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Effect of Perfluorodecanoic Acid on the Efflux of 2-Aminopurine From L5178Y Cells

12. PERSONAL AUTHOR(S): Paul W. Wigler, Ph.D., Professor

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Yr., Mo., Day)	15. PAGE COUNT
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16. SUPPLEMENTARY NOTATION: A summary report of activities and presentations is attached.

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB. GR.	Inhibition of purine efflux by perfluorodecanoic acid, high speed transport assay, uric acid, L5178Y cells, 2-aminopurine a fluorescent transport substrate.

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Transporters in the cell membrane of animal cells are susceptible to the action of toxic environmental agents. L5178Y mouse leukemia cells were treated with 150 µg/ml perfluorodecanoic acid (PFDA), a toxicant, in growth medium for 24 hr at 30°C. The PFDA-treated cells were transferred to fresh growth medium to demonstrate recovery of the capacity to transport a fluorescent purine, 2-aminopurine (AP). The membrane flux of AP was estimated with a high-speed kinetic assay. The cells were preloaded with 100 µM AP and rinsed twice in a cold buffer. The preloaded cells were resuspended in buffer at 21°C and AP flux was estimated continuously from the fluorescence emission of AP at 370 nm. At the beginning of the recovery period, AP efflux from PFDA-treated cells was markedly inhibited in comparison with the untreated controls. After 2 days at 37°C, the count of PFDA-treated cells doubled and the initial rate of AP efflux returned to approximately 60% of the controls. After 6 days at 30°C, the cell count increased 2.7-fold and AP efflux returned to approximately 60% of the controls. The results of experiments with untreated control

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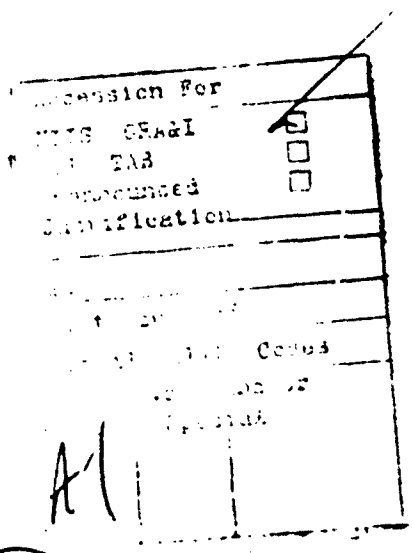
cells show that the initial rate of AP efflux is proportional to the intracellular AP concentration. A plot of the reciprocal of the rate of AP efflux against the concentration of an inhibitor (uric acid) in the external buffer is a straight line. These observations suggest the presence of a uric acid-sensitive purine channel in the membrane of L5178Y cells. The channel is inactivated by PFDA under conditions that do not appreciably change cell viability. The gradual recovery of the PFDA-inactivated purine channel can be demonstrated after the cells are transferred to fresh growth medium.



DEPARTMENT OF MEDICAL BIOLOGY
UNIVERSITY OF TENNESSEE MEMORIAL RESEARCH CENTER
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Paul W. Wigler, Ph.D., Professor

SUMMARY REPORT OF ACTIVITIES AND PRESENTATIONS:

101. Summary dates: July 1983 to August 1, 1984.

1011. Teaching: I taught MB 5430 "Metabolism of Drugs" for two credit hours in the fall term of 1983, from September through December. This was equivalent to 20 contact hours.

1012. Service: Member, Education Committee of DMB.

1013. Scholarly Activities:

10131. Presentations delivered at meetings.

A) P.W. Wigler and Y.B. Shah, July 26, 1983, "Effects of Perfluorodecanoic Acid on Transport in L5178Y Cells." Review of Air Force Sponsored Basic Research in Biomedical Sciences, University of California, Irvine, Calif. Paper #10.

B) P.W. Wigler and Y.B. Shah, November 11, 1983, invited symposium paper, "Toxicant Effects on the Efflux Rate of Flucrescent Substrates from L5178Y Cells." American Chemical Society 35th Southeast Regional Meeting, Charlotte, NC. Paper #230, symposium title "Structure and Transport in Membranes."

C) P.W. Wigler and Y.B. Shah, December 10, 1983, "Effects of Perfluorodecanoic Acid on a Purine Transporter." Southeastern Regional Chapter of the Society of Toxicology, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

10132. Publications.

A) Annual Report for AFOSR Grant 82-0261, August 1, 1982 to August 1, 1983. Submitted on October 11, 1983. "Effect of Chemicals on the Cell Membrane Transport of Nucleosides." (See DD Form 1473)

B) Research Progress Report for AFOSR Grant No. 82-0261 B and C, February 15, 1984. "Effect of Perfluorodecanoic Acid on the Efflux of 2-Aminopurine from L5178Y Cells."

10133. Grants.

A) Grant No. 82-0261 from the Air Force Office of Scientific Research titled "Effects of Chemicals on the Cell Membrane Transport of Nucleosides" was extended for one year on August 1, 1983. The budget was increased from $102,000 to $191,006.

B) Renewal application for grant AFOSR 82-0261, March 12, 1984.

10134. Continuing Education and Visiting Seminars.

A) Seminar to the Department of Biochemistry, University of Tennessee, Knoxville, January 13, 1984. "Toxicant Effects on Transport of a Fluorescent Purine."

B) Seminar to staff of U.S. Army Medical Research Institute of Chemical Defense, Aberdeen P.G., Maryland, April 18, 1984. "Effect of Perfluorodecanoic Acid on Efflux of 2-Aminopurine from L5178Y Cells."

C) Seminar to members of the Life Sciences Directorate, AFOSR, Bolling AFB, Washington, DC, April 20, 1984. "Toxicant Effects on Transport of a Fluorescent Purine in L5178Y Cells."

D) Seminar to Faculty of the Department of Medical Biology, May 30, 1984. "Kinetics of Cell Membrane Transport."

10135. Membership in Professional Societies.

Accepted in January 1984 by the Society of Toxicology as an Associate Member. Retained membership in the American Society of Biological Chemists and the American Chemical Society.

10136. Collaborative Associations.

Established a collaborative research project with Leroy B. Townsend, Ph.D., Professor, Dept. of Medicinal Chemistry, The University of Michigan, on 11/23/83.

1014. Goals for the 1984-85 Academic Year:

10141. Teaching.

I shall offer MB 5430 in the spring quarter of 1985.

10142. Presentations to be Delivered.

A) P.W. Wigler, Informal Research Review, Wright-Patterson AFB, Dayton, Ohio, October 29, 1984. Title: See 10142 B.

B) P.W. Wigler and Y.B. Shah, "Recove., of a Purine Transporter from the Action of a Toxicant, Perfluorodecanoic Acid" for the Fifth Annual Meeting of the American College of Toxicology, Arlington, VA, November 27-29, 1984.

AFOSR Grant Number 82-0261 B&C

RESEARCH ANNUAL REPORT

15 September 1984

EFFECT OF PERFLUORODECANOIC ACID ON THE EFFLUX OF 2-AMINOPURINE FROM L5178Y CELLS

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Controlling Office: USAF Office of Scientific Research/NL

Bolling Air Force Base, DC 20332

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101. PERIOD OF REPORT: 1 A[illegible]t 1983 to 1 August 1984

102. TABLE OF CONTENTS: AFOSR 82-0261B&C

103. ABSTRACT: by Paul W. Wigler, Ph.D.

The apparatus (and methodology) for a high speed kinetic assay of purine efflux has been developed. The procedure is based on a flow system with a membrane filter to remove preloaded L5178Y cells and a sensitive rapid detector of the fluorescence emission of a buffer that contains a transport substrate, 2-aminopurine (AP). The rate of AP efflux is proportional to the intracellular AP concentration. When uric acid is dissolved in the external buffer, AP efflux is inhibited. This observation suggests that uric acid interacts rapidly with a binding site in the AP channel. A plot of the reciprocal of the rate of AP efflux against the uric acid concentration is linear at constant intracellular AP concentration. The slope of the foregoing plot has been determined by regression analysis. Perfluorodecanoic acid (PFDA), adenine, hypoxanthine, theophylline,

caffeine, or xanthine in the external buffer have no direct effect on the rate of AP efflux, in comparison with controls.

L5178Y cells were given a prior incubation with 200 µg/ml PFDA at 30° for 24 hr. These cells were preloaded with 100 µM AP and the excess substrate was removed by rinsing the cells with cold buffer. The prior incubation of the cells with PFDA produces a total inhibition of the efflux of AP. On the other hand, a prior incubation of the cells with PFDA plus 50 mU/ml bovine insulin produces approximately 40% inhibition in comparison with controls. These findings suggest that the purine channel exists in an active and inactive form; PFDA treatment inhibits formation of active channel. Insulin appears to stabilize the active channel and protect against the effects of PFDA.

In recent experiments, L5178Y mouse leukemia cells were treated with 150 µg/ml PFDA for 24 hr at 30° and preloaded with 100 µM AP. AP efflux from the PFDA-treated cells was markedly inhibited in comparison with the controls. The PFDA-treated cells were transferred to fresh growth medium, to demonstrate recovery of the capacity to transport AP. After two days at 37°, the count of PFDA-treated cells doubled and the initial rate of AP efflux returned to approximately 60% of the controls. After one day at 30°, the cell count increased 13% and there was no detectable change in the rate of AP efflux. After six days at 30°, the cell count increased 2.7-fold and AP efflux returned to approximately 60% of the controls. PFDA-treated L5178Y cells lose the capacity to transport AP in comparison to untreated control cells; the defect in transport is stable for one day at 30°. The capacity to transport AP returns to 78% of the level of controls after nine days at 30°.

104. THEORY AND EXPERIMENTAL DESIGN:

Two different types of experiments were performed to show the potential effects of the reaction of PFDA, insulin, or purines (hypoxanthine, caffeine, theophylline, adenine, xanthine, and uric acid) on the transporter for AP in the membrane of L5178Y cells. The first type of experiment was designed to show whether the reactants could produce a rapid and direct effect on the function of the carrier. The second type of experiment was designed to show a potential slow and indirect effect on the transporter of AP.

1041. Direct Effects on the Transport System for 2-Aminopurine.

In a typical experiment of this type, living cells are incubated with a nonmetabolizable substrate at a temperature sufficient to permit substrate influx through the cell membrane but below the optimal temperature for cell growth; a temperature of 30° is usually appropriate. After the substrate is preloaded to the equilibrium concentration, the cells are cooled to 4° and sedimented in a refrigerated centrifuge. The preloaded cells are resuspended and centrifuged twice in cold (4°) buffer to remove excess substrate and the fluorescent compounds in the growth medium. The preloaded cells are then resuspended in a warm buffer (21°) that contains a known concentration of inhibitor. The efflux rate is then determined immediately by a filtration-flow fluorescence procedure (see Annual Report to AFOSR 82-0261, 1 August 1983).

Recent experiments indicate that the rate of AP efflux is proportional to the AP concentration. Furthermore, a plot of the reciprocal of the rate of AP efflux against the concentration of uric acid in the external buffer

is linear. These findings indicate that the catalyst for AP transport is a non-saturable membrane channel with a binding site for uric acid.

To derive a rate equation for the trans inhibition of a non-saturable membrane channel, the following mechanism is proposed:

$$S_i + T \underset{b}{\overset{a}{\rightleftharpoons}} TS \xrightarrow{c} T + S_o \qquad (1)$$

$$TS + I \rightleftharpoons ITS \qquad (2)$$

In the foregoing equations, the intracellular substrate is represented by S_i and the transporter is represented by T. The transporter-substrate complex is TS and the substrate in the external buffer is S_o. The inhibitor is represented by I, and the transporter-inhibitor complex is ITS. A rate equation was derived from equations 1 and 2 for the special case of a non-saturable channel, defined as $0 \approx 1/c$.

$$v = \frac{c\,[T_t]\,[S_i]}{K_S + [I]\,[S_i]/K_I} \qquad (3)$$

The parameters in equation 3 are: v is the initial velocity of AP efflux; K_S is $(b + c)/a$; $[T_t]$ is the total concentration of transporters; and K_I is the inhibitor dissociation constant. The intracellular concentration of S_i (and I in the external buffer) are indicated in brackets. From equation 3, it can be seen that v is proportional to $[S_i]$ when [I] equals zero. When equation 3 is rearranged into linear form equation 4 is obtained:

$$\frac{[T_t]}{v} = \frac{K_S}{c[S_i]} + \frac{[I]}{c\,K_I} \qquad (4)$$

From equation 4, it can be suggested that a plot of 1/v against [I] should be a straight line with a slope of $1/cK_I$, at constant $[S_i]$. From the foregoing equations it may be concluded that the experimental

parameters can be determined from the effects of AP and uric acid on the efflux of AP.

1042. Indirect Effects on the Transport System for 2-Aminopurine.

In this experiment, the L5178Y cells are given a prior incubation for 24 hr with PFDA dissolved in the growth medium. The "damaged" cells are then isolated by centrifugation and most of the medium is removed by aspiration. The cells are then preloaded with the substrate. The excess substrate and medium is removed with cold buffer and the rate of efflux is determined by the filtration-flow fluorescence procedure.

105. EXPERIMENTAL.

The experiments to show the potential direct (rapid) effects of the reactants PFDA, insulin, or purines will be described separately from the experiments that show an indirect (delayed) effect.

1051. Direct Effects on a Purine Transport System.

The PFDA was obtained from Aldrich Chemical Co. and the other reagents were obtained from Sigma Chemical Co. In these experiments a stock solution of PFDA in ethanol was prepared and diluted into the buffer; the final concentration of ethanol was 0.01%. An inoculum of 100 L5178Y cells (gift of Dr. C. F. Arlett, Cell Mutation Unit, Brighton, England) was added to 9.0 ml McCoy's 5A medium plus 1.0 ml horse serum (10FM. Andersen et al., 1982). The suspension was incubated at 37° until the cell count was 2 x 10^5 cells/ml (approximately five days). The medium contained 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were centrifuged at 1000 RPM for 10 min and 9.0 ml of the supernatant was removed by pipet. A solution of AP in medium was added to different final concentrations of AP and the 1.0 ml suspension was incubated with mild agitation for 2 hr at 30°.

The cell suspension was cooled to 4°, centrifuged at 1000 RPM for 10 min, and the supernatant was discarded. The cell pellet was resuspended in cold phosphate-buffered saline, pH 7.4 (PBS, 4°) twice and centrifuged twice to remove excess substrate. The preloaded cell pellet was resuspended in warm (21°) PBS buffer that may contain different reagents (PFDA, hypoxanthine, uric acid, bovine insulin, adenine, caffeine, theophylline, or xanthine). The 3.0 ml cell suspension was quickly poured into a filtration-flow apparatus with a 5 micron Millipore filter to remove the cells. The supernatant was drained into a quartz flow cell; the flow rate was regulated at 1.0 ml per min by a Buchler Polystaltic Pump, downstream from the flow cell. Ultraviolet light at 312.5 nm from a Hg-Xe lamp was used for excitation of the AP in the solution, and the emission of AP was estimated at 370 nm with an SLM-Aminco 8225 Photon-Counting Fluorescence Spectrophotometer and a PT 9635QA Photomultiplier Tube. The light emission was determined at different time intervals and the initial velocity of efflux was determined from an apparent zero order plot up to 14 sec. The volume of the cell suspension decreased 0.25 ml during the determination of the initial rate.

1052. Indirect Effects of PFDA on a Purine Transport System.

The L5178Y cells were grown in McCoy's 5A medium plus 10% horse serum as in section 1051. Suspensions of 37 x 10^6 cells in 10 ml medium that contain 10% serum were prepared with the following treatment conditions: a) 100, 150, or 200 µg/ml PFDA final concentrations; b) 50 or 100 mU/ml bovine insulin final concentrations; c) PFDA at 100, 150, or 200 µg/ml plus insulin at 50 or 100 mU/ml; and d) no additions (controls). (Different treatment conditions are shown in Annual Report of August 1983, Fig. 6 and Table 1). The foregoing suspensions were given a prior incubation with

mild agitation for 24 hr at 30° and the cells were subsequently counted in each suspension, with a Neubauer Hemacytometer. The cells were centrifuged at 1000 RPM for 10 min and 9.0 ml of the supernatant was removed by pipet. A solution of AP was added to a final substrate concentration of 100 μM and the 10 ml suspension was incubated for 2 hr at 30°. The cell suspension was cooled to 4°, centrifuged at 4°, and the supernatant was discarded. The pellet was resuspended and centrifuged twice in cold PBS as in section 1051. The preloaded cells were resuspended in warm PBS (21°) and the 5.0 ml cell suspension was poured into the filtration-flow fluorescence apparatus to estimate the initial rate of AP efflux.

The emission values were converted to the concentration of AP from a plot of emission intensity versus AP concentration from 0.02 to 0.32 μM, performed each day. The data were also corrected to a cell count of 10^{12} cells per liter (10^{9} cells/ml).

106. RESULTS AND DISCUSSION

1061. Direct Effects on AP Efflux.

Determinations of the initial velocity of membrane transport require a high-speed kinetic procedure (Wohlhueter et al., 1976). A filtration-flow apparatus was constructed from scrap metal and routine laboratory parts. An assembly that consists of a hypodermic barrel and a membrane filter holder is mounted on top of the cell compartment lid of the fluorescence spectrophotometer with a tripod. The filter effluent drains into a quartz fluorescence flow cell through medical plastic tubing. The outflow plastic tubing from the quartz cell passes through a pump to regulate the rate of flow. Excitation light illuminates the flow cell from behind, and the emission light is detected at a right angle with a high sensitivity

photomultiplier tube. It is possible to measure the emission light at 2 sec intervals.

There is an inherent advantage to flux measurements from a small volume (the cells) into a large volume (the buffer) in the direction of efflux. Furthermore, an assay based on fluorescence can detect substrate molecules at very low concentrations. Thus, the assay for membrane transport described in this report is one of the best methods of estimation of substrate flux ever developed.

At the time that this project was initiated, the mechanism for the efflux of AP was unknown. It seemed that two possible mechanisms could be considered: transport by a non-saturable membrane channel; or transport by a saturable carrier. If the efflux of AP is saturable, however, further studies would be needed to determine whether an energy source is needed for the efflux of AP.

The non-saturable channel can be distinguished from a saturable carrier by a kinetic analysis of the effect of different intracellular AP concentrations on the rate of AP efflux. A plot of the reciprocal of the initial rate of AP efflux against the reciprocal of [AP] was reported in the Progress Report of 15 February 1984. Extrapolation of the double reciprocal plot to the intercept gave a 1/v value of approximately zero. This observation indicates that the maximum velocity for this reaction may be infinite; this finding suggests that AP is transported by a non-saturable process.

To comprehend these results, the same data were plotted with the rate of AP efflux against the intracellular AP concentration. This plot showed that the efflux rate is proportional to the internal AP concentration (see equation 3); the v value at the intercept is approximately zero. Thus, the

effect of different AP concentrations on the efflux rate also suggests a non-saturable process. This experiment has been repeated with additional intracellular AP concentrations, but the data has not been tabulated.

The mechanism of AP efflux was also investigated further by a study of the potential trans inhibitory effects of several different purines. In these experiments, it was observed that an extracellular concentration of 500 μM adenine, xanthine, caffeine, or theophylline does not significantly affect the rate of efflux of AP (at an intracellular concentration of 100 μM) in comparison with the controls. The direct effect of hypoxanthine on purine flux was tested with five different concentrations of hypoxanthine (in duplicate), from 200 μM to 1500 μM. The rate of AP efflux was not affected, within the error of the experiments, by hypoxanthine in the external buffer.

A preliminary trans inhibitor experiment was performed with L5178Y cells preloaded with 100 μM AP and suspended in solutions of uric acid in PBS buffer at 21°. The resultant suspensions were quickly poured into the flow-filtration apparatus and the effluent buffer was excited with light at 312.5 nm (a wavelength of high emission for the mercury lamp). The emission data was corrected for a cell count of 10^{12} cells per liter, based on counts of each cell suspension. The effect of time on the increase in emission at 370 nm (the peak for AP) was determined.

The results of the preliminary experiment were given in the Progress Report of February 15, 1984. The experiment was repeated with higher substrate concentrations; the efflux of AP at 300 μM and 600 μM AP was determined with five different concentrations of uric acid in the external buffer (from 200 μM to 800 μM). The uric acid concentration is tabulated with the reciprocal of the initial efflux velocity of AP in Table I.

** ** A148835 (U) FIELD/GROUP ******
UNCLASSIFIED TITLE
EFFECT OF PERFLUORODECANOIC ACID ON THE EFFLUX OF 2-AMINOPURINE FROM L5178Y CELLS.
ABSTRACT
(U) TRANSPORTERS IN THE CELL MEMBRANE OF ANIMAL CELLS ARE SUSCEPTIBLE TO THE ACT+MSG DI4 DROLS PROCESSING-LAST INPUT I GNOREDUSE LEUKEMIA CELLS WERE TREATED WIT

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USE ANIMALS CELLS

CELL MEMBRANE
USE CELLS(BIOLOGY) MEMBRANES(BIOLOGY)

LEUKEMIA CELLS
USE CELLS LEUKEMIA

TRANSPORTERS
USE CARGO VEHICLES

PHRASES NOT FOUND DURING LEXICAL DICTIONARY MATCH PROCESS

L5178Y CELLS

Inspection of these data indicate two straight lines (see equation 4). The data from the uric acid experiment were evaluated by an analysis of least squares and a test for parallel lines.

TABLE I
Effect of Uric Acid Concentration on the Reciprocal of the Initial AP Efflux Rate, at two AP Concentrations and 21°

Uric acid (μM)	$1/v$ ($\times 10^3$) (μM^{-1} sec 10^{-12} cells liter)	AP (μM)
0	0.591	300
0	0.537	300
200	0.804	300
200	0.690	300
400	1.017	300
400	1.019	300
600	1.303	300
600	1.258	300
800	1.751	300
0	0.376	600
0	0.342	600
200	0.578	600
200	0.561	600
400	0.766	600
400	0.973	600
600	1.183	600
600	1.212	600
800	1.344	600
800	1.509	600

For the results at [AP] = 300 μM, the slope = 1.384×10^{-6} (± 7.5% SE) μM^{-2} sec 10^{-12} cells liter. The results at 600 μM show 1.382×10^{-6} (± 6.0% SE) μM^{-2} sec 10^{-12} cells liter. A statistical test for parallelism suggests that the data are consistent with two parallel lines (courtesy of Dr. Ralph O'Brien, Division of Biostatistics, UTMRCH).

Guanine and isoguanine could not be tested with this system because these compounds are insoluble at a 500 μM concentration in aqueous buffer at pH 7.4. Insulin had no direct effect on efflux.

In a separate experiment, PFDA was tested as a potential trans (direct) inhibitor of AP efflux. The pellet of preloaded cells was resuspended in a PFDA solution in PBS at 21° and the suspension was poured into the flow-filtration apparatus. The results indicate that PFDA at 200 µg/ml in the external buffer has no direct effect on AP efflux in comparison with the controls (no additions).

1062. Indirect Effects of PFDA on AP Efflux and Cell Viability.

L5178Y cells were given a prior incubation with 100 µg/ml PFDA in medium plus serum for 24 hr at 30°. The cells were subsequently preloaded with AP to a final concentration of 100 µM and the cells were rinsed in cold buffer to remove excess substrate. The cells were resuspended in warm buffer and the suspension was quickly poured into the flow device. The efflux of AP from these PFDA-treated cells was strongly inhibited.

In another experiment, all the cells received a prior incubation in medium plus serum for 24 hr at 30°, additions of PFDA and insulin were used in different combinations. The cell count of the control suspensions increased from 3.7×10^6 cells/ml to 4.4×10^6 cells/ml in 24 hr at 30°. PFDA at 200 µM concentration produced a stable cell count in comparison with the count at zero time (see Annual Report, August 1983). The addition of insulin, however, produced a small stimulation of cell division. The L5178Y cells were also tested for a capability to exclude Trypan Blue; the exclusion test is used as a criterion of cell viability. Apparently, the treatment of cells with PFDA for 24 hr at 30° does not produce a significant increase in nonviable cells.

The cells were preloaded with AP to a concentration of 100 µM and excess substrate was removed with two cold rinses. The data were converted from emission to the concentration of AP with a standard curve. (An

excitation of 312.5 nm was used in these experiments.) The prior incubation with 200 µg/ml PFDA at 30° produces a total inhibition of AP efflux but the addition of 50 mU/ml bovine insulin provides a substantial degree of protection for the purine channel.

The effect of incubation of L5178Y cells with 150 µg/ml PFDA in growth medium at 30° for 24 hr, on the efflux of AP, is shown in Fig. 1. After the prior incubation with PFDA, the cells were preloaded to a final intracellular concentration of 100 µM AP; efflux is indicated by the filled triangles in Fig. 1. Although the inhibition of AP efflux is not complete at the 150 µg/ml PFDA concentration, the AP efflux rate is decreased to 16% of the controls.

Another cell sample was treated with 150 µg/ml PFDA under the same conditions. The cells were rinsed twice with growth medium and the cells were incubated again for 24 hr at 30° with fresh medium without PFDA. The cells were preloaded with AP and rinsed in cold PBS. As shown by the filled squares of Fig. 1, the efflux rate for AP is the same after the second 24 hr incubation as observed for the first 24 hr incubation (within the error of the experiment). The efflux rate for the 48 hr control appears to agree with the rate for the 24 hr control (filled and open circles).

The results of Fig. 1 indicate that at 30° the damage to the cell membrane produced by PFDA is a stable lesion. The effect of PFDA on the cell count, for the same experiment as Fig. 1, is shown in Table II. In the presence of 150 µg/ml PFDA for 24 hr at 30°, the cell count remains approximately constant. When the cells are rinsed with fresh medium and incubated again for 24 hr with fresh medium, the cells start to divide again.

TABLE II

Effect of PFDA on Cell Count at 30°*

Time (hr)	Treatment	Cells in 20 ml ($\times 10^{-6}$)	24 hr incr. in per cent
0	Zero time	156	0
24	Control	170	9
48	Control	198	17
0	Zero time	156	0
24	PFDA (150 µg/ml)	153	-
48	PFDA first 24 hr	173	13

*At the end of 24 hr, the cells were rinsed twice with McCoy's 5A medium and incubated again in medium without PFDA for another 24 hr.

As an estimate of cell viability, the Trypan Blue exclusion test was performed on the cells in the experiment of Fig. 1 and Table II. The results are presented in Table III. The results of Table III indicate that treatment of L5178Y cells with 150 µg/ml PFDA for 24 hr has no effect on cell viability in comparison with the controls. It seems reasonable to conclude that damage to the purine channel is an "early" event in the lysis and destruction of L5178Y cells by PFDA. Furthermore, the capacity of the channel to transport AP does not recover during a subsequent 24 hr incubation in fresh medium.

TABLE III

Effect of PFDA on Cell Viability at 30°

Time, hr	Treatment	Viable cells per cent
0	Zero time	93.3
24	Control	96.0
0	Zero time	93.3
24	150 µg/ml PFDA	97.0

Additional experiments were performed to determine whether the capacity to transport AP (of PFDA-treated cells) would recover when the cells are incubated again in the absence of PFDA. L5178Y cells were incubated for 24

hr at 30° with 150 µg/ml PFDA in McCoy's 5A medium plus horse serum. The cells were sedimented twice by centrifugation and resuspended twice in fresh growth medium. The cells were then incubated in fresh growth medium at 30° for 9 days. Aliquots of the cell suspension were removed on day 2, 3, 6, 7, and day 9, and the cell count was estimated. Each aliquot of cells was preloaded with 100 µM AP, cooled to 4°, and rinsed twice in cold buffer. The initial rate of AP efflux was determined for each aliquot by the AP efflux technique. The results are shown in Table IV.

TABLE IV

Recovery of PFDA-Treated L5178Y Cells In McCoy's 5A Medium at 30°

Time (Days)	Change in Cell Count		AP Efflux of PFDA-Treated (Per Cent of Controls)
	Controls	PFDA-Treated	
0	+ 1.0 X	+ 1.0 X	-
2	+ 1.4 X	+ 1.5 X	25
3	+ 1.5 X	+ 1.6 X	23
6	+ 1.7 X	+ 2.7 X	56
7	+ 1.8 X	+ 2.7 X	71
9	+ 2.3 X	+ 4.0 X	78

From the cell count data in Table IV, it may be observed that PFDA-treated cells incubated again in fresh medium multiply faster than the untreated control cells. This difference in the rate of cell division is small up to the third day, but a relatively large yield of PFDA-treated cells is noticeable on the sixth day. In addition, there is a steady increase in the capacity of PFDA-treated cells to transport AP during the incubation in fresh growth medium for 9 days at 30°. The initial rate of AP efflux (per 10^{12} cells) increases up to 78 per cent of the controls on the ninth day.

Another recovery experiment was performed at 37°. For the experiment reported in Table V, the cells were treated with 150 µg/ml PFDA in growth medium at 30° for 24 hr. The cells were rinsed twice and incubated again in growth medium at 37° for 4 days. Aliquots of the cell suspension were removed on day 2 and day 4. On the fourth day, the yield of PFDA-treated cells was greater than the yield of control cells. This finding is consistent with the results of Table IV. In addition, the results of Table V show that the initial rate of AP efflux per 10^{12} cells returns to 76 per cent of the controls on the fourth day.

Table V

Recovery of PFDA-Treated L5178Y Cells in McCoy's 5A Medium at 37°

Time	Change in Cell Count		AP Efflux of PFDA-Treated
(Days)	Controls	PFDA-Treated	(Per cent of controls)
0	+ 1.0 X	+ 1.0 X	-
2	+ 2.2 X	+ 2.1 X	61
4	+ 3.3 X	+ 4.1 X	76

The results of Tables IV and V indicate that the PFDA-treated cells recover the capacity for cell division and the capacity for AP efflux at approximately the same time. It may be interesting to determine whether AP efflux will recover when the cells are incubated under conditions that prevent cell division. One approach will be to demonstrate the recovery of the purine channel at 30° in McCoy's 5A medium with no serum added. Another possibility may be to incubate PFDA-treated cells in a minimal essential medium and assay for AP efflux during an extended time period. We want to determine whether the AP channel recovers only in daughter cells. If the parent cells are incapable of AP efflux, it could be concluded that PFDA produces a stable lesion of the purine channel.

107. COMPARATIVE APPLICATIONS AND EQUIPMENT:

The purpose of this section is to discuss future experiments and to summarize our results. We have developed the apparatus and methodology for a high-speed kinetic assay of purine efflux, based on fluorescence. Most of the transport assays in current use depend on isotope-labeled substrates. Isotope counts are based on levels of radioactive atoms; however, the cpm may not be proportional to the substrate concentration. Uziel and Sulkirk (1979) have suggested that the specific radioactivity of extracellular uridine must be known, to correlate cpm with the concentration of uridine excreted from growing cells. Fluorescence has the advantage that emission from a fluorescent substrate is directly proportional to the substrate concentration.

A fluorescence technique for the assay of membrane transport has been reported by Eidelman and Cabantchik (1983). Human red blood cells were preloaded with a fluorescent substrate (NBD-taurine). (The fluorescence of the substrate is quenched by the hemoglobin inside the cells.) The preloaded erythrocytes were cooled and rinsed twice with a cold isotonic solution; the cell suspension was mixed with warm medium and the fluorescence of the suspension was measured continuously with a fluorescence spectrophotometer (Eidelman and Cabantchik, 1983). Apparently, light scattering from the erythrocytes in the light beam does not interfer with an accurate determination of the emission of NBD-taurine in the extracellular medium. The rate curves shown by Eidelman and Cabantchik (1983) are plotted over a 30 min time interval; thus, the efflux of NBD-taurine may be a relatively slow reaction. In addition, the NBD-taurine method may not be applicable to leukocytes (such as L5178Y cells) but is limited to erythrocytes.

The trans inhibitory effect of uric acid (Annual Report, August, 1983) suggested that the latter purine binds to the AP carrier. This finding was confirmed by further experiments with different uric acid concentrations. In addition, experiments shall be performed with other potential transport inhibitors, such as colchicine.

The primary metabolic site of action of PFDA is unknown at the present time. All available evidence suggests that inhibition of an enzyme in the biosynthesis of saturated lipids is the primary site. Alterations in the lipid composition and fluidity of the cell membrane would produce significant secondary metabolic changes. If the primary site of PFDA action is lipid synthesis, a direct inhibition of the purine carrier by PFDA would not be expected. When efflux of AP from L5178Y cells was estimated with PFDA in the external buffer, the initial rate observed was the same as the controls within the experimental error (Wigler and Shah, 1983). On the other hand, the prior incubation of L5178Y cells with PFDA for 24 hr produced an inhibition of AP efflux. These observations are consistent with the concept that changes in membrane lipids decrease the levels of active purine channel.

In the experiments from this laboratory, the L5178Y cells survive a 24 hr treatment with 150 µg/ml PFDA at 30°. It seems reasonable to determine whether the decrease in active purine channel represents an early state in cell destruction. Cell counts were performed prior to the efflux experiment in Fig. 1. The results of Table II show that L5178Y cells do not undergo cell division in 150 µg/ml at 30° for 24 hr. On the other hand, the cell count of the PFDA-treated cells does not decrease significantly. The AP efflux of PFDA-treated cells, which received a prior incubation at 30°, is inhibited in comparison with the controls (see Fig.1).

These observations suggest that a decreased level of active channel is an early event in the destruction of leukocytes by PFDA.

The potential effects of environmental agents on receptors in the cell membrane has been discussed by Cook et al. (1976). One possible mechanism for the inhibition of AP efflux is a modification in the turnover of the transporter protein. PFDA may be an inhibitor of channel protein synthesis in the cell membrane. Another hypothesis suggests that the composition of lipids surrounding the channel would influence transport activity. This is an attractive idea because of the other known effects of PFDA treatment. In this mechanism, the presence of an excessive level of unsaturated phospholipids would inhibit formation of active purine channel.

The physiological function of the purine channel is to facilitate the excretion of the purine end products of catabolism. If the concentration of these compounds increases because the efflux channel has been damaged by a toxicant, the cellular metabolism is subject to feed-back inhibition (Wigler, 1978). Uric acid, an end product of purine catabolism, is toxic to cells at elevated concentrations. Damage to the purine channel for efflux may be one significant reason for the loss of cell viability due to PFDA treatment.

The Annual Report of August, 1983, indicates the presence of an insulin receptor in L5178Y cells. The physiological effects of insulin-receptor interaction have been extensively studied in the last three years (Seals and Czech, 1981). Apparently, insulin binding to the receptor at the membrane surface leads to the release of peptide mediators. These mediators may stimulate or repress intracellular enzymes or transporters in the membrane.

A 24-hr treatment with 200 µg/ml PFDA at 30° produces complete inhibition of the AP efflux reaction. The combination of 50 mU/ml insulin plus 200 µg/ml PFDA preserves approximately 60% of the AP transport capacity of the L5178Y cells. One possible explanation of this observation is that an insulin mediator could stimulate the synthesis and deposition of saturated lipids in the cell membrane to restore the unsaturated/saturated ratio and the membrane fluidity to normal physiological levels. Another possibility is than an insulin mediator may inhibit the synthesis of unsaturated fatty acids.

One possible explanation for the inhibition of AP efflux by the prior incubation with PFDA is an inability of the treated L5178Y cells to preload with the fluorescent substrate. The effect of a 24-hr PFDA-treatment on AP preloading has been determined. The L5178Y cells were treated with 200 µg/ml PFDA for 24 hr at 30° and the cells were subsequently preloaded with 100 µM AP for 2 hr at 30°. The suspension was cooled to 4° and the cell pellet was rinsed twice with cold PBS. The cells were disrupted with a glass homogenizer and the resultant suspension was mixed with PBS. Insoluble cell debris was sedimented by centrifugation; the supernatant was analyzed for soluble fluorescent compounds with excitation by light at 312.5 nm and emission at 370 nm. The intensity of emission of the solute from PFDA-treated cells is approximately the same as the emission of the controls. Thus, the PFDA-treated cells are preloaded with AP to the same level as the controls.

There is an experiment in progress at the present time to show the effect of PFDA treatment on the lipids in L5178Y cells. The fatty acids of L5178Y cells are analyzed in collaboration with Dr. K.D. Lin by the following procedure: The control cells and PFDA-treated (150 µg/ml) cells are

centrifuged and resuspended in fresh medium; the cells are rinsed 3 times to remove protein, lipid, or phenol red from the culture medium.

An 0.2 ml sample of packed cells (about 200 x 10^6 cells) are transferred to a flask and shaken with 8 ml chloroform and 8 ml methanol. The mixture is placed in a 100° water bath to extract the membrane and cytosol lipid. The mixture is filtered through a fluted filter paper and poured into a 60 ml separatory funnel. Ten ml of saline is added and the mixture is shaken. The lower layer is filtered through fluted filter paper into a beaker. The solution is evaporated to dryness on a constant temperature hot plate at 67-70° with a nitrogen flush. The total residue is dissolved in 2 ml of 1% alcoholic KOH and heated on a steam bath for 15 min. The hydrolysate is adjusted to pH 2.0 with conc. HCl, after the addition of 3 ml H_2O. The free fatty acids are extracted with 15 ml petroleum ether. The petroleum ether extract is dried under nitrogen flush and residues taken up in 0.2 ml of ethanol for the lipid analysis.

A Bendix model 2500 gas chromatograph is equipped with on-column dual-flame ionization detectors, a temperature programmer, and a 6-foot U-shape 4 mm ID glass column packed with a 3% OV-25 on chromosorb W (HP) 80/100 mesh. The sample is methylated on the column with methelude (Pierce) and the fatty acid methyl esters are separated with a temperature gradient from 100° to 280°. Fatty acid methyl ester mixtures of 0.1 ml per ml of ethylacetate are used as standards to establish the elution position and to quantitate the amounts based on the peak height. If differences in fatty acid content are noted for PFDA-treated cells and the controls, the experiment will be repeated with purified fractions of the cell membranes.

In early March, we received a cooled PMT housing for the 9635 QA, a digital graphics plotter, a data acquisition processor, a dot matrix

printer, and a disk drive. The new equipment is now operating according to specifications.

108. FIGURE 1

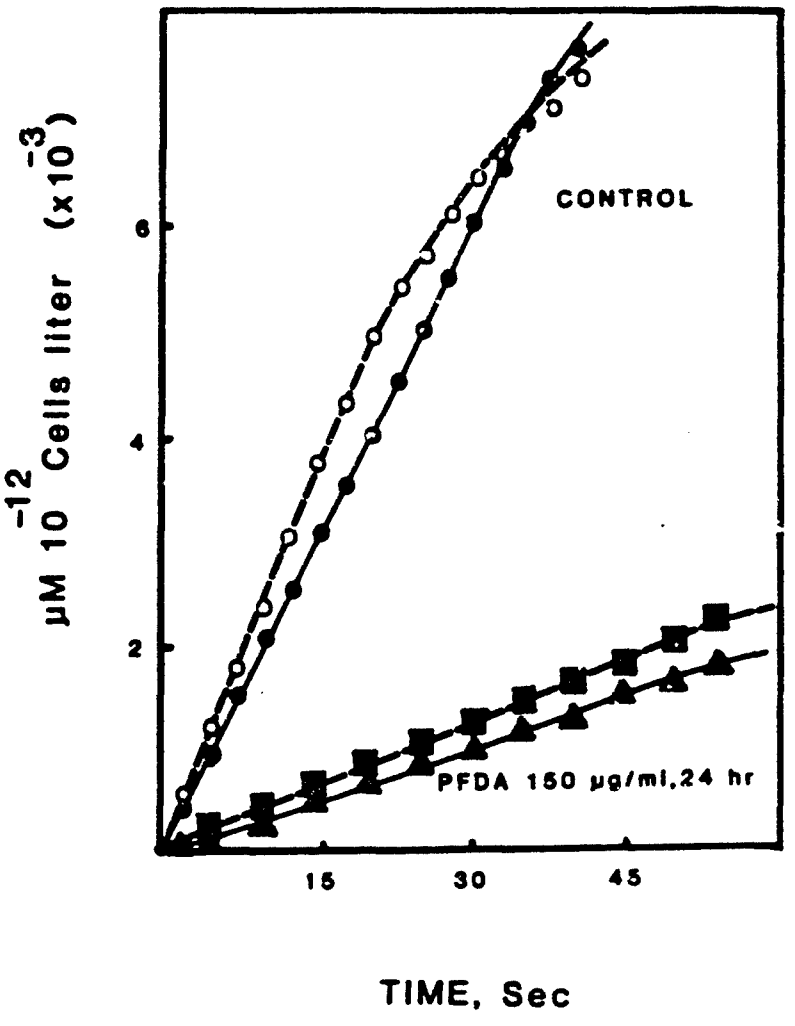

Fig. 1: The effect of a 24-hr treatment with 150 µg/ml PFDA at 30° on the efflux of AP from L5178Y cells: The cells were incubated for 24 hr in medium at 30°, preloaded with 100 µM AP, and the efflux of AP determined by the flow-fluorescence technique (●); cells were incubated twice for 24 hr in medium, preloaded with AP, and AP efflux determined (○); cells were incubated 24 hr in medium that contained 150 µg/ml PFDA, preloaded with AP, and AP efflux determined (▲); cells were incubated for 24 hr in medium plus 150 µg/ml PFDA, rinsed, incubated for 24 hr with fresh medium without PFDA, preloaded with AP, and the AP efflux determined (■).

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Recovery of a Purine Transporter from the Action of a Toxicant, Perfluorodecanoic Acid, P.W. Wigler* and Y.B. Shah*, Department of Medical Biology, University of Tennessee Memorial Research Center, Knoxville, TN 37920 (Intr. by H. Witschi)

Transporters in the cell membrane of animal cells are susceptible to the action of toxic environmental agents. L5178Y mouse leukemia cells were treated with 150 µg/ml perfluorodecanoic acid (PFDA) in growth medium for 24 hr at 30°. The PFDA-treated cells were transferred to fresh growth medium to demonstrate recovery of the capacity to transport a fluorescent purine, 2-aminopurine (AP). The membrane flux of AP was estimated with a high-speed kinetic assay. The cells were preloaded with 100 µM AP and rinsed twice in a cold buffer. The preloaded cells were resuspended in buffer at 21° and AP flux was estimated continuously from the fluorescence emission of AP at 370 nm. At the beginning of the recovery period, AP efflux from PFDA-treated cells was markedly inhibited in comparison with the untreated controls. After 2 days at 37°, the count of PFDA-treated cells doubled and the initial rate of AP efflux returned to approximately 60% of the controls. After 6 days at 30°, the cell count increased 2.7-fold and AP efflux returned to approximately 60% of the controls. The results of experiments with untreated control cells show that the initial rate of AP efflux is proportional to the intracellular AP concentration. A plot of the reciprocal of the rate of AP efflux against the concentration of an inhibitor (uric acid) in the external buffer is a straight line. These observations suggest the presence of a uric acid-sensitive purine channel in the membrane of L5178Y cells. The channel is inactivated by PFDA under conditions that do not appreciably change cell viability. The gradual recovery of the PFDA-inactivated purine channel can be demonstrated after the cells are transferred to fresh growth medium. (Supported by Grant No. 82-0261 from the Air Force Office of Scientific Research.)

Microtubules and Movement in the Foraminifer, Allogromia. J.L. Travis* and R.D. Allen, Department of Biological Sciences, Dartmouth College, Hanover, N.H. (Intr. by M. Lubin).

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The reticulopodial networks (RPN) of the foraminifera provide excellent models for the study of the role of cytoplasmic microtubules (MT) in the structure and function of motile systems. Allogromia latticollaris, a benthic foram,

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