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Research Article

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HUMAN LEUKOCYTE METABOLISM *IN VITRO*. I. INCORPORATION OF ADENINE-8-C^{14} AND FORMATE-C^{14} INTO THE NUCLEIC ACIDS OF LEUKEMIC LEUKOCYTES

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The studies of Winzler and co-workers have shown that isolated human leukocytes are capable of incorporating various labeled substrates into the cell "gross protein fraction" (nucleic acids and proteins) (1, 2); they have also recently shown that such leukocytes can incorporate C^{14}-labeled formate and adenine into their nucleic acid bases (3, 4). The present studies were undertaken to explore further the *in vitro* nucleic acid metabolism of the morphologically different types of human leukemic leukocytes. Since the nucleic acid purines may be derived either from preformed purines or from purines synthesized by the cells (5, 6), both pathways of purine utilization were assessed; preformed purine incorporation was studied with labeled adenine, while nucleic acid purine incorporation of formate-C^{14} was used to evaluate utilization of purines synthesized by the *de novo* pathway (6). Synthesis of deoxyribose nucleic acid (DNA) thymine could be evaluated concurrently in studies using formate-C^{14}, since formate is a source of the methyl carbon required for the conversion of a uracil compound to thymine (6–8).

EXPERIMENTAL PROCEDURE

1. Materials. Chromatographically pure adenine-8-C^{14} and formate-C^{14} were purchased from Isotopes Specialties Company and California Corporation for Biochemical Research. Samples of unlabeled purines, purine precursors, and pyrimidines were obtained from Mann Research Laboratories and the California Corporation for Biochemical Research and their purity confirmed by paper chromatography using two or more solvent systems.

2. Leukocyte isolation and incubation. Siliconized (Siliclad, Clay-Adams, Inc.) glassware and heparin, 0.02 mg per ml of blood or leukocyte suspension, were used during cell separation and incubation. Venous blood was mixed with 0.5 vol of cold 5 per cent dextran in 0.9 per cent saline and allowed to stand in the cold until red cell sedimentation was complete. The supernatant suspension was transferred to a graduated centrifuge tube and aliquots taken for total and differential leukocyte counts. The leukocytes were sedimented by centrifugation at 4° C at 150 G; the platelet-rich supernatant was discarded and the leukocytes resuspended, in a concentration of about 20,000 per mm^3, in the incubation medium. This consisted of Hanks' solution, pH 7.4, and human serum in equal parts (8). Human serum was obtained from blood bank pilot tubes and pooled to minimize the poorly defined effects of individual sera on cell metabolism (9, 10). Prior to use, the Hanks'-serum mixture was supplemented with glucose, 1 mg per ml, and the pH determined and readjusted to pH 7.4, if necessary.

Siliconized 25-ml incubation flasks were prepared in advance to contain 10 μc of the radioactive substrate, 2,500 U of penicillin G, and 1 mg of streptomycin, by freeze-drying solutions of these materials. Since the specific activities of the radioactive substrates differed, their molar concentrations in the incubation media differed from one series of experiments to the next. These concentrations are assumed to be in excess of total cell utilization, since cell purine or pyrimidine specific activity was found in preliminary experiments to be independent of the number of cells incubated, within a range of 2 to 10×10^8 leukocytes.

Ten-ml aliquots of the leukocyte suspension, usually containing approximately 2×10^8 leukocytes, were pipetted into each flask and incubation carried out in a metabolic shaker at 37° C and 100 oscillations per minute in room air. An average period of 1.5 hours was required for the separation and preparation of the leukocytes for incubation.

Unlabeled purines, purine precursors, and pyrimidines were added in dry form, in view of their limited solubility at physiological pH, by accurately weighing these materials into individual incubation flasks prior to the addition of the cell suspension.

3. Leukocyte fractionation. After incubation the samples were chilled and contaminating erythrocytes lysed, by the gramicidin-lysolecithin technique of Athens and coworkers (11). The leukocytes were then washed three times in 0.9 per cent saline by resuspension and centrifugation at 4° C.

The addition of unlabeled carrier adenine during the fractionation process in certain of the adenine-C^{14} experiments resulted in no decrease in the specific activity of the nucleic acid purines isolated subsequently; therefore, contamination of the cell purines by the extracellular radioadenine was assumed to be negligible. After freezing, the cells were disrupted by thawing and resuspension in

cold 5 per cent trichloroacetic acid. The precipitated proteins and nucleic acids were dried and the lipids removed by suspension and sedimentation successively in ethanol and ether. From this precipitate the mixed nucleic acids were extracted into 10 per cent NaCl (1 ml per 1×10^8 cells) at neutral pH in a boiling-water bath, with phenol red as an internal indicator. This method of nucleic acid extraction was used in preference to the conventional Schmidt-Thannhauser procedure (12) to minimize contamination of the nucleic acids by the parent substrate and intracellular free nucleotides of potentially high specific activity. The nucleic acids were precipitated from the saline extract by the addition of 3 vol cold ethanol. In certain experiments this step was repeated and resulted in no significant change in the specific activity of the nucleic acid bases. In appropriate experiments the mixed nucleic acids were fractionated into ribose nucleic acid (RNA) and DNA by hydrolysis of RNA to its acid-soluble nucleotides (12) in 0.1 N NaOH, followed by reprecipitation of DNA, in the cold, with HCl. The RNA nucleotides were recovered by precipitation as their barium salts [saturated $Ba(OH)_2$ to pH 11] in cold ethanol (13). The mixed nucleic acids, DNA, or barium nucleotides of RNA were hydrolyzed to the free bases with concentrated perchloric acid and the bases separated by two-dimensional descending paper chromatography, using isopropanol-HCl-water and butanol-ammonia solvent systems (8).

The adequacy of DNA-RNA separation with this method was evaluated by the orcinol and diphenylamine reactions (14) and nucleic acid standards prepared by other means (15). These studies showed that up to 10 per cent of the nucleic acid phosphorus of the DNA fraction was RNA and that 10 to 20 per cent of the nucleic acid phosphorus of the RNA fraction was actually DNA. Since comparable results were obtained by Elion, Bieber and Hitchings (16) using similar methods, these data will not be reported in detail. Further evidence of cross contamination was observed when the purines and pyrimidines were separated by paper chromatography: DNA thymine was regularly detectable in digests of the RNA fraction and small amounts of RNA uracil were often present on chromatograms of the DNA fraction. This method of nucleic acid fractionation was used despite these failings, since larger numbers of leukocytes would have been required for more precise methods of separation (15).

4. Specific-activity determinations. After location of the compounds on the chromatograms under ultraviolet illumination, 1-inch paper discs containing the appropriate nucleic acid bases were cut out, mounted in metal planchets and the contained radioactivity measured in a gas-flow counter of 25 per cent counting efficiency. Self-absorption by the paper disc was found to be constant and reduced over-all counting efficiency to 10 per cent; self-absorption by the nucleic acid bases themselves was not appreciable for the quantity of material analyzed (10 to 150 μg or 0.1 to 1 μmole). Sufficient counts were recorded to reduce the statistical error of counting to less than 3 per cent. The background radioactivity of paper blanks cut from adjacent areas of the chromatograms was determined and subtracted from the sample counting rate; counting rates of less than twice the chromatographic background were considered to be insignificant. After counting, the base contained in each paper disc was quantitatively eluted into a measured volume of 0.1 N HCl and its concentration estimated in a Beckman DU spectrophotometer, using the ultraviolet extinction coefficients of Salzman, Eagle and Sebring (17). Completeness of elution and the accuracy of the extinction coefficients were verified experimentally with appropriate standards.

5. Statistical methods. Differences between the results of replicate incubations were generally proportional to the counting rate of the samples. A cumulative plot of these data formed a skewed rather than a normal distribution curve but closely approximated a normal distribution curve when differences between the logarithms of the numbers were plotted instead. Accordingly, the variance of differences between the duplicates was estimated by the formula

$$Sd = \sqrt{\left[\Sigma d^2 - \frac{(\Sigma d)^2}{n}\right] \Big/ (n-1)}$$

when d = the difference between the logarithms of the duplicate specific activity measurements, and n = the number of duplicates to be compared in the individual experiment. Comparisons between the logarithms of the means of duplicate incubations in the same study were made after estimating t by the formula

$$t = \frac{\log \bar{x} - \log \bar{y}}{Sd/\sqrt{2} \times \sqrt{\frac{1}{2} + \frac{1}{2}}}$$

using $n - 1$ degrees of freedom.

Repeated studies made on comparable cell samples from the same patient on different days showed greater variations than were found between simultaneous duplicate incubations. Since practical considerations prevented the estimation of this day-to-day variation in most instances, statistical evaluation of differences between studies done on different days has not been attempted.

RESULTS

Rate of uptake and nucleic acid distribution of the labeled compounds. To determine the period of incubation required for significant incorporation of the labeled compounds into the leukocyte nucleic acids, samples of chronic myelocytic (CML) and chronic lymphocytic leukemic (CLL) leukocyte suspensions were incubated in duplicate for periods of 2 to 8 hours. Adenine-C^{14} was incorporated into nucleic acid adenine and converted to nucleic acid guanine by both cell types; incorporation increased linearly with time during incubations of 6 to 8 hours (Figure 1). Formate-C^{14} was incorporated into the DNA thymine of CML leukocytes in

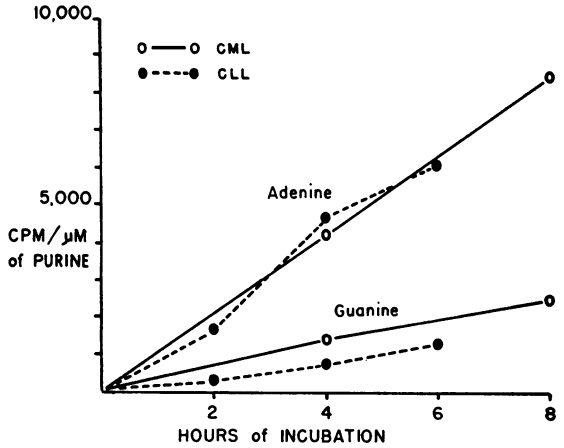

FIG. 1. TIMED INCORPORATION OF ADENINE-C^{14} INTO LEUKOCYTE NUCLEIC ACID PURINES. Chronic myelocytic leukemic leukocytes O——O; chronic lymphocytic leukemic leukocytes ●——●. Each data point is the mean of duplicate incubations; samples of each cell type were incubated simultaneously. Radioadenine specific activity, 3.82 mc per mmole.

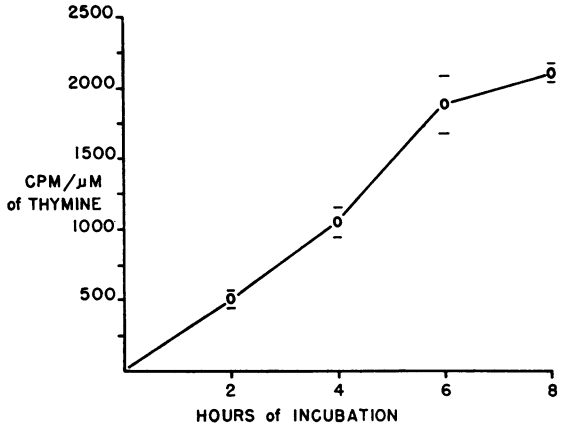

FIG. 2. TIMED INCORPORATION OF FORMATE-C^{14} INTO DNA THYMINE BY CHRONIC MYELOCYTIC LEUKEMIC LEUKOCYTES. Horizontal dashes indicate the range between duplicate incubations. Radioformate specific activity, 1.51 mc per mmole.

a similar linear fashion over this period of time (Figure 2). Comparisons of the incorporation of these compounds into the nucleic acid bases by different leukocyte populations during a 6-hour period of incubation are shown in Tables I and II.

Adenine-C^{14} was incorporated into nucleic acid adenine and, to a limited extent, converted to nucleic acid guanine by each of the leukocyte populations studied. Increasing specific activity of the nucleic acid purines tended to parallel the percentage of immature cells in the differential formulas of the different leukocyte populations.

Formate-C^{14} was not incorporated to a measurable extent into the nucleic acid purines of mature granulocytes, CLL lymphocytes or CML granulocytes. Subsequent experiments using formate-

TABLE I

In vitro nucleic acid purine incorporation of adenine-8-C^{14} by different leukocyte populations *

Leukocyte source	Leukocyte differential							Specific activity in	
	Blasts	Promy.	Myelo.	Metam.	PMN	Lym.	Mono.	Adenine	Guanine
	%	%	%	%	%	%	%	*cpm/μmole purine*	
Polycyth. vera			1	33	57	5	4	4,780	510
Leukemia									
Chronic lymphocytic					15	85		6,090	1,320
Chronic myelocytic	5	5	30	28	32			8,580	1,310
			53	40	7			10,680	2,980
Acute myeloblastic	75				1		24	21,630	7,710
	99				1			24,630	14,350
	100†							44,490	18,490
	100†							57,200	17,480
Acute lymphoblastic	100							14,420	1,100
	96				4			21,130	7,060

* Portions of a single sample of radioactive adenine having a specific activity of 3.82 mc/mmole were used in each experiment, in a concentration of 10 μc (approximately 2,664,000 cpm) per 10 ml of leukocyte suspension. Duplicate cell samples were incubated for 6 hours and analyzed separately; specific activity is expressed as an average of the results of duplicate determinations, rounded off to a significant figure. Duplicate determinations differed by less than 10 per cent. Leukocyte differential: Promy. = promelocytes; Myelo. = myelocytes; Metam. = metamyelocytes; PMN = polymorphonuclear leukocytes; Lym. = lymphocytes; Mono. = monocytes.

† Studies done on the same patient's cells, 2 weeks apart.

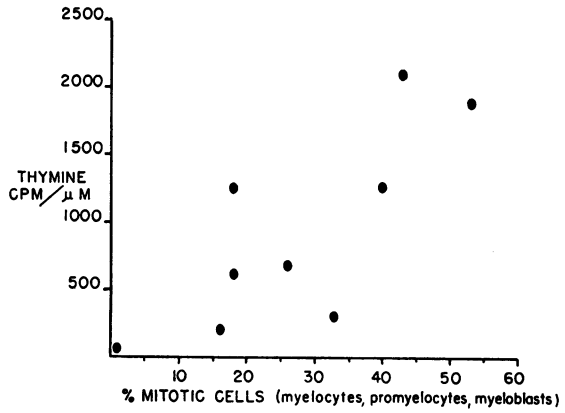

FIG. 3. RELATION OF THYMINE-FORMATE INCORPORATION TO MITOTICALLY ACTIVE GRANULOCYTES. Chronic myelocytic leukemic leukocyte populations incubated 6 hours under comparable conditions. Each data point is the mean of duplicate incubations. Radioformate specific activity, 1.51 mc per mmole.

C^{14} of higher specific activity (9.5 mc per mmole) were no more successful in demonstrating nucleic acid purine-formate incorporation by these cells. The incorporation of formate into DNA thymine by mature granulocytes and CLL lymphocytes was similarly insignificant. The extent of thymine incorporation in CML leukocytes appears to be related generally to the presence of cells capable of mitotic division (Figure 3).

The formate incorporation pattern of acute leukemic leukocytes differed substantially from that of the other leukocyte populations studied. Significant incorporation of formate into nucleic acid purine was regularly observed. This was unrelated quantitatively to the incorporation of formate into DNA thymine by the same cells. Thymine incorporation of formate by acute leukemic leukocyte populations was also unrelated to the percentage of morphologically immature cells. Indeed, there was no significant incorporation of formate into the thymine of two of the four acute lymphoblastic leukemic (ALL) leukocyte populations studied (Tables II and III). In a third, however, thymine specific activity was quite high, measuring four to six times that of the purines (Table II). These cells were isolated from the

TABLE II

In vitro nucleic acid purine and thymine incorporation of formate-C^{14} by different leukocyte populations *

Leukocyte source	Leukocyte differential							Base specific activity		
	Blasts	Promy.	Myelo.	Metam.	PMN	Lym.	Mono.	Adenine	Guanine	Thymine
	%	%	%	%	%	%	%		*cpm/μmole*	
Polycyth. vera			1	33	57	5	4	†	†	50
Leukemia										
Chronic lymphocytic	98				1		1	†	†	†
Chronic myelocytic	1	2	13	43	40	1		†	†	180
	3	6	24	26	39	1		†	†	290
		1	17	37	44	1		†	†	620
		4	39	35	22			†	†	630
	14		4	8	71	2	1	†	†	630
	8		10	15	63	4		†	†	1,260
	5	5	30	28	32			†	†	1,270
	1	1	51	17	28	2		†	†	1,880
			43	16	37	4		†	†	2,100
Acute myeloblastic	85	9			2	4		130	360	790
	99			1				350	670	300
	100‡							2,440	3,930	620
	100‡							1,480	3,250	830
	100‡							2,950	3,560	1,580
Acute lymphoblastic	83				17			250	470	440
	100							680	760	†
	96§				3	1		220	390	1,270

* The conditions of these experiments were the same as those presented in Table I and in many instances were run concurrently. Ten μc of formate-C^{14} having a specific activity of 1.51 mc/mmole, was used as substrate. Abbreviations as in Table I.

† Insignificant radioactivity (less than twice background) present at 10% counting efficiency with sample size of 0.2 to 1 μmole.

‡ Successive studies of the same patient's leukocytes during a 15-day period, listed chronologically.

§ Atypical disease (see text).

TABLE III

Relative incorporation of labeled substrates into RNA and DNA *

Leukocyte source	Substrate and substrate specific activity	Base specific activity				
		RNA		DNA		
		Adenine	Guanine	Adenine	Guanine	Thymine
	mc/mmole	*cpm/mole*				
Leukemia						
Chronic lymphocytic	Adenine 1.8	14,810	5,220	180	†	†
Chronic myelocytic	Adenine 1.8	13,860	2,560	1,020	450	†
Acute myeloblastic	Adenine 5.3	11,200	2,990	580	170	†
Acute myeloblastic	Formate 5.0	280	205	†	†	800
Acute myeloblastic‡	Formate 9.5	395	290	†	†	5,000
Acute lymphoblastic	Formate 9.5	2,880	5,270	†	†	†

* Experimental conditions as in Tables I and II.
† Insignificant radioactivity (less than twice background) at 10 per cent counting efficiency with sample size of 0.1 to 1 μmole.
‡ Acute phase of CML.

blood of a child with an atypical syndrome that more closely resembled lymphosarcoma than the usual clinical picture of childhood acute leukemia.

The leukocytes of one patient with acute myeloblastic leukemia (AML) were restudied several times over a period of weeks, in the absence of changes in the leukocyte differential pattern or complicating antileukemic therapy. Reasonable agreement between the results of repeated studies with both adenine-C^{14} and formate-C^{14} was found. However, greater differences in mean specific activity measurements were found between consecutive or serial studies than between simultaneous duplicate incubations. This degree of day-to-day variation is typical of that found in studies of the leukocytes of other patients with CML, AML and ALL.

Relative incorporation into RNA and DNA. The specific activities of the RNA purines after incubation with adenine-C^{14} were found to be at least ten times those of the DNA purines of both chronic and acute leukemic leukocytes. Apparent DNA purine incorporation of this magnitude could be wholly accounted for by contamination of the DNA fraction with highly labeled RNA purine (see Methods). Labeled formate incorporation into the nucleic acid purines of acute leukemic leukocytes was entirely in the RNA fraction; the specific activity of the RNA purines after formate incubation was probably too low to produce measurable contaminating radioactivity in the DNA purines. The results of representative studies of the relative incorporation of these labeled substrates into the RNA and DNA bases are presented in Table III.

Influence of unlabeled compounds on purine incorporation of adenine-C^{14} and formate-C^{14}. Relative utilization of extracellular purines other than adenine was assessed in several leukocyte populations by determining the dilution effect of unlabeled purines on radioadenine and formate incorporation. The effect of these unlabeled compounds on adenine-C^{14} incorporation into the nu-

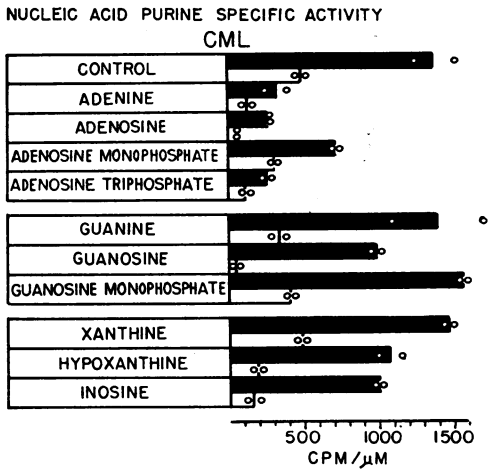

FIG. 4. INFLUENCE OF UNLABELED PURINES ON ADENINE-C^{14} INCORPORATION INTO NUCLEIC ACID PURINES. Chronic myelocytic leukemic leukocytes, 6-hour incubation. Solid bars, adenine; open bars, guanine. Data points indicate the range between duplicate incubations. Unlabeled purine concentration, 1 μmole per ml. Radioadenine specific activity, 1.8 mc per mmole.

cleic acid purines of CML leukocytes is shown in Figure 4. The effect of unlabeled adenine reflects simple dilution of the labeled substrate, independent of the metabolic processes of the cell, and is presented for comparison with the effects of equal concentrations (1 μmole per ml) of the other compounds. Adenosine and adenosine triphosphate (ATP) equaled adenine in their diluent effect on adenine-C^{14} incorporation into both nucleic acid adenine and guanine. Adenosine monophosphate (AMP) was less effective than either adenosine or ATP ($p < 0.001$). Guanine, guanosine monophosphate and xanthine were without influence on adenine-C^{14} incorporation. Guanosine decreased the conversion of adenine-C^{14} into nucleic acid guanine ($p < 0.001$) but had no significant effect on nucleic acid adenine incorporation. Hypoxanthine and its riboside, inosine, decreased radioadenine incorporation into nucleic acid guanine ($p < 0.001$) but insignificantly affected incorporation into nucleic acid adenine.

The effects of equimolar concentrations (1 μmole per ml) of unlabeled adenine, guanine, hypoxanthine and xanthine on the incorporation of radioformate into the RNA purines of isolated acute leukemic leukocytes are shown graphically in Figure 5. This concentration of adenine completely suppressed purine incorporation of formate-C^{14} in AML leukocytes and similarly inhibited this process in ALL leukocytes, although slight guanine incorporation was detectable in the latter cells. Guanine, hypoxanthine and xanthine were less effective than adenine ($p < 0.001$), but substantially decreased both adenine and guanine incorporation of labeled formate in AML leukocytes. These compounds were without influence on this process in the ALL leukocyte population similarly studied.

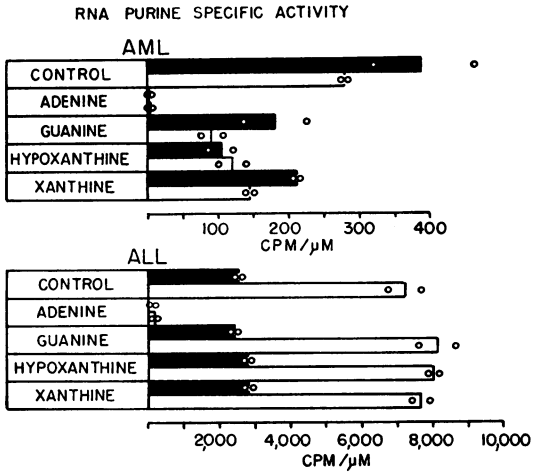

FIG. 5. INFLUENCE OF UNLABELED PURINES ON *de novo* RNA PURINE SYNTHESIS. AML, acute myeloblastic leukemic leukocytes; ALL, acute lymphoblastic leukemic leukocytes; conditions as in Figure 4. Radioformate specific activity, 9.5 mc per mmole.

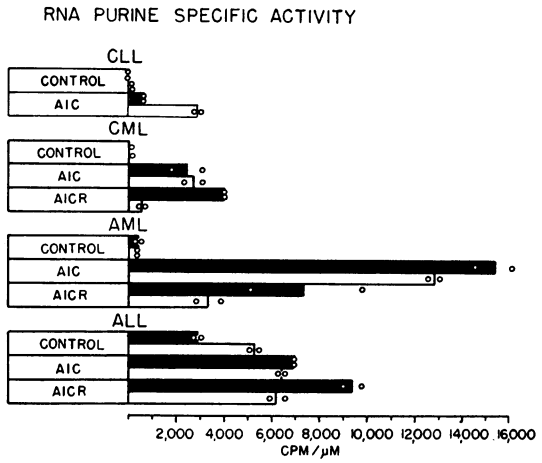

FIG. 6. INFLUENCE OF AIC AND AICR ON *de novo* RNA PURINE SYNTHESIS. CLL, chronic lymphocytic leukemic leukocytes; CML, chronic myelocytic leukemic leukocytes; AML, acute myeloblastic leukemic leukocytes; ALL, acute lymphoblastic leukemic leukocytes; AIC, 5-amino-4-imidazole carboxamide; AICR, AIC riboside; conditions as in Figure 5.

The purine precursors, 5-amino-4-imidazole carboxamide (AIC) and its riboside (AICR), in concentrations of 1 μmole per ml, significantly increased labeled formate incorporation into the RNA purines, of CLL, CML, AML and ALL leukocytes ($p < 0.001$) (Figure 6). DNA purine incorporation did not occur under these circumstances. Concurrent thymine incorporation of formate-C^{14} was not altered by the presence of either the preformed purines or the purine precursors.

Influence of unlabeled pyrimidines on formate-C^{14} incorporation into DNA thymine. The effects of added unlabeled pyrimidines and their ribosides and ribotides on the incorporation of labeled formate into thymine were studied in chronic and acute leukemic leukocytes. The results of the most complete of several studies using CML leukocyte suspensions are presented in Figure 7. Of the free pyrimidine bases, uracil, orotic acid and cytosine may have slightly stimulated this process. However, the effect of uracil, which was the most

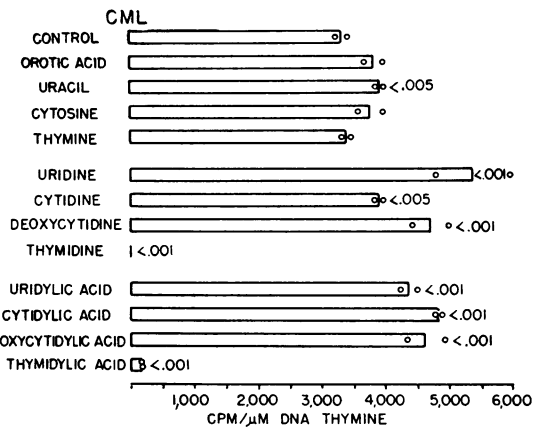

FIG. 7. INFLUENCE OF UNLABELED PYRIMIDINES ON THYMINE-FORMATE INCORPORATION INTO CHRONIC MYELOCYTIC LEUKEMIC LEUKOCYTES. Conditions as in Figure 6. Unlabeled pyrimidine concentration, 1 μmole per ml; p values refer to comparisons with the control.

pronounced of the pyrimidine bases assessed, is considered to be of doubtful statistical significance. Thymine was without measurable effect. The pyrimidine nucleosides, uridine and deoxycytidine, substantially enhanced formate incorporation into DNA thymine, while thymidine completely inhibited this process. The corresponding nucleotides produced similar effects.

Uridine, which was consistently most effective in stimulating formate incorporation into DNA thymine in CML leukocytes, was without effect on the incorporation of formate into the DNA thymine of CLL, AML and ALL leukocytes in single experiments with each of these cell types. The RNA purine incorporation of formate by acute leukemic leukocytes was similarly unchanged by the added uridine.

DISCUSSION

The interpretataion of isotope-incorporation studies involving cell synthetic reactions requires consideration of the concentration of precursors, intermediate compounds and reaction products present in the system. Both extracellular and intracellular pools of these compounds are of importance in *in vitro* studies of cell synthesis.

In the present studies the extracellular pools of compounds related to nucleic acid synthesis either were kept constant or were varied in a known fashion. Therefore, differences in the specific activities of the leukocyte nucleic acid bases presumably reflect variations in the size of the metabolic activity of the intracellular pools of these compounds.

The size of these intracellular pools was not measured in the present studies, but an estimate of this factor can be obtained from the analyses of human leukocytes published by Davidson, Leslie and White (18), Rigas and co-workers (19), and Will, Glazer and Vilter (20). Their results suggest that cell content of DNA is approximately the same from one leukocyte population to another. RNA content, on the other hand, varies significantly: cell RNA concentrations are greatest in morphologically immature leukocytes and decrease with maturation. Thus, the RNA/DNA ratios of acute leukemic and CML leukocyte populations range between 0.60 and 0.45 (19, 20), while those of normal leukocytes and the mature lymphocytes of CLL are between 0.35 and 0.30 (18, 19).

The over-all size of the acid-soluble cell nucleotide pool generally parallels cell RNA content. In mature granulocytes and lymphocytes, this pool is about one-third the size of the DNA pool. In CML and acute leukemic leukocytes the total soluble nucleotide pool is about one-half the size of the DNA pool, when expressed in terms of nucleotide phosphorus (19).

The data of Wells and Winzler (3) and Shapira and co-workers (4) and our own preliminary data, as well as analogous studies with cells other than human leukocytes (21), indicate that the nucleic acid purines are derived from this pool of soluble intracellular nucleotides. Therefore, the incorporation of labeled compounds into the nucleic acid purines probably is influenced by their transport through this cell compartment. More specific information concerning the size, composition and turnover of this pool in leukemic leukocytes is not now available and is under investigation in this laboratory.

Purine metabolism. Extracellular radioadenine is readily incorporated into the nucleic acid purines of the various human leukemic leukocytes. The present studies indicate that, within the limits of the analytical procedures, *in vitro* leukocyte incorporation of adenine into the purines of the mixed nucleic acids represents incorporation only into RNA. In the studies in which the specific activity of mixed RNA and DNA adenine or guanine was determined, the measurements reflect both RNA purine radioactivity and the extent to which

the radioactive purines are diluted by nonradioactive DNA purines. Therefore, differences in purine incorporation between the various leukocyte populations may be related only to the amount of RNA relative to DNA in the total nucleic acid fraction extracted from these cells. This seems to be the case in the various leukocyte populations studied for their incorporation of labeled adenine, although the number of observations is small. The specific activity of the mixed RNA/DNA purines tended to parallel the morphological immaturity of these cells; this pattern coincides generally with the biochemical measurements of cell RNA content described above, as well as with less exact estimations of cell RNA content obtained by histological techniques (22).

The present adenine-C^{14} incorporation data may be compared with the results of similar studies reported recently by Shapira and colleagues (4), who found that the rate of incorporation of radioadenine was approximately the same in normal, CLL and CML leukocytes. This supports the conclusion that differences in the specific activity of the mixed RNA/DNA purines in the different leukocyte populations are due not to variations in incorporation rate, but result from differences in cell nucleic acid pool size. In contrast to the results of the present studies, however, these workers describe significant DNA incorporation of labeled adenine by both CLL and CML leukocytes. This discrepancy is probably due, at least in part, to differences in the methods used for the fractionation of DNA and RNA.

As was the case with adenine-C^{14}, nucleic acid purine incorporation of formate-C^{14} in the present studies represented incorporation solely into RNA purines. This is considered to be evidence of *de novo* purine synthesis. Identification of the chemical locus of formate incorporation into the purine ring requires consideration of the steps involved in the biosynthesis of cell purines, summarized in Figure 8 (21). Glycine is the nucleus of the purine ring. Formate may contribute either the 8-carbon, condensing with glycine amide ribotide (GAR) to yield formyl-GAR in an early step in ring synthesis, or the 2-carbon needed for completion of the purine ring from AIC ribotide.

The site of formate incorporation into the nucleic acid purines was not completely defined in the present experiments. However, glycine-C^{14} incorporation into the soluble nucleotide and nucleic acid purines of normal, CLL, CML and acute leukemic leukocytes can not be demonstrated *in vitro* (23). This indicates that these cells are incapable of the earlier steps of *de novo* purine synthesis, and that utilization of formate for condensation with GAR is unlikely under these conditions.

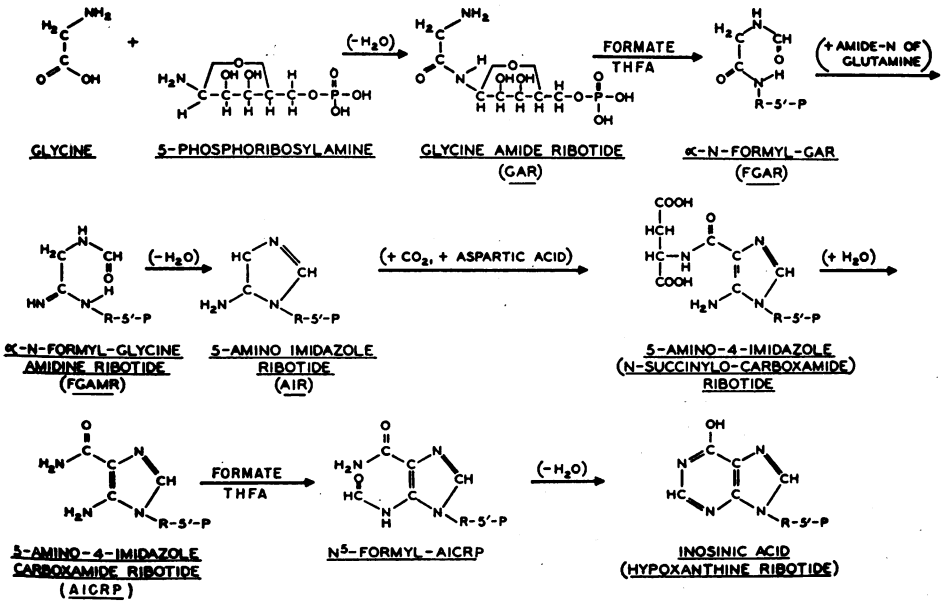

FIG. 8. *De novo* PURINE BIOSYNTHESIS. Modified from Wyngaarden (25).

The substantial enhancement of *in vitro* purine incorporation of formate by AIC or AICR observed in several different leukocyte populations suggests that formate is incorporated largely into the 2-carbon position lacking in these compounds. This effect of AIC further suggests that *de novo* purine synthesis in isolated human leukocytes is limited to some extent by the availability of this immediate precursor of the cell purines.

In contrast to the results of Wells and Winzler (3), purine incorporation of formate by CLL and CML leukocytes could not be demonstrated in the absence of added AIC in the present studies, despite the use of formate-C^{14} of high specific activity.

The effect of exogenous purines on nucleic acid incorporation of adenine-C^{14} and formate-C^{14} was studied to assess the relative absorption of the various purines by the cells and their effects on *de novo* purine synthesis. The results of these experiments indicate that adenine and its derivatives are more readily incorporated by leukemic leukocytes than are the other purines studied. Similar *in vitro* studies of other cell types have shown this comparatively greater avidity for exogenous adenine (21).

Guanosine, hypoxanthine and inosine significantly influenced labeled adenine incorporation into CML leukocytes and would therefore appear to have been incorporated. Guanine and xanthine were without influence on radioadenine incorporation into CML leukocytes, but some incorporation of exogenous guanine and xanthine must have taken place in AML leukocytes, since inhibition of *de novo* purine synthesis was seen in the presence of these purines.

Adenosine was a more effective diluent of radioadenine incorporation than was its monophosphate nucleotide (AMP); similarly, guanosine was more effective than was guanosine monophosphate (GMP). This suggests that AMP and GMP were less readily incorporated than were their respective nucleosides. These results are in agreement with the similar observations made with mouse ascites carcinoma cells by Williams and LePage, who concluded that extracellular nucleotides are incorporated only after conversion to their respective nucleosides (24). The greater dilution of labeled adenine incorporation by ATP than by AMP suggests that incorporation of the triphosphate occurs by a more direct route.

In mouse ascites carcinoma cells, nucleic acid incorporation of labeled formate is decreased by the addition of exogenous preformed purines. This may be due either to an increase in the size of the intracellular pool of soluble purine nucleotides, with resultant dilution of purine nucleotides synthesized by the *de novo* pathway, or to inhibition of the *de novo* pathway by a feedback mechanism (21). The addition of exogenous purines produced similar effects on purine incorporation of formate by AML leukocytes in the present study. The differing patterns of incorporation produced by the various purines are of interest, since they may be explained in part by pathways of intermediary purine metabolism delineated in other systems, as summarized by Wyngaarden (25) (Figure 9). Adenine was a more effective inhibitor of *de novo* purine synthesis than were the other purines studied. This is probably due to preferential incorporation of adenine by these human leukocytes, since a similar preference for adenine has been noted in several other cell types (21). The pattern of purine synthesis seen in the presence of exogenous adenine or guanine suggests that extracellular preformed purines are incorporated into their respective nucleic acid purines in preference to those arising by the *de novo* pathway.

Exogenous hypoxanthine affected incorporation of *de novo* purine into adenine and guanine equally. This may have been the result of an enlargement of the intracellular pool of hypoxanthine ribotide through which the newly synthesized purine passes before entering either of the nucleic acid purines. Exogenous xanthine produced effects compatible with the irreversible nature of the enzymatic oxidation of hypoxanthine ribotide to xanthine ribotide. This allows utilization of xanthine for nucleic acid synthesis only after its conversion to guanylic acid (25). Thus, the purine ring of xanthine readily enters nucleic acid guanine but enters nucleic acid adenine only after the conversion of guanylic to adenylic acid by way of hypoxanthine ribotide. Therefore, exogenous xanthine would be expected to dilute *de novo* purine incorporation into nucleic acid guanine, but might have little effect on nucleic acid adenine incorporation. This was seen to be the case in the AML leukocyte population studied.

Exogenous guanine, hypoxanthine and xanthine were without apparent effect on *de novo* purine

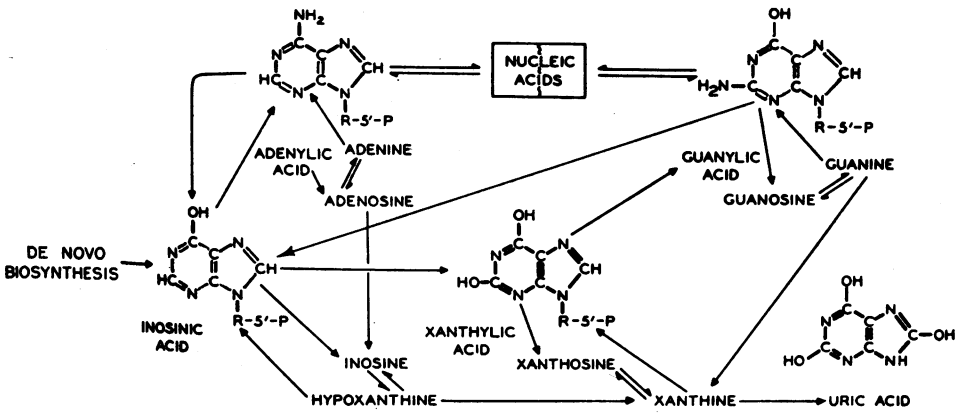

FIG. 9. INTERMEDIARY PURINE NUCLEOTIDE METABOLISM. Modified from Wyngaarden (25).

synthesis in the ALL leukocyte population studied. The pattern observed suggests that the exogenous purines, other than adenine, were insignificantly incorporated by these cells. Similar studies with leukemic cells from mice have shown that 6-mercaptopurine-resistant cells are unable to use exogenous purines (26, 27). In view of this, it is of interest that these ALL leukocytes were from a patient whose disease had become resistant to 6-mercaptopurine.

Pyrimidine metabolism. Current concepts of the biochemical pathways involved in pyrimidine synthesis and interconversion are summarized in Figure 10 (28). The pyrimidine nucleus is built up from simple precursors by a series of enzymatic reactions yielding orotic acid; several of the enzymes that catalyze specific reactions in this sequence have been demonstrated in human leukemic leukocytes by Smith, Baker and Sullivan (29). Orotic acid is converted to uridylic acid (UMP), which may then be incorporated into RNA or converted to cytidylic (CMP) or deoxyuridylic (d-UMP) acids. Exogenous UMP, uridine and uracil first enter an intracellular pool of UMP (30, 31), while exogenous CMP, cytidine and cytosine are similarly incorporated into intracellular CMP (31).

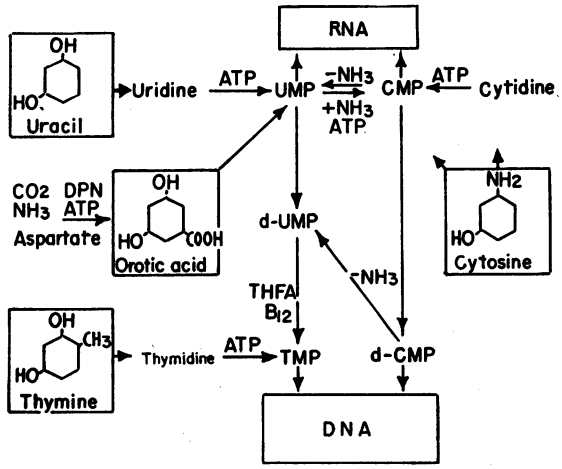

FIG. 10. BIOCHEMICAL PATHWAYS OF PYRIMIDINE SYNTHESIS AND INTERCONVERSION. Modified from Bolinder and Reichard (28).

Formate is used in the synthesis of thymine as a source of the carbon needed for the methylation of d-UMP in its conversion to thymidylic acid (TMP), a reaction catalyzed by thymidylic synthetase (7). The d-UMP required for this synthesis may be derived either from UMP or from deoxycytidylic acid (d-CMP); the conversion of d-CMP to d-UMP is catalyzed by a specific deaminase. Elevated levels of both thymidylic synthetase and d-CMP-deaminase have been found in tissues having increased rates of cell division (32).

In the present studies, formate incorporation into thymine by CML leukocytes appears to have been related to the number of mitotically active granulocytes in the incubated cell population. This suggests that *in vitro* thymine synthesis accurately reflects DNA synthesis. Concurrent studies of tritiated thymidine incorporation performed by Craddock using several of these same leukocyte populations tend to confirm this relationship (33). However, the substantial influence of added precursors or products on thymine synthesis in CML leukocytes suggests that radioformate incorporation into DNA thymine may not be a completely valid measurement of cell DNA synthesis.

The nucleosides and nucleotides of uracil and

cytosine enhance thymine synthesis, probably by contributing to an intracellular pool of d-UMP. Comparison of these compounds in CML leukocytes suggests that uridine enters this pool more readily than do deoxycytidine and cytidine.

Thymidine and TMP substantially inhibit thymine synthesis in CML leukocytes, probably by enlarging an intracellular pool of TMP, with resultant dilution of *de novo* TMP synthesis or inhibition of thymidylic synthetase. The negligible effect of the free pyrimidine bases on thymine synthesis suggests that these compounds either are poorly incorporated by the cell or are less readily incorporated than are their respective nucleosides and nucleotides.

General significance of in vitro leukocyte nucleic acid incorporation of labeled precursors. The results of these investigations imply that, in general, mechanisms demonstrated in other systems for the regulation of purine and pyrimidine biosynthesis and metabolism (5–7, 13, 21, 24, 25, 30–32) are operative in human leukemic leukocytes. Several speculations concerning the significance of certain aspects of these studies are suggested by other investigations.

Greulich, Cameron and Thrasher have recently observed that the mitotic activity of mouse intestinal epithelium is increased by the administration of thymidine. In view of this and other evidence, these workers have postulated that thymine synthesis, which is bypassed by the addition of thymidine, is rate limiting for DNA synthesis (34). If this be so, the increased thymine synthesis observed upon addition of potential precursors of d-UMP to incubated CML leukocytes suggests that, in these cells, thymine synthesis is in turn limited by the supply of such precursors. This appears not to have been the case in other leukocyte populations, since uridine, the most influential of the d-UMP precursors tested in CML leukocytes, failed to enhance thymine synthesis in CLL, AML and ALL leukocytes. This may have been due to failure of the cells to absorb uridine, limitation of cell thymidylic synthetase activity, deficiencies of B_{12} or folic acid cofactors of this enzyme (21, 28), or its inhibition by intracellular TMP.

The low rates of thymine synthesis observed in several of the acute leukemic leukocytic populations have been associated with similar low rates of tritiated thymidine incorporation (33). These results and those of previous studies by Craddock (10) and Gavosto, Mariani and Pileri (35) indicate that the rate of DNA synthesis of some acute leukemic cell populations may be quite low. To explain this finding and the apparent failure of such cells to undergo normal maturation, Gavosto and colleagues have postulated the presence of a functional defect of DNA synthesis (35).

The negligible *in vitro* thymine synthesis found in mature granulocytes and lymphocytes was expected from previous studies (3), their lack of *in vitro* and *in vivo* thymidine incorporation, and other evidence of the absence of DNA synthesis in these cells (10, 36). However, the incorporation of preformed purine into RNA by these mature leukocytes indicates that synthesis of this nucleic acid does occur. It is unlikely that this represents net synthesis of RNA, since RNA content decreases progressively with increased cell maturity (18–20, 22). Thus, this process may reflect mere turnover of cell RNA. RNA turnover may be related, in turn, to cell synthesis of enzymes or other proteins, in view of the theoretical dependence of protein synthesis on concurrent RNA synthesis and the parallel rates of RNA and protein metabolism found in normal marrow cells (37).

The present studies suggest that RNA turnover in dividing cells may also reflect the utilization of RNA nucleotides for DNA synthesis. The most direct evidence of this is the enhancement of thymine synthesis produced in CML leukocytes by precursors of d-UMP which are normally present in substantial amounts only in cell RNA. A less direct indication of this is the imbalance between DNA thymine and DNA purine synthesis or incorporation in these same cells, which could be balanced by conversion of the RNA purine nucleotides to deoxynucleotides. Previous evidence in favor of the transformation of RNA to DNA in maturing cells has been summarized by Lajtha (22). If this transformation truly exists, the postulated functional defect of DNA synthesis in acute leukemic leukocytes may result from the inability of these cells to use their RNA nucleotides for DNA synthesis.

SUMMARY

1. The incorporation of C^{14}-labeled adenine and formate into the nucleic acid bases of isolated hu-

man leukemic leukocytes of various types has been studied *in vitro*.

2. Adenine-C^{14} was incorporated into both nucleic acid adenine and guanine by each of the leukocyte populations examined. Within the limits of the analytical procedure, nucleic acid incorporation was entirely into RNA. Incorporation into the mixed nucleic acid purines was related generally to the RNA content of the different leukocyte types.

3. RNA incorporation of labeled adenine by mature leukocytes appears to represent turnover rather than net synthesis of this nucleic acid fraction.

4. Leukocyte nucleic acid purine incorporation of formate-C^{14} *in vitro* reflects only the utilization of formate carbon for closure of the purine ring of an otherwise complete precursor, 5-amino-4-imidazole carboxamide (AIC) ribotide, and subsequent incorporation of the labeled purine into RNA adenine and guanine. *De novo* synthesis of DNA purines could not be demonstrated. In the absence of exogenous AIC, *de novo* synthesis of RNA purines was observed only in acute leukemic leukocytes. Supplementary AIC enhanced *de novo* purine synthesis several-fold in acute leukemic leukocytes. The *de novo* purine synthesis induced in chronic lymphocytic (CLL) and chronic myelocytic (CML) leukocytes by supplementary AIC was less extensive than that of acute leukemic cells.

5. In the absence of exogenous pyrimidines, the extent of formate-C^{14} incorporation into DNA thymine by different leukocytes agreed generally with other indices of DNA synthesis. In CML leukocytes, however, thymine synthesis was enhanced by exogenous uridine and other pyrimidine nucleosides and nucleotides. A similar effect of exogenous uridine in CLL and acute leukemic leukocytes was not seen.

6. The effects of exogenous pyrimidines on thymine synthesis in CML leukocytes and of exogenous AIC on *de novo* purine synthesis indicate that leukemic leukocyte nucleic acid synthesis is determined, in part, by the availability of precursors of the nucleic acid bases.

7. In CML leukocytes, utilization of RNA nucleotides for DNA synthesis is suggested by the nucleic acid incorporation pattern of these cells *in vitro*. The low rate of DNA synthesis found in some acute leukemic leukocytes in this study and in previous investigations may reflect a block in the conversion of RNA to DNA.

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