MEMBRANE TRANSPORT BY MURINE LYMPHOCYTES

I. A Rapid Sampling Technique as Applied to the Adenosine and Thymidine Systems*

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During the process of an immune response the small lymphocyte undergoes drastic alterations in morphology and activity. As the original stimulus arises outside the cell, many of these changes are thought to originate at the cell surface. One alteration that has been postulated to occur at the plasma membrane is in permeation of both electrolytes, such as potassium and calcium (2, 22, 26–28, 36, 37), and nonelectrolytes, such as sugars (2, 23), amino acids (2, 3, 5, 12, 13, 16, 19, 20, 38), and nucleosides (4, 24).

Permeability is a property of the membrane and may encompass a number of processes. What has probably been measured in the published studies is uptake, which reflects a complex process of which only one component is translocation across the plasma membrane. To measure carrier-mediated translocation one must measure influx during the time interval before back flux becomes significant. In addition, a carrier-mediated transport system is one which is defined as demonstrating saturation of uptake with increasing substrate concentration and also chemical specificity (7, 9).

Here we describe a rapid sampling technique for animal cells in suspension which we have developed for the small lymphocyte. Using this method, we have described the uptake of thymidine and the uptake and transport systems of adenosine by murine bulk nonadherent spleen cells.

Materials and Methods

Preparation of Cells. Murine nonadherent spleen cells were prepared in the standard fashion (21, 23, 34, 35) except that all manipulations were carried out in LMGB,[1] which consists of 10 mM sodium phosphate buffer, pH 7.5, containing 0.9% NaCl, 0.1% bovine serum albumin, 5 × crystallized (Sigma Chemical Co., St. Louis, Mo.), and 5 mM glucose. CD-1 male mice, 6–8-wk old, were obtained from Charles River Breeding Farms, Wilmington, Mass. Mice were sacrificed by cervical dislocation, and the spleens were immediately excised and placed in 30 ml chilled LMGB. Cells were gently teased from the spleen capsules, filtered through Nytex bolting cloth (Tobler, Ernst, and Trafer, Elmsford, N. Y.), and centrifuged at 500 *g* for 10 min at 4°C. The pellet was washed once with LMGB and resuspended in 30 ml LMGB. 5-ml aliquots of the suspension were layered over 3 ml Ficoll-Hypaque (a mixture of 12 parts 9% [wt/vol] Ficoll, Sigma Chemical Co. and 5 parts 34% [wt/vol] Hypaque, Sterwin Chemical Co., New York) and spun at room tempera-

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[1] *Abbreviations used in this paper:* LMGB, leukocyte medium with glucose and bovine serum albumin; PCA, perchloric acid; TCA, trichloroacetic acid.

ture for 20 min at 400 *g*. Only the cells that banded at the interface were removed and incubated in plastic Petri dishes at 37°C for 30–45 min. Nonadherent cells were centrifuged, counted, and resuspended in LMGB to the desired concentration, usually 1×10^7 cells/ml. Using morphological and adherence criteria, we obtained about 97% lymphoid cells. Whenever the percentage of contaminating cells exceeded 5%, the suspension was discarded. Viability by trypan blue exclusion, whether at this time or after experimentation was finished, was about 97%.

Measurement of Transport. Rapid separation of lymphocytes from medium was accomplished by centrifugation through oil in a fashion similar to that originally described for beef heart mitochondria (14) and later applied to *Escherichia coli* cells (18) and to thymocytes (1). Transport was measured by mixing 0.1 ml of prewarmed LMGB containing 1×10^6 cells with 0.2 ml of prewarmed substrate at 3/2 the desired concentration and 5–7 μCi tritiated substrate/ml. A 0.2-ml Biopet (BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.) was used for dispensing cells into the incubation vessel. The radioisotope was then added to the cells and rapidly mixed. After incubation at 37°C for the appropriate length of time, 0.2 ml was layered into a 400 μl microfuge tube containing 40 μl 10% perchloric acid as the bottom layer and a 150 μl layer of silicone oil (a mixture of 4 vol Dow Corning 550 fluid and 6 vol Dow Corning 510 fluid, 50 centistokes, Dow Corning Corp., Midland, Mich.). The tube was spun at 10,000 rpm for 30 s by means of an Eppendorf microfuge. The cells passed through the oil layer and into the acid layer; separation of the cells from the medium presumably occurred immediately as they traversed the top meniscus of the oil layer (see time studies below). Only radioactive material which was cell associated penetrated the oil layer, since centrifugation of isotope in the absence of cells gave negative results. The tip of the tube was then sliced into a scintillation vial for counting. Cellular material was digested with 1.0 ml of 0.5 N KOH, containing phenol red, for more than 30 min and then neutralized with 10% perchloric acid. 10-ml scintillation cocktail (Scintiverse, Fisher Scientific Co., Pittsburgh, Pa.) was added and the samples counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) with efficiency corrections for each vial. Efficiencies ran between 29 and 45%.

Purification of Radioactive Compounds. Radioactive materials were purchased from New England Nuclear, Boston, Mass. or from Schwartz-Mann Div., Becton Dickinson and Co., Orangeburg, N. Y. Tritiated compounds were purified before use by means of ascending paper chromatography on Whatman no. 1 paper in one of several systems described by Fink et al. (11).

Metabolism Studies. Metabolism of adenosine and thymidine was studied in several ways. Cells were incubated at the desired substrate concentration with 25 μCi/ml radioactive substrate and after the desired time interval separated from the medium as described above. The perchloric acid extracts from 10 samples were pooled and neutralized immediately with KOH. Aliquots of both the neutralized perchloric acid (PCA) extracts of centrifuged cells and of cell supernatant fluids were chromatographed on Dowex 2-X8 ion exchange resin (formate form) in a 0.5-cm diameter × 21-cm high column. Four successive eluates of 5.0 ml water, followed by four successive eluates of 5 ml of 4 M ammonium formate in 2 M formic acid, were collected. [^{14}C]sucrose and [^{14}C]thymidine monophosphate were used as standards.

In other studies, uptake of thymidine over a 20-s interval was compared with uptake and metabolism over a 2-h period. 1-ml cells at 1×10^7 cells/ml was mixed with 2 ml of isotope solution and incubated at 37°C with agitation for 2 h. A 0.2-ml aliquot was removed for centrifugation through oil into PCA in order to determine total uptake; to the remainder was added 3.0 ml 10% trichloroacetic acid (TCA). Alternately, cells were incubated for 2 h as described above. A portion was removed to determine total uptake; 10 × 0.2-ml portions were also removed and spun through oil into 10% TCA. The TCA extracts were carefully pooled and treated in the following manner (29): each pool was centrifuged at 2,000 *g* for 10 min and the precipitate washed once with cold 5% TCA. The precipitate was resuspended twice in 100% ethanol at 65°C for 5 min each time to remove isotope incorporated into lipid. It was then resuspended in 5% TCA and heated to 90–95°C for 15 min. The resultant supernate contains material of nucleic acid or polynucleotide origin (10, 29). The precipitate contains protein (29). Calculated recoveries at each step indicated that significant amounts of isotope were ethanol extractable, while variable, small numbers of counts remained in the final precipitate. Therefore, in all the studies reported here hot ethanol and hot TCA extractions were performed, and the values reported are those obtained from the hot TCA supernatant fluid.

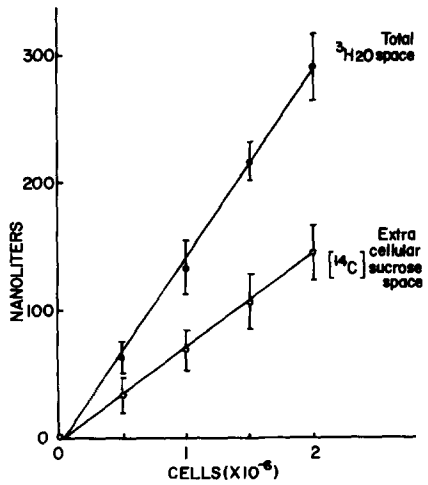

FIG. 1. Total and extracellular space in centrifuged cell pellets. Cells at various concentrations (0.5–2 × 10^7 cells/ml) were incubated in LMGB containing ^3H_2O (1.25 mCi/ml) and [^{14}C]sucrose (2.5 μCi/ml) for 1 min at 37°C, centrifuged through oil as described in Materials and Methods, and the bottoms of the microfuge tubes containing the PCA phase immediately dropped into scintillation fluid. The ^{3}H and ^{14}C radioactivity of the incubation medium was determined from counting 10-μl aliquots of the supernatant fluid. ^3H_2O rapidly equilibrates with internal cellular water, while the cells are impermeable to [^{14}C]sucrose. The lines are calculated by regression and analysis of points which are the averages of three to five experiments carried out in quadruplicate. The bars represent ± the standard deviation of the mean.

Results

Rapid Separation of Cells from Medium. To characterize transport in mouse spleen cells, we developed a technique capable of separating cells from medium over time intervals as short as 4 s (see Materials and Methods). When cells were centrifuged through oil into 10% sucrose, the number of cells recovered in the pellet was greater than 98%, and fewer than 1% of the applied cells were recovered in the supernatant fluid. The quantitative recovery of cells and virtual exclusion of the incubation medium is also shown by the fact that both total and extracellular water spaces in the bottom layer varied directly with the number of cells centrifuged (Fig. 1). In most uptake experiments 6.7 × 10^5 cells in 200 μl medium were layered over silicone oil. That number of cells contained 52.3 nl intracellular water and cocentrifuged with 50.1 nl medium, the total space (102.4 nl) representing 0.05% of the original 200 μl vol. When no cells were present, no radioactivity was found in the acid fraction. In addition, the amount of substrate recovered in subsequent transport studies in the pellet varied directly with the numbers of cells in the incubation medium (not shown).

Adenosine Uptake, Time Dependence. To measure unidirectional flux, it is necessary to measure uptake when that process is linear with time and extrapolates to zero (6). A plot of uptake vs. time at sufficiently low adenosine concentrations (1 mM or less) has a line that extrapolated through the origin (Fig. 2). The linearity of uptake at low concentrations (50 μM) of substrate lasted for more than 5 min. The uptake values plotted in this graph are the carrier-medi-

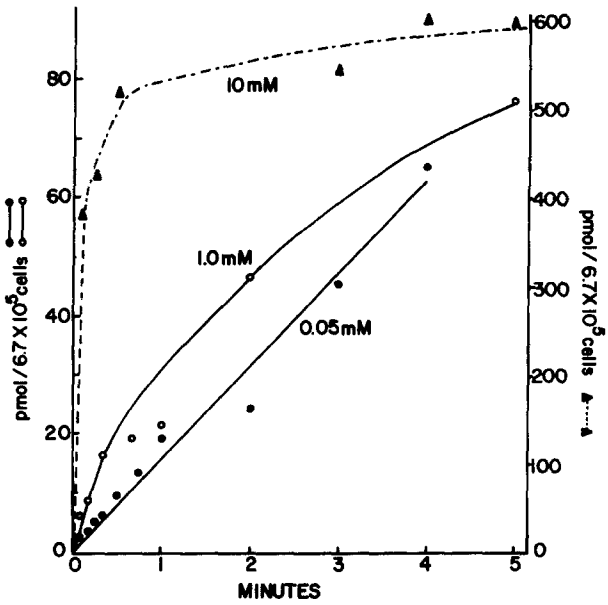

FIG. 2. Time dependence of adenosine uptake. Each vessel contained 0.1 ml of cells at 1×10^7 cells/ml to which was added 0.2 ml of LMGB containing adenosine (75 μM, 0.15 mM, and 15 mM, 6 μCi/ml). Samples of 0.2 ml were taken at indicated times, centrifuged, and the cell pellets counted as described in Materials and Methods. The contribution for nonspecific adsorption and diffusion was subtracted from the values at two lower adenosine concentrations. Each point was determined in quadruplicate.

ated component: the radioactivity ascribable to mechanical trapping and diffusion has been subtracted (see concentration dependence studies below) by incubating cells with 10 mM adenosine for each sampling time. Those values that represent mechanical trapping, adsorption, and passive diffusion decreased with time from 28% at 5 s to 3% at 4 min.

In contrast to the time-course at low adenosine concentrations, the interval of linearity at higher concentrations was shorter (Fig. 2). At 1.0 mM adenosine, carrier-mediated uptake was linear for about 30 s. Thus, in subsequent experiments cell samples were routinely incubated for 20-s intervals in order to work within the time of initial rates of uptake and thereby measure unidirectional flux.

Adenosine Uptake, Kinetics. Carrier-mediated transport systems show saturation with increasing substrate concentration. When the dependence of transport was determined in relation to the substrate concentration, the resulting data could be resolved into a nonsaturating component (adsorption-diffusion) (Fig. 3) and into two saturable systems (Fig. 4). As seen in Fig. 3 at adenosine concentrations above 0.5 mM, the amount of radioactivity that was cell-associated reached a minimum level. That level is thought to come from mechanical trapping of medium which cocentrifuges with the cells and from nonspecific, nonsaturable passive diffusion and/or adsorption. Since saturation occurred above 0.5 mM, it was appropriate to use 10 mM in all subsequent experiments for determination of the nonspecific component in our data. Therefore, the radioactivity that was cell associated when the external medium concentration

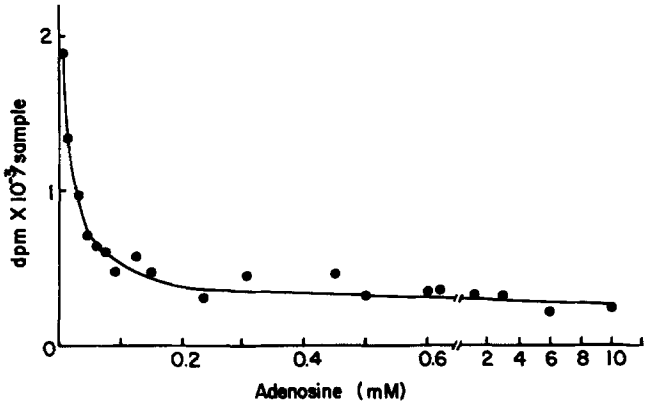

FIG. 3. Saturation of adenosine uptake. Each reaction vessel contained 0.1 ml cells at 1 × 10^7 cells/ml to which was added 0.2 ml LMGB with adenosine at concentrations increasing from 3.3 μM to 10 mM. The radioactive adenosine was constant at 5 μCi/ml of reaction mixture. After 20-s incubation, the cell-associated radioactivity was measured as described in Materials and Methods.

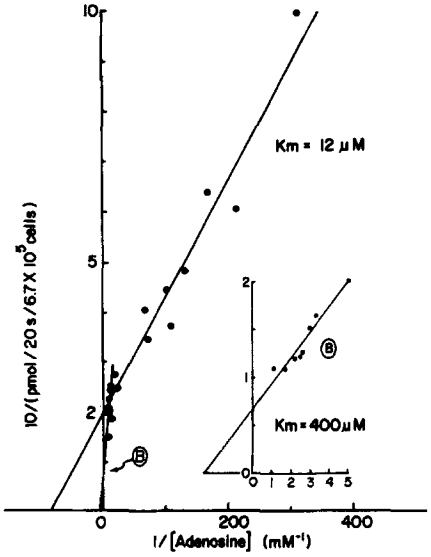

FIG. 4. Kinetics of adenosine uptake. Carrier-mediated uptake of adenosine over 20 s was measured as described in Materials and Methods, by subtracting the radioactivity measured at 10 mM adenosine from the total activity measured at each of the indicated concentrations (3.3 μM to 1 mM). Disintegrations per minute were then converted to picomoles substrate transported/20 s. The values pooled from 17 experiments and calculated by the method of least squares are represented as a Lineweaver-Burk plot. Insert (Fig. 4) is an expansion of the data obtained at adenosine concentrations greater than 0.2 mM.

was 10 mM was routinely subtracted from cell-associated radioactivity at lower concentrations, where carrier-mediated processes occurred.

After subtraction of the counts due to adsorption-diffusion, the resulting data could be resolved into two systems (Fig. 4). As seen in Fig. 4, which is a composite of 17 experiments, there was a high affinity system with a K_m of 12

μM. On graphic expansion of the experimental points (Fig. 4, insert), a second, low affinity system, with a K_m of 400 μM could be discerned. That the discontinuous derivative represents two different transport systems or at least very different forms of the same transport system was confirmed by the specificity studies described below.

Adenosine Transport, Specificities. A characteristic feature of carrier-mediated transport is specificity for the transport substrate and closely related chemical analogues. A number of compounds related to adenosine was tested as potential inhibitors.

The analogue 2-deoxyadenosine was found to inhibit adenosine uptake by mouse spleen lymphocytes. When the high affinity adenosine transport system was examined, the presence of 5 mM unlabeled 2-deoxyadenosine showed competitive inhibition kinetics (Fig. 5). At high adenosine concentrations (Fig. 5, insert), the line drawn in the Lineweaver-Burke plot extended through the origin. The apparent K_I for 2-deoxyadenosine was calculated to be 230 μM for the high affinity adenosine transport system. Thus, the two systems are affected differently by this analogue.

The other analogues tested had less effect. When cells were exposed to adenosine in the presence of 5-mM ribose, the transport of adenosine was not inhibited. Adenine (10 mM) decreased carrier-mediated transport measured at 1 mM adenosine by about 50%. When tested at adenosine concentrations of 50 μM or less, 5 mM adenine showed competitive inhibition of the high K_m adenosine system with an apparent K_I of 350 μM. Thymidine showed noncompetitive inhibition of the high affinity system with an apparent K_I of 525 μM. In the case of thymidine, the low affinity system was not inhibited.

Adenosine Uptake, Metabolism. We have carefully defined the term transport to mean saturable, unidirectional flux across the plasma membrane. However, after a 20-s exposure to either 10 or 100 μM adenosine, the substrate that was cell associated was 87% phosphorylated. Essentially no radioactive material that is adsorbable to negatively charged resin (Dowex 2-X8) was found in the supernate. If the capacity to phosphorylate greatly exceeds the rate of uptake, then membrane translocation can be considered rate limiting (25). Indeed, these cells were capable of phosphorylating 2.05 nmol adenosine taken up over a 20-s interval when the external concentration of substrate was 10 mM. This amount is more than 100 times the content of phosphorylated derivative found at 10 μM adenosine. Therefore, translocation may be the rate limiting step under these conditions.

Thymidine Uptake, Time Dependence. Not only was far less thymidine than adenosine taken up by these lymphocytes, but time dependence of uptake could not be shown by our method (Fig. 6). At thymidine concentrations greater than 80 nM, saturation with time occurred as quickly as the first sample could be taken (4 s). At 80 nM thymidine, time-dependent uptake was sometimes observed; however, a period when initial rates held was not obtainable in a reproducible fashion.

Thymidine Uptake, Kinetics. The amount of uptake of this nucleoside over a wide concentration range varied from experiment to experiment and was always well below the uptake of adenosine measured at similar concentrations. In

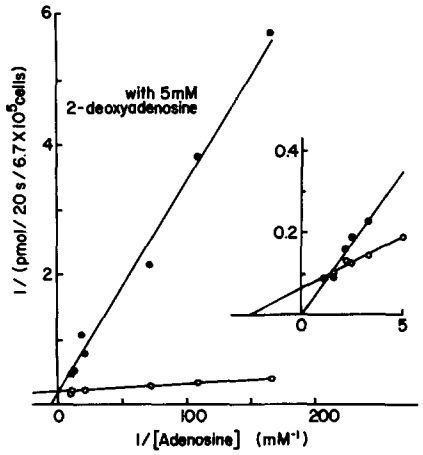

FIG. 5. Effect of 2-deoxyadenosine on adenosine uptake. The experiment was performed as in Fig. 4; 5 mM 2-deoxyadenosine was present where indicated.

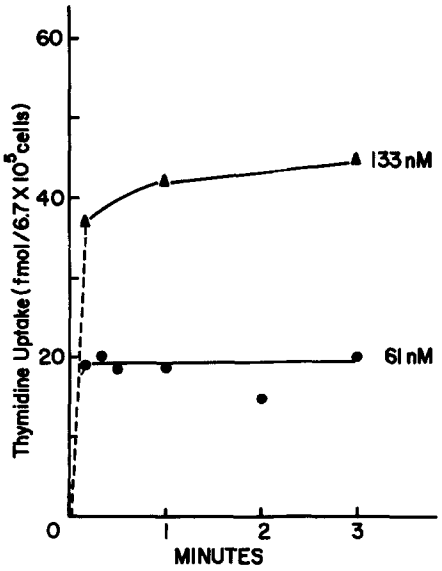

FIG. 6. Time dependence of thymidine uptake. The experimental conditions were as described in Materials and Methods.

addition, uptake measured over 20-s intervals was not saturated when tested at concentrations up to 10 mM thymidine (data not shown). Hence, carrier-mediated activity with definable K_m values could not be determined for thymidine.

Thymidine Uptake, Metabolism. As with adenosine, a portion of the thymidine which was cell associated at 20 s has been chemically altered. While 50% of the cell-associated thymidine could be accounted for as contributed by extracellular space, 38–47% of internalized substrate was phosphorylated as shown by ion exchange chromatography. No phosphorylated substrate was detected in the supernate. We were concerned with how much cell-associated thymidine was

TABLE I
Comparison of Uptake of [^{3}H]Thymidine with TCA Precipitable Counts

Measurement	Experiment		
	I	II	III
A. Total radioactivity in medium (dpm × 10^{-6} per 10^7 cells)	24.0	21.8	19.2
B. Radioactivity in hot TCA fraction after 2 h (dpm × 10^{-6} per 10^7 cells)	0.27	0.38	0.16
Percent of radioactivity in hot TCA fraction (A/B × 100)	1.1	1.7	0.8
C. Uptake/2 h (dpm × 10^{-6} per 10^7 cells)	0.122	0.168	0.072
Percent of radioactivity taken up in 2 h (C/A × 100)	0.5	0.7	0.3
Percent of radioactivity taken up in 2 h found in hot TCA fraction (B/C × 100)	221	226	222
D. Uptake/20 s (dpm × 10^{-6} per 10^7 cells)	0.015	0.014	0.008

Cells were incubated in bulk (3.0 ml, 3.3 × 10^6/ml) in the presence of 80 nM [^{3}H]thymidine, 4–6 μCi/ml. After 2 h, 0.2 ml was withdrawn to measure uptake by the oil microfuge technique (line C), and then 3.0 ml 10% TCA was added to the remaining suspension. Extraction, including two ethanol washes at 65°C, was carried out as described in Materials and Methods. The counts appearing in the nucleic acid fraction (hot TCA fraction) are reported in line B. Cells were also exposed to 80 nM [^{3}H]thymidine, 4–6 μCi/ml, in the standard fashion for 20 s and separated from the incubation mixture by the oil-microfuge technique. The values are reported as line D.

incorporated into TCA-precipitable material, since the availability of different size precursor pools may alter the apparent synthetic rates of macromolecules and in turn, the size of precursor pools may be altered by the transport process. Cells were incubated for 2 h in the presence of approximately 80 nM or 10 mM tritiated thymidine, the former being the lowest concentration obtainable within the limits of the highest specific activity of commercially available tritiated thymidine.

Total uptake of thymidine at 80 nM, as determined by centrifugation through oil into PCA, was compared with radioactivity associated with the nucleic acid fraction after TCA precipitation (see Materials and Methods and Table I). Total uptake at 2 h was also compared with radioactivity associated with the nucleic acid fraction after cells had been washed once with LMGB before addition of TCA (8) and after they had been separated from medium by centrifugation through oil into TCA (see Materials and Methods). Table II is a comparison of the results obtained by these techniques. If cells were directly precipitated with TCA, the number of counts recovered in the nucleic acid fraction greatly exceeded the counts that were cell associated by the oil centrifugation method. While the former represented only 1% of total radioactivity with which cells were incubated, it was twice the number of counts which were recovered by the rapid sampling method reported here.

TABLE II

Comparison of Uptake of [^{3}H]Thymidine with TCA Precipitable Counts before and after Centrifugation through Oil

Exp.	Uptake (dpm × 10^{-4} per 10^7 cells)	Radioactivity in hot TCA fraction (dpm × 10^{-4} per 10^7 cells)		
		Cells through oil into TCA	Standard TCA extraction	Standard TCA extraction after one wash
I	6.21 (100%)	3.88 (62.4%)	11.69 (188%)	11.07 (178%)
II	7.94 (100%)	2.04 (25.0%)	12.23 (153%)	12.92 (162%)

Cells were incubated in bulk as described in the legend for Table I for 2 h. 0.2-ml aliquots were withdrawn to determine uptake. They were then handled in one of three ways: (*a*) 10 × 0.2-ml aliquots were removed and spun by the oil microfuge technique into 10% TCA. The material in the bottom of the tubes was quantitatively recovered and pooled. Extraction was carried out as described in Materials and Methods. (*b*) 3 ml 10% TCA was added to the remaining cell suspension. (*c*) Cells were washed once and resuspended in 3.0 ml LMGB. 3 ml 10% TCA was then added and extraction carried out in a similar fashion.

Moreover, even if cells were washed once with LMGB before TCA was added, the number of counts in the nucleic acid fraction still greatly exceeded the cell-associated counts recovered by the rapid sampling method. On the other hand, if cells were centrifuged through oil into 10% TCA before further extraction, the amount of radioactivity in the nucleic acid fraction never exceeded the total radioactivity taken up. That percentage varied greatly from experiment to experiment and ranged from 25 to 65% of the total cell-associated counts. These percentages fall within the range of percent phosphorylated thymidine recovered after much shorter exposures to the nucleoside. Hence, the standard method for measuring thymidine utilization yields estimates under these conditions which can be as much as 100% too high.

Discussion

The development of a rapid sampling technique for suspended cells has allowed us to delineate two transport systems of bulk mouse spleen nonadherent white cells. This technique complements the rapid sampling techniques described for cells that are capable of adhering to a solid substratum (6, 7, 15, 30, 31, 32, 33). The silicone oil-centrifugation method results in the rapid separation of intact lymphocytes with samples taken as rapidly as 4 s of incubation time. When cells are centrifuged by conventional techniques, a layer of cells in the pellet is in contact with the incubation medium until the latter has been removed.

We chose to investigate transport of two compounds of critical importance in cellular metabolism, adenosine and thymidine. The former is a key intermediate of energy metabolism; the latter is incorporated into nucleic acid.

The kinetics of adenosine uptake by these cells demonstrate the importance of early sampling: uptake was linear with time for relatively long periods (5 min or

longer) only when the adenosine concentration was relatively low (50 μM); when the substrate concentration was increased, this period progressively decreased, lasting about 30 s at 1 mM adenosine. Hence, it is important to sample the incubation mixtures at times sufficiently short to reflect the initial uptake velocity for all substrate concentrations studied.

The concentration dependence of adenosine transport could be resolved into two carrier systems: a low affinity system (K_m = 400 μM), and a high affinity system (K_m = 12 μM). The latter system is relatively specific for this nucleoside. Of the chemical analogues tested, 2-deoxyadenosine competitively inhibited adenosine uptake with high inhibitor affinity. Adenine, thymidine, and purine, but not ribose, also showed some affinity for this system. Two carrier-mediated systems for adenosine have not been demonstrated in animal cells previously (6, 25). Whether each system can be accounted for by different lymphocyte classes remains to be explored.

Internal adenosine accumulation is the result of both transport and further metabolism, since more than 50% of the cell-associated radioactive material was phosphorylated; the remainder could be accounted for as being present in the extracellular fluid. The metabolism of adenosine may explain the relatively long period of linear uptake found at low adenosine concentrations, since membranes are impermeable to phosphorylated derivatives which would then be trapped inside the cell.

The uptake of thymidine is of interest because the incorporation of this compound into TCA precipitable material is a common assay for blastogenesis (17). In addition, thymidine analogues are among the most common and potent anti-cancer drugs in use. The bulk nonadherent cell population from the spleens of normal healthy mice took up thymidine in a different fashion from adenosine. Linearity of uptake with time occurred for less than 5 s at thymidine concentrations of 80 nM or greater, and uptake measured over a 20-s interval was not saturated at 10 mM thymidine. The thymidine system, therefore, cannot be described as carrier mediated. However, some of the thymidine that was cell associated could not be accounted for by the contaminating extracellular fluid because 10–20% of the total was phosphorylated while no phosphorylated thymidine derivatives were present in the supernatant fluid. In experiments now in progress, a thymidine carrier-mediated system can be demonstrated in cells from lectin-stimulated mice; hence the negative results obtained with cells from normal mice can be considered significant. The low thymidine uptake by unstimulated cells is consonant with these cells being in the resting state.

Our studies indicate the importance of comparing recoveries for incorporation into cellular material versus total precursor taken up. Using the oil centrifugation technique, we have shown that only by thoroughly stripping extracellular thymidine from cells before TCA is added can one have confidence in the apparent rate of nucleic acid synthesis as measured by TCA precipitable counts. To explain the phosphorylation and apparent incorporation into the nucleic acid fraction we have to hypothesize that a few cells are rapidly synthesizing DNA or that thymidine is incorporated into polynucleotides or involved in repair synthesis when lymphocytes are in the resting state. Bulk murine nonadherent cells are by no means a homogeneous population. Also, Cleaver and Boyer have

shown that only 16–18 bases are required for a polynucleotide to be precipitable by 5% TCA (10). Therefore, both possibilities remain to be explored.

Summary

We have developed a rapid sampling technique for animal cells in suspension for the purpose of measuring membrane transport in lymphocytes. The method involves rapid centrifugation of cells through a layer of silicone oil into perchloric acid after incubation periods as short as 4 s. Using this method we have described the uptake of thymidine and the uptake and transport systems of adenosine by murine bulk nonadherent spleen cells. The two uptake systems are markedly different. Adenosine was shown to be taken up by classical carrier-mediated diffusion, while thymidine was not.

In addition we have explored the metabolism of the two nucleosides under the conditions we employed for measuring transport or uptake. Both nucleosides are phosphorylated extensively. We also investigated the uptake and metabolism of thymidine over a 2-h interval, the standard time used to measure DNA synthesis in lymphocytes. Unless cells were separated from medium by centrifugation through oil before TCA addition, the TCA precipitable counts exceeded the total radioactive uptake. Hence the standard method for measuring thymidine utilization yields estimates under these conditions which can be as much as 100% too high.

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References

1. Andreasen, P., and B. Schaumberg. 1974. A rapid technique for separation of thymocytes from suspensions by centrifugation through silicone oil. *Anal. Biochem.* 59:610.
2. Averdunk, R. 1972. Uber die Wirkung von Phytohemagglutinin und Anti-lymphozytenserum auf den Kalium-, Glucose- und Aminosaure-Transport bei menschlichem Lymphozyten. *Hoppe-Seyler's Z. Physiol. Chem.* 353:79.
3. Baran, D., W. Peck, P. Frengley, and M. Lichtman. 1973. Cortisol-induced inhibition of amino acid transport in thymic lymphocytes. *Biochim. Biophys. Acta.* 307:627.
4. Barlow, S., and M. Ord. 1975. Thymidine transport in phytohaemagglutinin-stimulated pig lymphocytes. *Biochem. J.* 148:295.
5. van den Berg, K. and I. Betel. 1973. Early increase of amino acid transport in stimulated lymphocytes. *Exp. Cell Res.* 66:257.
6. Berlin, R. D. 1973. Temperature dependence of nucleoside membrane transport in rabbit alveolar macrophages and polymorphonuclear leucocytes. *J. Biol. Chem.* 248:4724.
7. Berlin, R., and J. Oliver. 1975. Membrane transport of purine and pyrimidine bases and nucleosides in animal cells. *Int. Rev. Cytol.* 42:287.
8. Beyer, C., and W. Bowers. 1975. Periodate and concanavalin A induced blast transformation of rat lymphocytes by an indirect mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 72:3590.
9. Christensen, H. N. 1969. Some special kinetic problems of transport. *Adv. Enzymol. Relat. Areas Mol. Biol.* 2:1.

10. Cleaver, J., and H. Boyer. 1972. Solubility and dialysis limits of DNA oligonucleotides. *Biochim. Biophys. Acta.* **262**:16.
11. Fink, K., R. Cline, and R. Fink. 1963. Paper chromatography of several classes of compounds; correlated R_f values in a variety of solvent systems. *Anal. Chem.* **35**:389.
12. Frengley, P., W. Peck, and M. Lichtman. 1974. Accelerated active transport of alpha aminoisobutyric acid by human leukemic leukocytes in an amino acid deficient environment. *Exp. Cell Res.* **88**:442.
13. Frengley, P., W. Peck, and M. Lichtman. 1975. Inhibition of time-dependent enhancement of amino acid transport by leukemic leukocytes: a possible index of the sensitivity of cell to drugs. *Blood Cells.* **1**:139.
14. Harris, E., and K. van Dam. 1968. Changes of total water and sucrose space accompanying induced ion uptake or phosphate swelling of rat liver mitochondria. *Biochem. J.* **106**:759.
15. Hawkins, R., and R. Berlin. 1969. Purine transport in polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **173**:324.
16. Helmreich, E., and D. Kipnis. 1962. Amino acid transport in lymph node cells. *J. Biol. Chem.* **237**:2582.
17. Ling, N. R. 1968. Lymphocyte Stimulation. North-Holland Publishing Co., Amsterdam.
18. Manno, J., and D. Schacter. 1970. Energy-coupled influx of thiomethylgalactoside into *Escherichia coli*. *J. Biol. Chem.* **245**:1217.
19. Mendelsohn, J., A. Skinner, and S. Kornfeld. 1970. Rapid induction of increased α-aminoisobutyric acid uptake by phytohemagglutinin stimulated lymphocytes. *Proc. 5th Leucocyte Cult. Conf.*
20. Mendelsohn, J., A. Skinner, and S. Kornfeld. 1971. The rapid induction of phytohemagglutinin of increased α-aminoisobutyric acid uptake by lymphocytes. *J. Clin. Invest.* **50**:818.
21. Noble, P., J. Cutts, and K. Carroll. 1968. Ficoll flotation for the separation of blood leukocyte types. *Blood.* **31**:66.
22. Parker, C. 1974. Correlation between mitogenicity and stimulation of calcium uptake in human lymphocytes. *Biochem. Biophys. Res. Commun.* **61**:1180.
23. Perper, R., T. Zee, and M. Mickelson. 1968. Purification of lymphocytes and platelets by gradient centrifugation. *J. Lab. Clin. Med.* **72**:842.
24. Peters, J., and P. Hausen. 1971. Effect of phytohemagglutinin on lymphocyte membrane transport. I. Stimulation of uridine uptake. *Eur. J. Biochem.* **19**:502.
25. Plagemann, P., D. Richey, J. Zylka, and J. Erbe. 1975. Cell cycle and growth stage-dependent changes in the transport of nucleosides, hypoxanthine, choline and deoxyglucose in cultured Novikoff hepatoma cells. *J. Cell Biol.* **64**:29.
26. Quastel, M., D. Dow, and J. Kaplan. 1970. Stimulation of K^{42} uptake into lymphocytes in phytohemagglutinin and role of intracellular K^+ in lymphocyte transformation. *Proc. 5th Leucocyte Cult. Conf.*
27. Quastel, M., and J. Kaplan. 1968. Inhibition by ouabain of human lymphocyte transformation induced by phytohaemagglutinin in vitro. *Nat. New Biol.* **219**:198.
28. Quastel, M., and J. Kaplan. 1970. Early stimulation of potassium uptake in lymphocytes treated with PHA. *Exp. Cell Res.* **63**:230.
29. Rendina, G. 1971. Experimental Methods in Modern Biochemistry. W. B. Saunders Co., Philadelphia, Pa.
30. Strauss, P. R., and R. D. Berlin. 1973. Effects of serum on membrane transport I. Separation and preliminary characterization of factors which depress lysine or stimulate adenosine transport in rabbit alveolar macrophages. *J. Exp. Med.* **137**:359.
31. Strauss, P. R. 1974. Effects of serum on membrane transport. II. Serum and the stimulation of adenosine transport, a possible mechanism. *J. Cell Biol.* **60**:571.

32. Tsan, M. F., and R. D. Berlin. 1971. Membrane transport in the rabbit alveolar macrophage. The specificity and characteristics of amino acid transport systems. *Biochim. Biophys. Acta.* **241**:155.
33. Tsan, M. F., and R. D. Berlin. 1971. Effect of phagocytosis on membrane transport of nonelectrolytes. *J. Exp. Med.* **134**:1016.
34. Unanue, E., W. D. Perkins, and M. J. Karnovsky. 1972. Ligand-induced movement of lymphocyte membrane macromolecules. I. Analysis by immunofluorescence and ultrastructural radioautography. *J. Exp. Med.* **136**:885.
35. Unanue, E., M. Karnovsky, and H. Engers. 1973. Ligand-induced movement of lymphocyte membrane macromolecules. III. Relationship between the formation and fate of anti-Ig-surface Ig complexes and cell metabolism. *J. Exp. Med.* **137**:675.
36. Whitney, R., and R. Sutherland. 1973. Characteristics of calcium accumulation by lymphocytes and alterations in the process induced by phytohemmagglutinin. *Proc. 7th Leucocyte Cult. Conf.*
37. Wright, P., M. Quastel, and J. Kaplan. 1973. Inhibition by ouabain of lymphocyte transformation. *Proc. 7th Leucocyte Cult. Conf.*
38. Yunis, A., G. Arimura, and D. Kipnis. 1963. Amino acid transport in blood cells. I. Effect of cations on amino acid transport in human leukocytes. *J. Lab. Clin. Med.* **62**:465.