical and pharmacological agents, are reported.

EXPERIMENTAL PROCEDURES

Materials

Sodium [¹³C]bicarbonate (50 mCi/mmol), [carbamyl-¹⁴C]citrulline (54.3 mCi/mmol), [¹¹C]ornithine (46 mCi/mmol), and [¹⁴C]carbamyl phosphate (4.9 mCi/mmol) were purchased from New England Nuclear, and sodium [¹³C]formate (59 mCi/mmol) was a product of Amersham/Searle. 5-Amino-[⁵-¹⁴C]imidazole 4-carboxamide HCl (5 mCi/mmol) was provided by Dr. J. Edwin Seegmiller, University of California, San Diego. Carbamyl phosphate, carbamyl aspartate, and all pyrimidine and purine bases, nucleosides, and nucleotides were obtained from Sigma. Phytohemagglutinin was purchased from Wellcome Research Laboratories, and Dowex ion exchange resins were products of Bio-Rad Laboratories. Ficoll was obtained from Pharmacia Fine Chemicals and Hypaque from Winthrop Laboratories. Eagle's minimal essential medium, modified autoclavable ME medium, RPMI-1640 medium, and fetal calf serum were purchased from Grand Island Biological Co. All other chemicals and media components were obtained from commercial sources and were of the highest grades available.

Methods

Cell Cultures—The following diploid human B-cell lymphoblast lines were used in these studies: WI-L2, derived from the spleen of an individual with hereditary spherocytosis (8), was obtained from Dr. Richard Lerner, Scripps Clinic and Research Foundation; RS760, the abbreviations used are: ME medium, Eagle's minimal essential medium; PHA, phytohemagglutinin; PP-rbose-P, 5-phosphoribosyl-1-pyrophosphate.

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1 The abbreviations used are: ME medium, Eagle's minimal essential medium; PHA, phytohemagglutinin; PP-rbose-P, 5-phosphoribosyl-1-pyrophosphate.
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derived from the peripheral blood lymphocytes of a normal individual, was provided by Dr. Seegmiller who also provided cell lines Ag9C185SCl and MTT107a, derivatives of WI-L2 which are severely (>99%) deficient, respectively, in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) (9) and adenine kinase (EC 2.7.1.20) (10) activities.

Suspension cultures of lymphoblasts were grown in 75-cm² Falcon flasks in modified autotaxia medium containing 50 mM sodium bicarbonate and supplemented with 2 mM glutamine, 1 mM sodium pyruvate, nonessential amino acids (0.1 mM of each), and 10% fetal calf serum. Cultures were incubated at 37°C under an atmosphere of 5% CO₂ in air on a rotary shaker and were subcultured twice weekly with 2 ml of fresh suspension medium. No evidence of mycoplasma contamination of any of the lymphoblast or fibroblast cultures was noted. In all cases, the results of determinations referred to growth rate represent measurements made on cells harvested in the course of at least 48 h of log phase growth, in polystyrene tubes (17 × 100 mm), 0.1 ml of 150 mM sodium bicarbonate was added. Tubes were then covered and incubated with shaking in a water bath at 37°C for 30 min to allow equilibration of bicarbonate. The assay was started by addition of 20 µl of sodium [¹⁴C]bicarbonate (0.4 µmol, 50 mCi/mmol). After 60 min at 37°C, reactions were terminated by addition of 0.1 ml of 6 N HCl, and stoppers were immediately replaced with rubber gaskets from which were suspended center wells containing 0.25 ml of 1 M hyamine solution. Tubes were stopped at 0°C and 30 min after stopping. Cell counts were performed on cultures inoculated at identical cell densities and growth conditions.

Preparation of Cells for Study—Lymphoblasts in log phase growth were harvested by centrifugation at 1506 x g for 5 min. The cells were washed once and resuspended at densities between 0.5 and 10 x 10⁶ cells per ml in a medium, free of bicarbonate and fetal calf serum, consisting of modified-autotaxia ME medium supplemented with 0.4% bovine serum albumin, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 2.6 mM triiodiomethane phosphate (pH 7.3) (suspension medium). Fibroblasts were removed from the suspension cultures by treatment with 0.02% sodium dodecyl sulfate (SDS) or in which individual radiolabeled purine bases or pyrimidine compounds were added (prior to heat treatment) to extracts of cells incubated under conditions previously described in detail (14). Each fibroblast strain was harvested for study at several points in the log phase of the growth cycle corresponding to 2 to 4 days after subculture at a density of 10⁶ cells per 75-cm² Falcon flask. No evidence of mycoplasma contamination of any of the lymphoblast or fibroblast cultures was noted. In all cases, the results of determinations referred to growth rate represent measurements made on cells harvested in the course of at least 48 h of log phase growth.

Suspension medium was adopted for [¹⁴C]bicarbonate incorporation studies in order to permit analysis to be performed in a completely defined medium. Although the serum-free suspension medium did not support long-term growth of lymphoblasts or fibroblasts, we found that growth of cultures, as assessed by cell number, was not significantly diminished during the first 24 h after introduction of this medium.

Preparation of Lymphocytes—On two occasions, lymphocytes were isolated from the peripheral blood of a normal donor by Ficoll-Hypaque density gradient centrifugation, as previously described (16). Three 50 ml lymphocyte cultures were prepared by resuspension of cells in RPMI-1640 medium at a density of 0.8 x 10⁶ cells per ml and were incubated at 37°C for 3 h. At this point (0 h), one culture was harvested by centrifugation. The rate of pyrimidine synthesis was measured immediately with the bicarbonate incorporation method, the rate of purine synthesis de novo was determined by each of the three methods described below. To one of the remaining cultures, PFA (1 µg/ ml) was added, and both cultures were incubated for an additional 72 h at 37°C prior to harvest and assay procedures.

Sodium [¹⁴C]Bicarbonate Incorporation into Purine and Pyrimidine Compounds—To 0.9 ml aliquots of cells in suspension medium

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Carbamyl Phosphate Concentrations—Triplicate cell suspensions containing $1 \times 10^7$ to $1 \times 10^8$ lymphoblasts or $1 \times 10^7$ to $4 \times 10^7$ fibroblasts in 1 ml of suspension medium with 15 mM sodium bicarbonate were incubated for 30 min at 37°C in covered polypropylene tubes ($17 \times 130$ mm). To one suspension from each set, 0.1 ml of cold 4.2 N perchloric acid was added and, after 10 min of storage in an ice water bath and 5 min of centrifugation at 1400 x g, the supernatant layer from each extract was neutralized by addition of 0.078 ml of 8 N KOH. To the remaining cell suspensions from each set, 0.1 ml of suspension medium with bicarbonate or an equal volume of the medium with an appropriate test compound (described under “Results”) was added, and incubation was continued for an additional 60 min prior to preparation of neutralized extracts of acid-soluble cellular material. Carbamyl phosphate concentrations in the neutralized cell extracts were determined utilizing a modification (18) of the method of Jones et al. (20) in which $[^14]C$-citritline was isolated and counted after incubation of extracts with $[^14]C$ornithine in the presence of an excess of partially purified ornithine transcarbamylase (EC 2.1.3.5) from Streptococcus faecalis (21).

RESULTS

Rates of Incorporation of $[^14]C$Bicarbonate into Pyrimidine and Purine Compounds—The rates of incorporation of sodium $[^14]C$bicarbonate into the pyrimidine and purine compounds of cultured human lymphoblasts and fibroblasts were constant for up to 120 min and were linearly related to the number of cells in the assay over ranges of at least 0.1 to 6.0 $\times 10^6$ cells per ml for lymphoblasts and of at least 0.2 to 3.0 $\times 10^6$ cells per ml for fibroblasts. These relationships are exemplified in Fig. 1 for lymphoblast line WI-L2 in which mean values of 14.2% and 10.5% of the total radioactivity incorporated into pyrimidine and purine compounds, respectively, appeared in the incubation medium. In experiments carried out to assess the possibility that label incorporation by fibroblasts in suspension might differ significantly from that of these cells in monolayer culture, rates of incorporation of $[^14]C$bicarbonate into the pyrimidine and purine compounds of fibroblasts assayed in suspension differed from those fibroblasts assayed in monolayer by no more than 8% in any study.

The methods employed here for isolation of pyrimidine and purine compounds permit determination of label in all pyrimidine compounds and precursors except carbamyl phosphate, and in all stable purine compounds distal to bicarbonate incorporation into the pathway of purine nucleotide synthesis de novo, measurement of the specific activities of intracellular carbamyl phosphate and bicarbonate pools under the conditions studied would thus allow assessment of the validity of equating rates of label incorporation and nucleotide synthesis de novo. While direct measurements of these specific activities of these compounds are not currently achievable, the following studies provide indirect evidence to support a close relationship between these rates in individual cell strains.

Intracellular carbamyl phosphate concentrations in three lymphoblast lines and two fibroblast strains under conditions of the experiments described below are shown in Table I. In all cases, concentrations of this compound were very low (0.4 to 2.1 pmol per $10^6$ cells) and were not substantially altered by incubation of 60 min with no additions or with certain effectors of bicarbonate incorporation. The pool of carbamyl phosphate was less than 0.1% of the hourly flux of labeled bicarbonate (based on the starting specific activity of $[^14]C$bicarbonate in the incubation medium) into pyrimidine compounds through this pool, suggesting an approximate turnover time of 4 s for the carbamyl phosphate pool. Thus, equilibration of the specific activity of this pool with that of the intracellular bicarbonate pool should be quite rapid, and the critical factor in relating rates of label incorporation and rates of synthesis in both pathways is the specific activity of the bicarbonate pool.

The constant rates of incorporation of labeled bicarbonate into pyrimidine and purine compounds during at least 120 min of incubation without additions suggest that the specific activity of the bicarbonate pool is relatively constant for this period and that rates of incorporation of label reflect rates of synthesis in this circumstance. The precise amount of pyrimidine or purine synthesized, however, cannot be stated without information regarding the specific activity of the intracellular bicarbonate pool. Thus, rates of incorporation under these conditions are stated as counts per min per $10^6$ cells per h and provide relative estimates of rates of synthesis of the respective classes of compounds. Evidence to suggest that the addition of the compounds described below which alter rates of label incorporation into one or both pathways do so by altering rates of synthesis rather than changing specific activities of the bicarbonate pool was provided by studies in which the order of addition of label and effector was varied (Table II). Regardless of whether addition of effector preceded addition of label by up to 30 min, followed addition of label by this time period, or the additions were simultaneous, the effects of an individual compound on incorporation of label into the two pathways were nearly constant. This finding held for each of the lymphoblast lines studied. In addition, several effectors showed selective alteration in incorporation into one or the other pathway, and incorporation in the presence of effectors was constant with time. Finally, when the concentration of $[^14]C$bicarbonate in the assay (with or without effectors) was varied over a 5-fold range (from 5 to 25 mM) with specific activity held constant, rates of incorporation of radioactivity remained constant. These findings are consistent with effects of these compounds on rates of synthesis rather than on rates of uptake of or equilibration of label into the bicarbonate pool.

In contrast to the apparent validity of the incorporation method in measuring relative rates of synthesis in individual cell lines and strains, differences in rates of incorporation of label into pyrimidine and purine compounds among different
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rates of pyrimidine and purine nucleotide synthesis in cultured pools have not been considered in previous studies comparing differences between cell strains in the labeling of precursor time) at the time of the labeling study, nearly linear relations of intracellular bicarbonate pools are unknown. While this must be borne in mind in interpreting the findings presented below, there is little to suggest a basis for differences in rates of incorporation into purine compounds in fibroblast strains are shown in Table II. Despite variation in growth rates among the cell lines, rates of incorporation into pyrimidines expressed in this way were similar in normal, hypoxanthine-guanine phosphoribosyltransferase-deficient, and adenosine kinase-deficient lymphoblasts. In contrast, the rate of incorporation into purine compounds for the four lymphoblast lines studied were obtained for 60 min after the final addition. Values are expressed relative to values of 1.00 for incorporation of $[^14]$Cbicarbonate into pyrimidine and purine compounds in the absence of effectors.

Considerable variation was observed in rates of $[^14]$Cbicarbonate incorporation into the pyrimidine and purine compounds of individual lymphoblast lines studied on multiple occasions over a period of several months. In the course of investigating factors which might account for this variation, it was found that despite apparently unaltered culture conditions, periodic changes in growth rate occurred for each cell line. However, when each determination of the rates of $[^14]$Cbicarbonate incorporation into pyrimidine and purine compounds was evaluated in relation to the log phase growth rate of the culture (expressed as the reciprocal of doubling time) at the time of the labeling study, nearly linear relationships between growth rate and rates of bicarbonate incorporation into these classes of compounds were established for each cell line, as shown in Fig. 2 for line WI-L2. Using the linear regression coefficients derived from these data, comparative rates of incorporation of label into pyrimidine and purine compounds for the four lymphoblast lines studied were calculated relative to an arbitrary doubling time of 30 h and are presented in Table III. Despite variation in growth rates among the cell lines, rates of incorporation into pyrimidines expressed in this way were similar in normal, hypoxanthine-guanine phosphoribosyltransferase-deficient, and adenosine kinase-deficient lymphoblasts. In contrast, the rate of incorporation into purine compounds in the hypoxanthine-guanine phosphoribosyltransferase-deficient line, Ag$^{9}$C135SC1, was more than twice those observed in normal lymphoblasts or in lymphoblasts deficient in adenosine kinase.

Rates of incorporation of $[^14]$Cbicarbonate into pyrimidine and purine compounds in fibroblast strains are shown in Table IV. In order to minimize possible effects of differences in passage number or state of culture confluence on rates of bicarbonate incorporation, all fibroblast strains were studied at several points during the log phase of a single growth cycle.

**TABLE I**

| Incubation time of cells | Addition | Lymphoblast line | Fibroblast strain |
|-------------------------|----------|-----------------|-----------------|
| min                     |          | WI-L2           | T.C.            |
| 30                      | None     | 0.89            | 1.46            |
| 90                      | None     | 1.11            | 2.10            |
| 90                      | 6-Azauridine | 1.60          | 0.53            |
| 90                      | Uridine  | 0.74            | 0.72            |
| 90                      | Adenosine| 1.00            | 0.40            |
| 90                      | Adenine  | 1.27            | 0.65            |
| 90                      | Inosine  | 1.30            | 0.55            |
| 90                      | Hypoxanthine | 1.34         |                 |

$^a$ Normal phenotype.

$^b$ Hypoxanthine-guanine phosphoribosyltransferase deficient.

$^c$ Adenosine kinase deficient.

$^d$ Excessive PP-ribose-P synthetase activity.

**TABLE II**

**Results of varying order of addition of label and effector on relative rates of $[^14]$Cbicarbonate incorporation into pyrimidine and purine compounds in three human lymphoblast lines**

Rates of $[^14]$Cbicarbonate incorporation were determined in duplicate as described under "Methods" except that order of addition (each at a final concentration of 0.1 mM) and label were varied as follows: A, effector added 30 min prior to label; B, effector and label added simultaneously; C, effector added 30 min after label. Incubations were carried out for 60 min after the final addition. Values are expressed relative to values of 1.00 for incorporation of $[^14]$Cbicarbonate into pyrimidine and purine compounds in the absence of effectors.

| Cell line | Phenotype              | Effector | Pyrimidines | Purines |
|-----------|------------------------|----------|-------------|---------|
| WI-L2     | Normal                 | Adenine  | A           | B       | C       |
| Ag$^{9}$C135SC1 | HGPRT$^a$ deficient | Adenine  | 0.64        | 0.60    | 0.58    |
| MTT107a   | Adenosine kinase deficient | Adenine  | 0.98        | 0.99    | 0.95    |
|           |                        | Uridine  | 0.67        | 0.61    | 0.58    |
|           |                        | Inosine  | 1.00        | 0.87    | 0.88    |
|           |                        | Uridine  | 0.34        | 0.38    | 0.26    |
|           |                        | 6-Azauridine | 1.56      | 1.59    | 1.49    |
|           |                        | Adenine  | 0.50        | 0.51    | 0.47    |
|           |                        | Uridine  | 0.89        | 0.91    | 0.89    |
|           |                        | Inosine  | 1.44        | 1.41    | 1.42    |
|           |                        | Uridine  | 0.54        | 0.48    | 0.52    |
|           |                        | 6-Azauridine | 0.46      | 0.44    | 0.44    |

$^a$ HGPRT, hypoxanthine-guanine phosphoribosyltransferase.
on at least two occasions between the seventh and eleventh passage in culture. Since much less variation in log phase growth rate was encountered either within individual fibroblast strains or among different strains (Table IV) than was the case for lymphoblast lines, no assessment of a relationship between growth and bicarbonate incorporation rates in fibroblasts comparable to that described for lymphoblasts seemed necessary. On the other hand, the design of these experiments permitted evaluation of the previously described (23) variation in the purine synthetic rates of individual fibroblast strains during the growth cycle. Fluctuation in apparent purine (and pyrimidine) synthetic rates in individual subconfluent fibroblast cultures was confirmed in these bicarbonate incorporation studies, and these variations were found to occur in cultures growing at constant logarithmic rates.

In view of these considerations, the comparative rates of incorporation of label into pyrimidine and purine compounds shown in Table IV are expressed as the mean value of determinations made at several points in the growth cycle with an appropriate indication of the variation among determinations. Four fibroblast strains derived from normal individuals were comparable in their rates of incorporation into both pyrimidines and purines. In a fibroblast strain severely deficient in hypoxanthine-guanine phosphoribosyltransferase, the mean rate of incorporation of label into purine compounds was more than 50% greater than the mean rates of this process in the normal strains, while the mean rate of incorporation into pyrimidine compounds in this mutant strain was not clearly increased. However, increased rates of $[^{14}C]$bicarbonate incorporation into both pyrimidine and purine compounds were observed in each of the four fibroblast strains derived from patients with excessive PP-ribose-P synthetase activity.

Relative rates of $[^{14}C]$bicarbonate incorporation into the pyrimidine and purine compounds of lymphoblast lines WI-L2 and Ag9C135SC1 during incubation with selected pyrimidine nucleosides and purine bases and nucleosides are shown in Table V. Substantial decrements in the purine synthetic rate of WI-L2 were observed in response to addition of 0.1 mM

**Figure 2. Relationships between rates of $[^{14}C]$bicarbonate incorporation into pyrimidine (□) and purine (○) compounds of lymphoblast line WI-L2 and the growth rate of the culture.** Cells were harvested during log phase growth at the rates indicated on the vertical axis, and $[^{14}C]$bicarbonate incorporation into pyrimidine and purine compounds was determined in duplicate as described under "Methods." Linear regression coefficients of 0.96 and 0.91, respectively, were calculated from the results of the nine experiments shown here for the relationships between pyrimidine and purine synthetic rates and culture growth rate.

**Table III**

Rates of incorporation of $[^{14}C]$bicarbonate into pyrimidine and purine compounds in cultured human lymphoblast lines

| Cell line | Phenotype | Range of log phase growth rates | Number of determinations | Rate of incorporation into | Pyrimidines | Purines |
|-----------|-----------|--------------------------------|--------------------------|---------------------------|-------------|---------|
| WI-L2     | Normal    | 17-42                          | 9                        | 4015 (0.96)               | 2875 (0.91) |
| RS760     | Normal    | 29-84                          | 6                        | 4424 (0.95)               | 3366 (0.98) |
| Ag9C135SC1| HGPRT" deficient | 10-29 | 5 | 3905 (0.92) | 7791 (0.96) |
| MTI'107a  | Adenosine kinase deficient | 22-37 | 5 | 5029 (0.89) | 2199 (0.94) |

"HGPRT, hypoxanthine-guanine phosphoribosyltransferase.

**Table IV**

Rates of incorporation of $[^{14}C]$bicarbonate into pyrimidine and purine compounds in cultured human fibroblast strains

| Phenotype | Strain | Growth rate | Number of determinations | Rates of incorporation into | Pyrimidines | Purines |
|-----------|--------|-------------|--------------------------|---------------------------|-------------|---------|
| Normal    | J.L.   | 25          | 6                        | 5,454 ± 913               | 5,289 ± 764 |
|           | O.N.   | 30          | 9                        | 5,964 ± 628               | 5,205 ± 456 |
|           | W.O.   | 25          | 9                        | 5,458 ± 824               | 3,975 ± 829 |
|           | M.G.   | 27          | 6                        | 5,987 ± 829               | 4,786 ± 979 |
|           | S.Mat. | 23          | 6                        | 6,608 ± 884               | 7,921 ± 781 |
| HGPRT" deficient | T.C. | 26          | 9                        | 10,169 ± 568              | 7,662 ± 615 |
| Excessive | R.A.   | 23          | 6                        | 11,384 ± 1932             | 8,507 ± 901 |
| PP-ribose-P synthetase activity | H.B. | 23          | 9                        | 8,864 ± 606               | 7,245 ± 692 |
|           | S.Mar. | 24          | 6                        | 8,743 ± 639               | 8,540 ± 844 |

*Mean ± 1 S.D. of number of determinations indicated.

"HGPRT, hypoxanthine-guanine phosphoribosyltransferase.
Rates of $[^{14}C]$bicarbonate incorporation were determined in duplicate as described under "Methods" except that additions of the appropriate compounds (each at a final concentration of 0.1 mM) were made just prior to addition of $[^{14}C]$bicarbonate. Incubations were carried out for 60 min. All values represent means ± 1 S.D. with the number of separate experiments indicated in parentheses and are expressed relative to mean values for incorporation of $[^{14}C]$bicarbonate into pyrimidine and purine compounds in the absence of additions.

### Lymphoblast line

| Addition       | WI-L2, relative rate of synthesis of | Ag/9C13SSC1, relative rate of synthesis of |
|----------------|-------------------------------------|------------------------------------------|
|                | Pyrimidines | Purines | Pyrimidines | Purines |
| None           | 1.00        | 1.00     | 1.00        | 1.00     |
| Adenine        | 0.63 ± 0.10 (4) | 0.39 ± 0.09 (6) | 0.56 ± 0.04 (4) | 0.28 ± 0.05 (6) |
| Adenosine      | 0.53 ± 0.12 (6) | 0.44 ± 0.09 (6) | 0.50 ± 0.10 (6) | 0.52 ± 0.12 (9) |
| Hypoxanthine   | 0.90 ± 0.04 (4) | 0.47 ± 0.08 (4) | 0.93 ± 0.06 (4) | 0.96 ± 0.04 (4) |
| Inosine        | 0.96 ± 0.09 (6) | 0.47 ± 0.11 (9) | 0.98 ± 0.08 (5) | 0.96 ± 0.09 (9) |
| Uridine        | 0.35 ± 0.10 (12) | 0.81 ± 0.06 (11) | 0.45 ± 0.09 (11) | 0.90 ± 0.07 (11) |
| 6-Azaauridine  | 1.47 ± 0.14 (12) | 0.77 ± 0.10 (10) | 1.52 ± 0.13 (9) | 0.86 ± 0.10 (9) |

### Presence of adenosine and adenine. Addition of the pyrimidine nucleoside analogue 6-azaauridine was associated with marked increases in the rates of $[^{14}C]$bicarbonate incorporation into the pyrimidine compounds of both lymphoblast lines, an effect accompanied by slight reductions in purine synthetic rates.

#### Distribution of $[^{14}C]$Bicarbonate Incorporated into Pyrimidine Compounds—Fig. 3 shows the elution profile of standards corresponding to carbamyl aspartate, orotate, dihydroorotate, pyrimidine nucleoside monophosphate, and UDP-glucose from Dowex 1-X8-Cl$^-$ ion exchange columns subjected to a stepwise NH$_4$Cl gradient. The pyrimidine compounds in cell extracts of lymphoblast line WI-L2, prepared under several different conditions of incubation, yielded the elution patterns shown in Fig. 4. With no additions to the standard assay procedure (Fig. 4A), the major part of $[^{14}C]$bicarbonate incorporated into pyrimidine compounds in 60 min appeared
as the pyrimidine nucleotide derivatives UMP and CMP and as UDP-glucose. Lesser amounts of labeling were detected in carbamyl aspartate, OMP, and the pyrimidine bases, orotate and dihydroorotate.

The increased incorporation of [14C]bicarbonate into pyrimidine compounds during incubation with 0.1 mM 6-azauridine was associated with a distinctly altered pattern of labeling (Fig. 4B). Incorporation of [14C] into UMP, CMP, and UDP-glucose was greatly diminished, while substantial increments in labeling of uridylate precursors occurred and included a slight increase in labeling of OMP. This pattern of incorporation of [14C]bicarbonate appears to be in accord with the previously described inhibition of orotidylic acid decarboxylase (EC 4.1.1.23) by the 6-azauridine derivative 6-azauridyl acid (27). A somewhat similar distribution of [14C]bicarbonate incorporation into pyrimidine compounds resulted from addition of 0.1 mM oxipurinol (Fig. 4C), nucleotide derivatives of which inhibit orotidylic acid decarboxylase (28–30). A mean increase in bicarbonate incorporation of 25% in response to this compound was accompanied by diminished labeling of UMP, CMP, and UDP-glucose and by increases in uridylate precursor labeling. Finally, severely diminished labeling of all pyrimidine compounds resulted from addition of 1.0 mM uridine (Fig. 4D), consistent with an inhibition of the pathway of pyrimidine synthesis at an early step by uridine nucleotides (31–33).

Stimulation of Lymphocyte Purine and Pyrimidine Synthetic Rates by PHA—Several studies utilizing previously described isotopic methods for estimating rates of either purine or pyrimidine synthesis have shown acceleration of these processes during exposure of mammalian lymphocytes to the plant lectin PHA (2, 34, 35). Lymphocyte transformation by PHA thus afforded an opportunity to compare the current method for simultaneous determination of purine and pyrimidine synthetic rates with alternative procedures for the separate estimation of each. Incubation of freshly isolated human peripheral blood lymphocytes with PHA for 72 h resulted in a 4-fold increment in the rate of [14C]bicarbonate incorporation into purine compounds (Table VI). The magnitude of this increase in the rate of incorporation in response to PHA is nearly identical with that reported by Hovi et al. (34) who used [14C]glycine to assess purine synthesis de novo. In contrast, [14C]formate incorporation either into total purines or into α-N-formylglycinamide ribotide in the presence of asa
erine, was increased 40- and 25-fold, respectively, in the presence of PHA (Table VI), values similar to those previously reported (35) using [14C]formate to estimate rates of purine synthesis. The different magnitudes of acceleration of labeled bicarbonate and glycine incorporation on the one hand, and labeled formate incorporation on the other, may reflect differences in transport or equilibration of the respective compounds in the presence of PHA (19, 36) rather than differences in the magnitudes of stimulation of purine synthesis. The nearly 8-fold increment in rate of label incorporation into lymphocyte pyrimidine compounds in response to PHA agrees closely with the finding of Ito and Uchino (2) whose estimates of pyrimidine synthetic rate were based on the incorporation of [14C]bicarbonate into acid-soluble uridine nucleotides.

**DISCUSSION**

The pathways of pyrimidine and purine nucleotide synthesis de novo share a requirement for 1 molecule of CO₂ for incorporation into the respective base moieties. In addition, few other reactions in cultured mammalian cells fix CO₂ into acid-stable products. For these reasons, we have chosen to study the incorporation of [14C]bicarbonate into pyrimidine and purine compounds as a method for simultaneous determination of the rates of operation of these pathways in individual human lymphoblast lines and fibroblast strains. The present studies which provide evidence for the validity and reproducibility of this method, suggest its potential usefulness in investigations directed at defining the biochemical basis of coordination of these pathways in intact cells.

Rates of incorporation of [14C]bicarbonate into pyrimidine and purine compounds of lymphoblasts and fibroblasts were constant for at least 2 h and were proportional to the number of cells added over wide ranges of cell densities. In addition, in normal cells and in cells deficient in hypoxanthine-guanine phosphoribosyltransferase, rates of bicarbonate incorporation showed the expected patterns of response to addition of compounds previously demonstrated by alternative methods to inhibit purine or pyrimidine, or both, biosynthesis (3, 5, 20).

That rates of incorporation of [14C]bicarbonate reflect rates of synthesis of pyrimidine and purine compounds in individual cell strains under these conditions is suggested by the demonstration that: 1) carbamyl phosphate concentrations were inconsequential with respect to the flux of label into the pyrimidine pathway in the presence or absence of effectors of incorporation; 2) the specific activity of the bicarbonate pool was apparently unchanged since rates of incorporation were constant with time and, in the presence of effectors, were independent of order of addition of label and effector.

Comparison of rates of incorporation of and patterns of distribution of [14C]bicarbonate into pyrimidine compounds under base-line conditions and in the presence of uridine, 6-



\[
\begin{array}{|c|c|c|c|}
\hline
\text{Incorporation} & \text{[14C]Formate into} & \text{[14C]Bicarbonate into} \\
& \text{FGAR} & \text{Pyrimidines} & \text{Pyrimidines} \\
\hline
\text{h} & \text{cpm per h per 10^6 cells} & \text{Per h per 10^6 cells} & \text{Per h per 10^6 cells} \\
\hline
0 & \text{none} & 343 & 687 & 738 & 423 \\
72 & \text{none} & 225 & 621 & 515 & 464 \\
72 & \text{PHA} & 5,801 & 26,048 & 2,124 & 3,608 \\
\hline
\end{array}
\]

* FGAR, α-N-formylglycinamide ribotide.

1 µg per ml.

**TABLE VI**

**Increases in rates of [14C]formate and [14C]bicarbonate incorporation into purine and pyrimidine compounds in human lymphocytes exposed to PHA**

Experimental procedures are described under "Methods." Values represent means of two separate experiments, each performed in duplicate.

**Rate of Incorporation of**

- **[14C]Formate into**
  - **FGAR**
  - **Purines**
  - **Pyrimidines**
- **[14C]Bicarbonate into**
  - **FGAR**
  - **Purines**
  - **Pyrimidines**

* 1 pg per ml.
the proliferative response to PHA. Although differences between cell strains in rates of labeled bicarbonate incorporation into pyrimidines and purines do not unequivocally reflect differences in rates of nucleotide synthesis, it is of interest that higher rates of incorporation of label into purine compounds were observed in fibroblasts derived from individuals with either severe deficiency of hypoxanthine-guanine phosphoribosyltransferase activity or excessive PP-ribose-P synthetase activity, both conditions associated with increased rates of purine nucleotide production in vivo and in vitro (3, 12, 13, 22, 38). Similarly, a lymphoblast line deficient in hypoxanthine-guanine phosphoribosyltransferase activity demonstrated a 2-fold greater rate of bicarbonate incorporation into purines than normal lymphoblasts, while no increase was seen in an adenosine kinase-deficient lymphoblast line previously reported to show a normal rate of purine synthesis \textit{de novo} (10). Of additional interest were the higher rates of \([1^{4}]\)bicarbonate incorporation into pyrimidine compounds in all four fibroblast strains from individuals with excessive PP-ribose-P synthetase activity in comparison to the rates in normal and hypoxanthine-guanine phosphoribosyltransferase-deficient cells. While the above findings, and the apparently selective increase in incorporation into purines but not pyrimidines in hypoxanthine-guanine phosphoribosyltransferase-deficient cells, may suggest that this method can be useful in assessing differences in nucleotides synthetic rates in different cell strains, the evidence is circumstantial, and further study will be required to validate this use of the method.

Close correlations between log phase growth rates and rates of \([1^{4}]\)bicarbonate incorporation into pyrimidine and purine compounds were observed for each of the four lymphoblast lines studied. As shown in Fig. 2 and Table III, much of the apparent variability demonstrated by individual cultures and some of the differences among cell lines in rates of incorporation of label could be ascribed to differences in growth rates. In addition to implications concerning a coordination of growth rate and pyrimidine and purine synthetic rates, this finding has obvious practical implications for comparative studies of the rates of these processes in cultured lymphoblasts. Thus, one factor contributing to the variability in published estimates of rates of purine synthesis \textit{de novo} both within individual lymphoblast lines (5, 9) and among lymphoblast lines (5, 9, 25) may be unappreciated differences in growth rates.

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