EFFECTS OF SERUM ON MEMBRANE TRANSPORT

II. Serum and the Stimulation of Adenosine Transport, a Possible Mechanism

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ABSTRACT

When monolayers of freshly obtained rabbit lung macrophages are exposed to the nucleoside analogue, showdomycin (sho), adenosine transport, measured over a 45 s interval, is irreversibly inhibited. Low doses of the drug or short periods of exposure, however, do not result in decreased transport, while higher concentrations or longer exposures result in exponential decline. The initial lag is not due to a long reaction time of sho with the transport carrier or to nonspecific sites absorbing the drug.

Previously it was shown that preincubation of monolayers with normal rabbit serum (NRS) results in increased adenosine transport. When monolayers are first exposed to sho so as to inhibit transport to varying degrees and then incubated with NRS, transport is increased over the inhibited level. Several experiments make it unlikely that serum removes the drug from the cell surface in a nonspecific fashion. Moreover, serum given before, during, or after sho alters the dose response curve so that no shoulder is seen. One way to explain these results makes use of target theory: the adenosine transport system could be comprised mainly of "coupled" or "clustered" sites of which only one is active at any time as well as "hidden" sites which are inactive. When a site in a group is irreversibly inactivated by sho, another in the group becomes activated. Serum might activate or uncouple all sites and also cause the appearance of hidden sites, which previously neither transported nor bound sho.

INTRODUCTION

Numerous authors have characterized transport of electrolytes and nonelectrolytes in a variety of mammalian cells in terms of kinetic parameters, sodium requirements, and specificity (4, 5, 7, 8, 15, 26, 33, 34). In addition, the activities of membrane transport systems are altered by pH,[1] temperature (1, 27), internal concentrations of various metabolites (15, 27), and the external medium bathing the cell (7, 13, 22, 32). Still there is little information regarding detailed regulatory mechanisms.

Recently we reported the effects of normal rabbit serum (NRS) on two transport systems in rabbit lung macrophages (32). The rate of lysine transport was depressed from the control and that of adenosine was enhanced by preincubating cells with NRS for 15-30 min. The substance(s) responsible for the depression was

[1] Taube, R., and R. D. Berlin. Manuscript in preparation.

(were) small and heat stable, while that (those) responsible for the enhancement was (were) larger and heat labile. In this study we have examined in greater detail the serum-induced stimulation of adenosine transport. In the previous publication, the kinetic data were insufficient to determine whether the number of transport sites, the rapidity of transport, or the affinity of the carrier for its substrate had been altered by previous exposure to serum. In this series of experiments the nucleoside analogue showdomycin (sho), originally described by Nishimura et al. (25), was used to irreversibly inhibit the nucleoside transport system. Both time and concentration curves had an interval at early times or low concentrations where transport did not decline. Thereafter, transport fell in an exponential fashion. Several experiments ruled out the possibility that lag was due to the reaction time of sho with the transport carrier or to nonspecific sites absorbing the drug. Serum treatment, while stimulating transport, abolished the interval over which transport rates were unaffected by sho. It also caused increased transport in cells already inhibited irreversibly with sho. One way to explain these results makes use of target theory. The assumptions required for application of this theory to the data are shown to be valid. From target theory it is inferred that the adenosine transport system might be composed of "coupled" sites of which only one is active at any time and "hidden" sites which are inactive. When a site in a group is irreversibly inactivated by sho, another becomes activated. Serum might activate all sites and also cause the appearance of previously hidden sites.

Coupling is consistent with certain models of enzyme activity where only one site acts on substrate at a time, even though all subunits are identical (21). Hidden sites are consistent with published accounts of regulation of surface elements in fused cells (11, 12) and in lymphocytes (37). Implicit in the model is the assumption of mobility of transport carriers within the plasma membrane as suggested by Singer and Nicolson (31). The assumption is supported by experiments dealing with the effect of NRS on prefixed cells. If the hypothesis is valid, this is the first demonstration of a functional constitutent of the cell surface that is regulated in this dual fashion.

MATERIALS AND METHODS

Previous publications have described how rabbit lung macrophages (34) and sera of plasma (32) were obtained. Cells were tested for their ability to transport adenosine by the cover slip technique developed by Hawkins and Berlin (14). Briefly, monolayers were formed from freshly obtained lung macrophages on circular 22-mm cover slips. Each monolayer contained 3×10^5 cells. The monolayers were exposed to the materials to be tested for the desired length of time at 37°C unless otherwise stated. Between exposures to different solutions, monolayers were rinsed through four changes of modified Hanks' solution (MH) (33) at room temperature unless a rinse at 4°C is indicated. Finally they were tested for the ability to transport adenosine (0.1 mM, 2–4 μCi/ml) at 37°C during a 45 s incubation period in MH. Every determination was run in triplicate or quadruplicate. The concentration of adenosine chosen for testing transport was its K_m value (32, 35). Isotopes were obtained from New England Nuclear, Boston, Mass., or from Schwarz-Mann Div., Becton, Dickinson and Co., Orangeburg, N. Y. No serum was present during the transport assay except presumed substances remaining adherent to the macrophage monolayer. Sho was also obtained from Schwarz-Mann.

Metabolism of [^{3}H]adenosine taken up during a 45 s incubation was determined by analysis of perchloric acid (PCA) extracts of cells by means of ascending chromatography. After the monolayers were exposed to isotope, they were frozen and thawed three times in 5% iced PCA which was then neutralized with KOH. Precipitated salt was removed with a Swinnex filter, Millipore Corp., Bedford, Mass., pore diameter 0.45 μm, and the extracts were stored frozen at −20°C. Ascending chromatography using Whatman paper #1 was performed in isobutyric acid:ammonium hydroxide:water, 66:1:33 with standards of adenosine, AMP, ADP, ATP, and inosine. The area of the unknown corresponding to each standard was cut and eluted with 0.05 N NaOH, and radioactivity in each eluate was determined. As inosine and AMP tended to run together, half the eluate from that fraction was rerun by ascending chromatography in the upper phase from a mixture of water:sec-butyl alcohol:tert-butyl alcohol, 48.4:43:8.6, as described by Fink et al. (10). Strips corresponding to the standards of inosine and AMP were eluted and counted as above. In one experiment [^{3}H]inulin was included in order to correct for the amount of fluid trapped in the extracellular space. Isotope trapped in the extracellular space would not be metabolized, and therefore that amount of [^{3}H]adenosine corresponding to the amount of trapped fluid was subtracted from counts recovered as adenosine. In normal cells this amounted to 5% total counts and in sho-inhibited cells to 25%. Recovery of total counts from the paper was 100% in all cases. In order to determine the total amounts of adenine nucleotides in buffer and serum-treated cells, high pressure liquid chromatography was performed on PCA extracts prepared as above with

liquid chromatograph, Varian Aerograph, LCS 1000 (Varian Associates, Palo Alto, Calif.) (2).

For fluorescence microscopy a Zeiss microscope equipped with FIIC primary filters manufactured by Optisk Laboratorium, Lyngby, Denmark was used. Fluorescein-conjugated concanavalin A was kindly supplied by Dr. Emil Unanue.

RESULTS

Sho Irreversibly Inhibits Adenosine Transport

Komatsu (17) reported that sho inhibited nucleoside transport in *Escherichia coli* if the analogue was present during the exposure to nucleoside. In rabbit lung macrophages, sho also inhibited adenosine transport when it was present during the 45 s exposure to [^{3}H-]adenosine. Fig. 1 is a Lineweaver-Burk plot showing that the inhibition was competitive in nature, as the two curves intersected at the ordinate. From this experiment it could be calculated that the K_i for half-maximal inhibition was 1.2 mM sho (see reference 33 for calculation). In contrast to the findings of Komatsu, however, after a 30 min preincubation with 0.1 mM sho, transport was inhibited irreversibly. Reversal in the absence of sho could not be demonstrated for at least 60 min. Under these conditions the kinetic data indicated noncompetitive kinetics for the effect of sho on adenosine transport. In the experiment shown in Fig. 2 the V_{max} for adenosine transport, determined from the intercepts at the ordinate, was

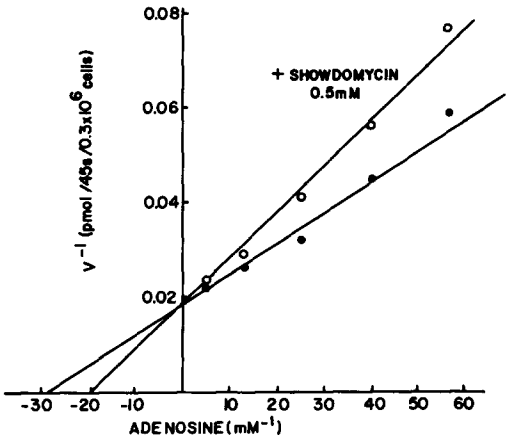

FIGURE 1 Lineweaver-Burk plot of [^{3}H]adenosine transport in the presence (○) or absence (●) of 0.5 mM sho. Transport was tested over a 45 s interval. Inhibition is competitive. The calculated K_i is 1.2 mM. Lines drawn by the method of least squares.

reduced to 5% of control value by ½ h exposure to 0.4 mM sho, while the K_m, calculated from the respective slopes, was unaffected. Thymidine, which has a high affinity for the adenosine carrier, (K_i = 0.18 mM [1]), completely prevented inhibition (Table I).

Komatsu and Tanaka (19) reported that in *E. coli* sho inhibits the metabolism of nucleosides. In contrast, using the time and concentrations indicated, this did not seem to occur to [^{3}H]-adenosine *taken up during a 45 s pulse* in lung macrophages. Adenosine transported during the test interval was metabolized by normal and drug-treated monolayers in a similar fashion in an experiment where transport was inhibited to 12.7% of control by ½ h preincubation with 0.3 mM sho (Table II). That 80% of adenosine taken up was metabolized to nucleotides in both treated and control monolayers provided evidence that membrane transport was rate limiting after exposure to sho as it was in the untreated cells. Also, when transport was inhibited to 8% of control by a 30 min exposure to 0.4 mM sho, transport was still linear for more than 1 min. Thus, sho irreversibly inhibited the transport of adenosine without interfering with its metabolism over the transport time and drug concentration ranges employed.

Time-Course of Inactivation of Nucleoside Transport

When cells were incubated with sho for varying periods of time, rinsed, and then tested for nucleoside transport, little or no decrease occurred until after a lag of several minutes. After this interval the transport rate declined exponentially (Fig. 3). Even at sho concentrations as high as 2.0 mM, well above the K_i concentration, there was a distinct lag before inactivation began. The lag could correspond to saturation by sho of cellular sites unrelated to the nucleoside transport system. However, when monolayers were preincubated for 30 min with 0.2 mM sho plus 0.4 mM adenosine so as to saturate such reactive groups while protecting nucleoside transport sites, then rinsed and incubated with 0.2 mM sho for varying lengths of time before assaying for transport, the time-course of inactivation showed the usual lag time.

The possibility that the lag was due to slow reaction leading to irreversible combination of sho with the nucleoside carrier was ruled out by

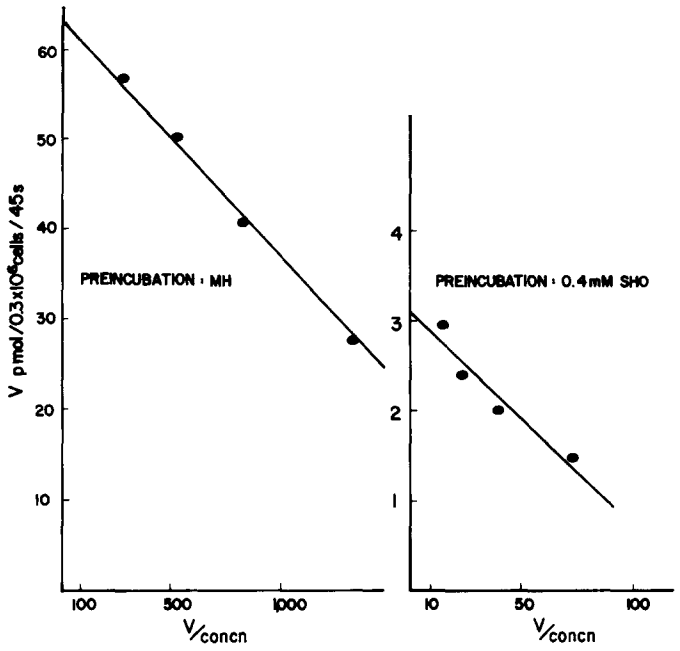

FIGURE 2 Woolf plot of [^{3}H]adenosine transport by monolayers preincubated for 30 min with or without 0.4 mM sho. Transport measured over a 45 s interval. Velocity is plotted against velocity divided by the concentration of adenosine. As the slopes are parallel but the intercepts differ, inhibition is noncompetitive. Lines drawn by the method of least squares.

TABLE I

Protection of Adenosine Transport Carrier against Sho

Sho (0.2 mM)	Thymidine (0.4 mM)	pmol adenosine transported/3 × 10^6/ cells/min
−	−	25.8
+	−	15.3
−	+	22.4
+	+	21.8

Monolayers were preincubated with or without sho in the presence or absence of thymidine.

an experiment in which cells were incubated with sho until inactivation had begun, rinsed, and then reincubated with sho. The second incubation led immediately to further inactivation (Fig. 4).

Dose Dependency of Inactivation of Nucleoside Transport

When monolayers were incubated with varying concentrations of sho for a uniform time period (30 min) and a plot of percent remaining transport vs. concentration prepared, this curve also showed a shoulder at low concentration (Fig. 5). This was made especially clear when contrasted with the dose dependence for monolayers treated with serum, as will be discussed in detail below.

TABLE II

Metabolism of [^{3}H] *Adenosine*

Preincubation (mM Sho)	% DPM recovered*		
	Nucleotides	Inosine	Adenosine
0	79.7	5.6	14.1
0.05	85.1	6.8	8.0
0.30	78.3	12.2	9.4

*PCA extracts were prepared after monolayers were preincubated for 30 min under the various conditions above and tested for the transport of adenosine (0.04 mM, 10 μCi/ml) for 45 s. Metabolites of [^{3}H] adenosine were separated by ascending paper chromatography as described in Methods. The strips corresponding to standards run simultaneously were eluted and counted. Control monolayers transported 17.8 pmol/45 s/3 × 10^5 cells.

Serum Alters the Response to Sho

Previously we (32) reported that when monolayers were exposed to NRS, adenosine transport was stimulated by 35 ± 2.3% (SE, 60 observations). In order to determine whether serum was recruiting new transport sites or affecting old ones, monolayers were preincubated for 30 min with different concentrations of sho to irreversibly

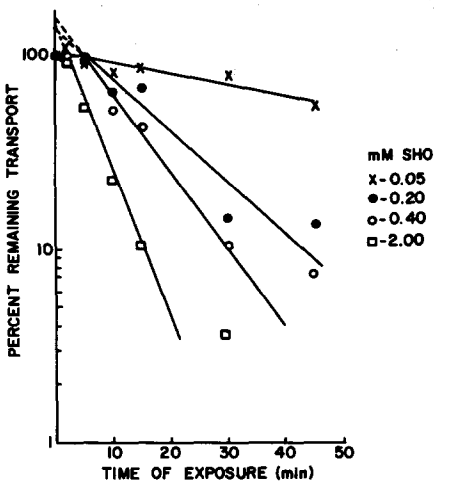

FIGURE 3 The effect of sho on adenosine transport, time dependence. A short lag precedes decline of transport. Dashed lines are the extrapolation to 0 min exposure to sho at each concentration of drug.

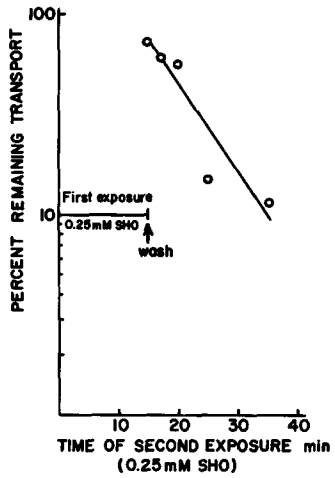

FIGURE 4 Time dependence of loss of adenosine transport due to sho when monolayers have previously been exposed to sho. Monolayers were treated with 0.25 mM sho or MH for 15 min, washed through four changes MH, and then further incubated with 0.25 mM sho or MH for the indicated period. No lag is seen before transport declines.

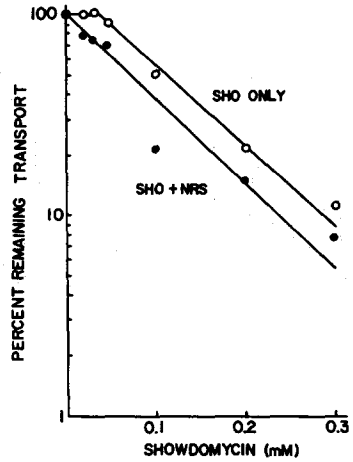

FIGURE 5 The effect of sho on adenosine transport with (●) or without (○) serum (NRS) preincubation. Monolayers were preincubated with MH or 5% NRS for 30 min, washed, and incubated with MH or Sho at the concentration indicated for 30 min. They were then washed and tested for the ability to transport [^{3}H]adenosine over a 45 s interval. Typical experiment of more than 12.

inactivate transport to varying degrees and then postincubated with MH or 5% NRS. Table III lists the data for the eight experiments graphed in Fig. 6. From the table one can see that treating monolayers with NRS rather than MH after transport has been inhibited by sho resulted in enhanced levels of adenosine uptake. The increment observed when monolayers were treated first with MH and then with NRS is designated $^{\Delta}$MH. This stimulation ranged from 18% to 178% (49 ± 25%, SE, eight observations) of controls treated in both intervals by MH. The increment observed when monolayers were treated first with various concentrations of sho and then with NRS is designated $^{\Delta}$Sho. This stimulation ranged from 15% to 840% (227 ± 99%, SE, eight observations) of controls treated first with sho and then with MH. Were the serum-induced increments dependent on the level of transport remaining when serum was applied, $^{\Delta}$Sho would have showed the same percentage increase of its control not treated with serum as $^{\Delta}$MH. The last column in Table III details the increase for that hypothetical case in each experiment. One can see that the observed values do not follow this prediction. Therefore, it was unlikely that the increment induced by NRS after sho was due to conformational change or to more rapid activity of transport sites that were left after inhibition.

TABLE III

Effect of Serum on Adenosine Transport in Monolayers Pretreated with Sho

Experiment	Sho (mM)	Serum (5%)	pmol adenosine transported/45s/ 3×10^5 cells	% control	Δ (pmol)	$\frac{\Delta Sho}{\Delta MH}$	Hypothetical* $\left(\frac{\Delta Sho}{\Delta MH}\right)$
1	—	−	14.6	100			
	—	+	19.7	136	5.1		
	0.05	−	14.5	99		0.73	1.00
	0.05	+	18.1	124	3.6		
2	—	−	14.1	100			
	—	+	20.5	138	6.4		
	0.05	−	15.0	107		0.36	0.89
	0.05	+	17.3	122	2.3		
3	—	−	15.7	100			
	—	+	43.6	278	27.9		
	0.1	−	7.0	45		0.27	0.45
	0.1	+	14.6	93	7.6		
4	—	−	18.7	100			
	—	+	23.2	120	4.5		
	0.2	−	5.7	30		0.57	0.24
	0.2	+	8.3	44	2.6		
5	—	−	64.7	100			
	—	+	76.1	118	11.4		
	0.5	−	0.5	1		0.37	<0.01
	0.5	+	4.7	7	4.2		
6	—	−	36.2	100			
	—	+	49.1	136	12.9		
	0.5	−	1.5	4		0.28	<0.01
	0.5	+	5.1	14	3.6		
7	—	−	40.6	100			
	—	+	54.5	132	13.9		
	0.5	−	1.8	4		0.32	<0.01
	0.5	+	6.0	14	4.2		
8	—	−	19.7	100			
	—	+	26.9	136	7.2		
	0.03	−	16.9	86		1.04	0.84
	0.03	+	24.4	124	7.5		
	0.3	−	2.0	10			
	0.3	+	5.4	28	3.4	0.47	0.01

*In each experiment monolayers were pretreated with the concentration of sho indicated for 30 min. They were rinsed and then incubated with or without serum for an additional 30 min. After rinsing again, they were tested for adenosine transport. Percent control refers to percent of transport by monolayers treated with neither sho nor serum. Δ_{Sho} is the difference in picomoles of transport between monolayers treated first with sho, then with serum and monolayers treated first with sho, then with buffer (MH). Δ_{MH} is the difference between monolayers treated first with MH, then with serum and monolayers treated in both intervals with MH. Hypothetical $\frac{\Delta_{Sho}}{\Delta_{MH}}$ is a ratio for Δ_{Sho} if it were the same percentage increment of the control treated only with sho as was Δ_{MH} for that experiment. It can be seen that the observed values are different from the hypothetical values.

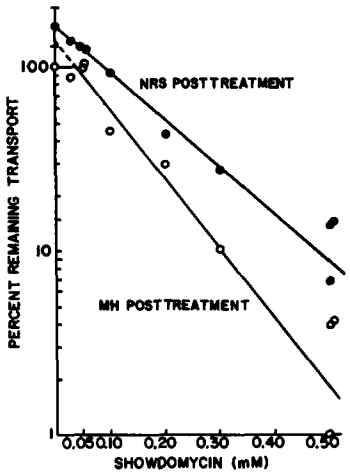

FIGURE 6 The effect of 5% serum (●) or buffer (○) posttreatment on the concentration dependence of sho inactivation of [^{3}H]adenosine transport. As indicated in Table III, monolayers were first treated with buffer or sho, rinsed, and then treated with buffer or 5% NRS. They were then rinsed again and tested for the ability to transport [^{3}H]adenosine. A pair of points (○, ●) represents one experiment.

However, as $^{\Delta}$Sho:$^{\Delta}$MH was a comparatively constant ratio over the range where sensitivity to sho was exponential (see Table III, $^{\Delta}$Sho/$^{\Delta}$MH), the increase due to serum after previous exposure to drug seemed related to the *absolute increment* of serum-induced stimulation incurred in the absence of previous exposure to sho. Fig. 6 presents the data as log percent remaining transport vs. concentration of sho. Note that the serum post-incubation resulted in a relationship lacking the shoulder region of concentration dependence. Also, when the upper curve was extrapolated back to 0 sho, the intercept lay within the observed range for serum-induced stimulation in the absence of sho.

Various aspects of the serum-induced stimulation in control cells (32) and sho-treated cells were similar. The increment in the latter depended on similar concentrations of serum as the initial serum-induced stimulation in the absence of previous exposure to sho (Table IV). 5% serum gave the maximal $^{\Delta}$Sho; 20% serum gave no more. Also, serum that was heat inactivated at 100°C for 20 min caused little increase in transport after sho, even at 20%. Finally, the time of post-incubation with 5% NRS required to give maximal $^{\Delta}$Sho was similar to the time required for the initial serum-induced stimulation (Fig. 7). In both cases incubations after the initial 15-20 min gave no further stimulation. Therefore, it seemed unlikely that serum was removing sho from the surface of cells in a nonspecific fashion, if at all.

In the converse experiment, when monolayers were first incubated with serum for 30 min and then incubated with different concentrations of sho for 30 min, the concentration dependence was exponential (Fig. 5). This was in contrast to the nonexponential concentration dependence observed for monolayers that had not been exposed to serum. The slopes of both inactivation curves were about the same, indicating that once inactivation had begun, the sensitivity of the transport carriers to sho was the same.

Serum Seems to Act at or Via a Site Different from the Nucleoside-Binding Site of the Carrier Molecule

When monolayers were exposed to sho for ½ h in the presence of serum, the concentration dependence of inactivation was linear, just as if exposed to sho *followed* exposure to serum. In addition, if monolayers were coincubated with serum and 0.4 mM adenosine or with serum and 0.4 mM thymidine, serum-induced stimulation still occurred (Table V). The concentrations of adenosine and thymidine employed were sufficient to protect transport against inactivation by 0.2 mM sho, but in this case they did not prevent stimulation.

Metabolism Does Not Appear Involved in the Serum-Induced Enhancement

To determine whether or not serum alters internal pools of adenine nucleotides and thereby alters the rate of adenosine transport, these were examined by high pressure liquid chromatography (2) (Table VI). No changes were observed in absolute or in relative amounts under conditions where transport was enhanced by 42.6%.

In addition, monolayers incubated with serum in the cold (4°C), washed either in the cold or the warm, and tested for transport in the warm (37°C) showed a similar increment of transport to those incubated and tested in the warm (Table VII). Hence, cellular functions such as phagocytosis, pinocytosis, or respiration that require physio-

TABLE IV

Effect of Various Concentrations of Serum and of Heat-Inactivated Serum on Monolayers Pretreated with Sho*

Experiment	Sho (mM)	Serum (%)	pmol adenosine transported/45 s /3 × 10^5 cells	% control	Δ	$\frac{\Delta Sho}{\Delta MH}$
1	—	0	36.2	100		
	—	1	42.8	118	6.6	
	—	5	49.1	136	12.9	
	—	20	42.5	117	6.3	
	0.5	0	1.5	4		
	0.5	1	3.5	9	2.0	0.30
	0.5	5	5.2	14	3.7	0.29
	0.5	20	5.0	13	3.5	0.55
2	—	0	40.6	100		
	—	1	54.5	133	13.9	
	—	5	52.9	130	12.3	
	—	20	54.8	134	14.2	
	0.5	0	1.8	4		
	0.5	1	6.8	16	5.0	0.36
	0.5	5	6.0	14	4.2	0.34
	0.5	20	6.0	14	4.2	0.30
3	—	0	22.1	100		
	—	5	35.6	161	13.5	
	—	5*	20.9	94	− 1.2	
	—	20*	23.3	105	+ 1.2	
	0.4	0	3.3	15		
	0.4	5	6.0	27	2.6	0.19
	0.4	5*	3.2	15	− 0.1	—
	0.4	20*	3.7	17	+ 0.4	0.15

*Serum heat-inactivated at 100°C for 20 min (32). Experiments performed as in Table III.

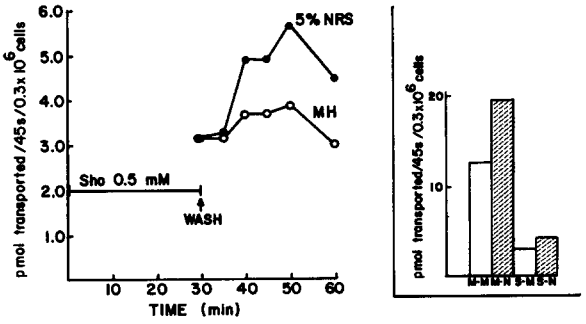

FIGURE 7 Time dependence of serum-induced increases in [^{3}H]adenosine transport after exposure to sho. Monolayers were first exposed to 0.5 mM sho, washed, and then exposed to either NRS or buffer (MH) for the indicated times. After a second washing, the monolayers were tested for the ability to transport [^{3}H]adenosine over a 45 s interval. The *inset* presents the comparison from the same experiment of monolayers treated with buffer for two 30 min consecutive periods (M-M), those treated with buffer followed by NRS (M-N), those treated with sho followed by buffer (S-M), and those treated with sho followed by NRS (S-N).

logical temperatures could not be responsible for the observed alterations. Recalling that the serum-induced stimulation requires 15 min, however, we do not rule out a multistep process, where the final step might be extremely rapid (see Discussion).

TABLE V

Effect of Thymidine or Adenosine Coincubation on Serum-Induced Stimulation

Serum (5%)	Adenosine (0.4 mM)	Thymidine (0.4 mM)	pmol adenosine transported/ 45s/0.3 × 10^6 cells	% control (without serum)
−	−	−	19.5	
+	−	−	26.4	135
−	+	−	16.3	
+	+	−	24.8	152
−	−	+	24.0	
+	−	+	31.4	130

Monolayers were incubated with or without serum in the presence or absence of adenosine or thymidine.

Glutaraldehyde Interferes with Serum-Induced Stimulation

While intracellular metabolic events were apparently not involved in the serum-induced stimulation of adenosine transport, surface mobility may be required. If monolayers were exposed for 10 min to 10-50 μM glutaraldehyde, concentrations that left adenosine transport unaffected, the capacity to demonstrate serum-induced stimulation of adenosine transport was reduced to about half (Table VIII). Paraformaldehyde, on the other hand, was unable to prevent the response to serum, even at 300 μM where base transport rates were altered. Thus the mere interaction of an aldehyde with superficial amino groups was insufficient to modify the response to serum.

Both glutaraldehyde and paraformaldehyde at higher concentrations are commonly used fixatives (29, 30) and have been shown to interfere with the mobility of lectin-binding sites in the membranes of normal and transformed fibroblasts (23, 30). By means of fluorescein-labeled con-

TABLE VI

Adenine Nucleotides in Cells Preincubated with Buffer (MH) or NRS

Treatment (30 min)	pmol adenosine transported/45s /0.3 × 10^6 cells	Nucleotide (nmol/10^8 cells)			ATP:ADP:AMP
		ATP	ADP	AMP	
MH	28.2	107.2	43.7	10.8	2.5:1.0:0.2
5% NRS	40.2	109.5	46.0	17.2	2.4:1.0:0.4

TABLE VII

Temperature Independence of Preincubation

Experiment	Preincubation	Temperature of preincubation (°C)	pmol adenosine transported/45s/0.3 × 10^6 cells	% control
1	MH	37	12.7	
	5% NRP		16.9	133
	MH	4	14.6	
	5% NRP		19.9	136
2	MH	37	46.2	
	5% NRP		58.3	126
	MH	4	44.5	
	5% NRP		58.7	132

Cells were preincubated with buffer (MH) or normal rabbit plasma (NRP) at the temperature indicated, washed at that temperature, and tested for transport at 37°C.

TABLE VIII

Effects of Aldehydes on Serum-Induced Stimulation of Adenosine Transport

Experiment	Treatment I (10 min)	Treatment II (30 min)	% control (no aldehyde, no serum)	% control (if aldehyde, no serum)
	μM			
1	MH	MH	100	
	MH	NRS	164	
	PFA, 300	MH	89	100
	PFA, 300	NRS	141	158
	GLUT, 5	MH	102	100
	GLUT, 5	NRS	136	133
2	MH	MH	100	
	MH	NRS	169	
	GLUT, 20	MH	101	100
	GLUT, 20	NRS	134	133
3	MH	MH	100	
	MH	NRS	180	
	GLUT, 10	MH	103	100
	GLUT, 10	NRS	157	153
	GLUT, 100	MH	54	100
	GLUT, 100	NRS	73	124
4	MH	MH	100	
	MH	NRS	170	
	GLUT, 10	MH	101	100
	GLUT, 10	NRS	155	153
	GLUT, 50	MH	114	100
	GLUT, 50	NRS	174	152

Monolayers were treated with buffer (MH), paraformaldehyde (PFA), or glutaraldehyde (GLUT), rinsed, and then treated with either MH or 5% NRS.

canavalin A (FCCA), it was possible to demonstrate in experiments parallel to those described above that exposure of monolayers to 20 μM glutaraldehyde but not to 300 μM paraformaldehyde was sufficient to interfere with the overall redistribution of fluorescence. Monolayers were treated with the appropriate fixative or with MH for 10 min at 37°C, washed, exposed to FCCA at 4°C for 10 min, rinsed, wet mounted at room temperature, and examined at various intervals up to 1 h. Within 5 min, fluorescence due to FCCA in unfixed cells appeared in a ring in the perinuclear region and as clumps or patches over the nucleus. Cells fixed with 1% (100 mM) glutaraldehyde before the application of FCCA retained a diffuse distribution over cytoplasm and nucleus, though occasional patches of dye were seen over cytoplasm and nucleus. Cells fixed with 300 μM paraformaldehyde and then exposed to FCCA resembled the unfixed controls: fluorescence rapidly disappeared from the cytoplasm of most cells and appeared as a perinuclear ring and/or stippled over the nucleus. Cells exposed first to 20 μM glutaraldehyde and then to FCCA retained much diffuse cytoplasmic fluorescence, even 1 h after FCCA was removed.

However, some cells acquired a perinuclear ring and some patching occurred. Thus, the low concentration of glutaraldehyde that reduced the capacity of cells to respond to serum was sufficient to alter the gross mobility characteristics of the cell surface, while high concentrations of paraformaldehyde did not affect the serum response and also had no effect on the mobility of FCCA.

DISCUSSION

Preincubation of rabbit lung macrophages with NRS for 15 min stimulates adenosine transport (32). Here it is shown that serum does not seem to act at or via the nucleoside-binding site of the carrier molecule, as inclusion of either thymidine or adenosine with NRS during preincubation does not alter the induced stimulation. Serum does not alter adenine nucleotide pools. As its effect is observed even if cells are preincubated and washed at 4°C, temperature-sensitive cellular functions such as phagocytosis and pinocytosis are apparently not responsible. Cytoplasmic and mitochondrial protein syntheses are probably not involved in the response, as exposure to 5 μg/ml cycloheximide and/or up to 500 μg/ml chloramphenicol do not alter the serum-induced stimulation (unpublished results).

In order to determine whether the activity of old transport sites was altered or if new sites were recruited in response to serum, an inhibitor of adenosine transport, sho, was used. When rabbit lung macrophages are exposed to sho, using the time and concentrations described, sho acts as an irreversible inhibitor of adenosine transport but does not alter the metabolism of adenosine *taken up during the 45 s transport period.* This observation is in contrast with the drug's effects on *E. coli* where sho inhibits nucleoside metabolism (17, 18, 19). Our evidence indicates that sho acts on the adenosine transport system at or via the transport site, as coincubation with thymidine or adenosine prevents the inhibition of transport. Even so, concentration (Figs. 5, 6) and time (Fig. 3) dependence curves show shoulders at early times and at low concentrations.

Serum can induce an increment in adenosine transport after adenosine transport has been reduced by sho. The increment does not seem to be due to nonspecific removal of sho from the cell surface as the optimal serum concentration (Table IV) and incubation time (Fig. 7) are the same for the serum-induced stimulation regardless of whether sho has been applied. In both cases exposure to higher concentrations of serum, heat-inactivated serum, or longer times does not alter the response. The percentage increase when sho-treated cells are then treated with serum is much greater than the percentage increase due to serum in control cells. In fact, the absolute amount of the increment is independent of transport remaining after sho inhibition. It can, however, be correlated with the magnitude of serum stimulation in control cells (Table III and Fig. 6). As sho under control conditions irreversibly inhibits adenosine transport and as the serum-induced increment is independent of transport remaining when cells are pretreated with sho, whatever changes that serum causes are unlikely to involve the transport sites remaining after sho. In other words, conformational change in the remaining transport carriers or more rapid movement by the carriers is unlikely to be primarily responsible for serum-induced changes. Moreover, exposure to serum alters the relationship of sho inhibition of adenosine transport to sho concentration so that the nonresponsive portion, as indicated by the shoulder, is not seen.

The irreversible inhibitor need not be a nucleoside analogue. Preincubation with *p*-chloromercuribenzosulfonic acid, a sulfhydryl reagent known to inactive adenosine transport (34), followed by exposure to serum, also leads to an increase over controls treated with the sulfhydryl reagent followed by buffer. Again, the time and concentration requirements for the increment in inhibitor-treated cells are the same as for controls (unpublished data).

The data pertaining to sho sensitivity and the effect of serum thereon are presented as log percent remaining transport vs. concentration of sho or vs. time of exposure to sho. From the plots one can see that transport diminishes in an exponential fashion except in the case of cells not exposed to NRS, where a shoulder is seen at early times and at low concentrations. Data which assume such an appearance can be interpreted on the basis of target theory so long as they satisfy the requirements of randomness of hit and discreteness of target (9). One might consider individual transport carriers as individual targets, thus satisfying the discreteness requirement. A plot of data from various experiments (Fig. 8) of the time required to inactivate 37% of transport vs. the reciprocal of the dose of sho (D_{37}) shows that the time-dose

relationship is constant. That is, the interaction of sho with the transport carrier can be considered a random event. Under these conditions one can consider the probability P of a given drug molecule interacting with a given transport site, as in footnote 2.

Presented in this form, log percent-remaining transport appears to be a function of concentration of drug (c), time of exposure (t), and sensitivity of transport sites to sho (α). When a shoulder is observed as well, as is the case here, target theory offers two models: a single-hit, multitarget model and a multihit, single-target model (9).[3]

Single-hit, multitarget models involve a clustering or coupling of targets. In terms of these data, then, some adenosine transport sites would exist in the plasma membrane coupled and some uncoupled. The percentage coupled must be small, as the extrapolation number for the inactivation curves (and the amount of serum-induced stimulation seen in the absence of sho) is in the neighbor-

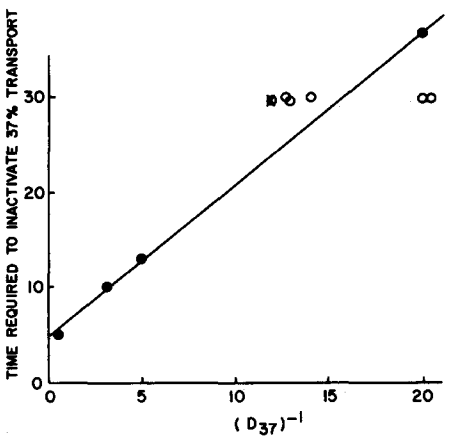

FIGURE 8 The relationship between time required to inactivate 37% of transport and dose of sho (D_{37}). Closed circles are points taken from Fig. 3. Open circles are points taken from five different experiments similar to the one presented in Fig 5. The X represents D_{37} from Fig 6. Data from serum-treated monolayers are not included.

[2] The probability that after n hits any target will have been hit m times and not hit $(n-m)$ times will be

$$P\{m\} = \frac{n!}{m!(n-m)!}p^m q^{n-m}. \quad (1)$$

For p very small, such that np is small, this can be approximated by the *Poisson* distribution:

$$P\{m\} = \frac{\lambda^m e^{-\lambda}}{m!}, \text{ where } \lambda = np. \quad (2)$$

Relating target theory to the deterministic description of exponential events, then, if a process exhibits exponential reduction, the incremental change in transport, dN, can be described mathematically for a single variable by

$$dN = -c_1\alpha dt \text{ (Eq. 3)}$$

which, when integrated, can be expressed as

$$N = N_o e^{-c_1\alpha t} \text{ (Eq. 4)},$$

where c_1, is the initial concentration of drug, α is the sensitivy of transport to drug, N is the transport remaining at time t, and N_o is initial transport. This can be related to unhit targets by setting $m = 0$ in the Poisson description above, then,

$$P\{0\} = \frac{N}{N_0} = e^{-c_1\alpha t}. \quad (5)$$

[3] The following models have been considered and rejected for the reasons given.

Model	Reason for rejecting
(*a*) Serum-induced increment due entirely to covered sites in the membrane which serum uncovers or causes to be uncovered. Covered sites cannot transport, nor can they bind sho.	No shoulder should be seen on the concentration vs. log per cent remaining transport or on the time of exposure to drug at constant concentration vs. log per cent remaining transport.
(*b*) Affinity changes of the carrier for adenosine account for the serum-induced stimulation.	No shoulder should be apparent, as for *a*. Also experiments where cells are treated first with sho and then with serum should show increment depending on remaining transport, and do not.
(*c*) Serum-induced changes due to unclustering only.	No increment should be seen in the ranges where inactivation is exponential.
(*d*) Serum-induced changes due to unclustering. Uncoupled sites have a higher affinity for adenosine.	Same objection as for *c*.

hood of 1.5. Only one transport site per group transports at any time; the others are inactive but have the potential ability to transport. There is precedence for this type of behavior with the enzyme alkaline phosphatase in *E. coli* (21).

However, all sites in a group can bind sho, and when one site is inactivated, another is recruited. Hence, at low concentrations of sho or at early times, sho will appear to have no or little effect on transport. At somewhat higher concentrations of sho, when all sites save the last in the group are inactivated, the curve takes on an exponential downward appearance. Serum uncouples or unclusters sites so that each transports independently of the others. Thus, every sho molecule hits a functioning target so that every hit is registered as a loss in transport. Therefore, there is no shoulder on the dose response curve. Experiments dealing with cells which are first exposed to sho and then to serum can be interpreted either as rescue of certain sites or as expression of a class of sites which never interacted with the drug. We do not favor a rescue hypothesis because serum should then rescue more carriers, perhaps by competition-displacement, when monolayers treated with sho are then treated with very high concentrations of serum (20%), and this does not occur. In support of expression of sites that never saw drug, there are several reported instances of activities present at the surface of cells which are uncovered by enzymatic treatment (11, 12, 24). Treatment of rabbit lung macrophages with proteolytic enzymes does not alter the rate of adenosine transport (35). However, some other enzymatic activity of serum may be involved, especially as the substance(s) in serum responsible for the enhancement of adenosine transport is (are) nondialyzedable and inactivated by heating at 100°C (32).

The second class of models predicted by target theory, the multihit, single target, would entail the following. As these data have a shoulder when plotted as log percent control vs. sho or vs. time of exposure to sho, they can be interpreted (from the intercept of the exponential region of the curves with the ordinate) such that each transport site requires more than one sho molecule to irreversibly inactive it. The intercept on the ordinate would indicate that more than 1 but less than 1.5 sho molecules are needed per transport carrier for inactivation. Serum pretreatment would alter that requirement so that only one sho molecule is required. The experiments where cells are first treated with sho and then with serum would be interpreted as above, that is, as either rescued or new sites.

It is impossible from the data at hand to distinguish unequivocally which model is the more appropriate. However, the ratio of shoulder to transport levels at various times and concentrations, e.g., the narrow shoulder, would strongly suggest the former (9). Moreover, a tentative choice in favor of the first can be made based on the aldehyde experiments. Exposure to low concentrations of glutaraldehyde (10–50 μM) but not formaldehyde (300 μM) for 10 min reduces the capacity of these cells to demonstrate serum-induced stimulation of adenosine transport without affecting base transport rates. Moreover, 20 μM glutaraldehyde like 100 mM (1%) glutaraldehyde but not 300 μM paraformaldehyde interferes with the mobility of fluorescein-conjugated concanavalin A over the surface of rabbit lung macrophages. Thus, these data would tend to support a model involving serum-induced movement of proteins on the cell surface. That the serum-induced changes also occur when the preincubation temperature is 4°C does not exclude a multistep process involving rotation or movement through very small distances: macrophage monolayers are rapidly warmed to 37°C when transport is tested. Rotation of rhodopsin in frog retina occurs within microseconds and is also prevented by cold or by previous exposure to glutaraldehyde (3, 6).

This model, involving both coupled and hidden sites, demonstrates a means by which cells can respond to environmental stimuli rapidly and effectively. Indeed, other activities that go on at the plasma membrane such as the accumulation of potassium in phytohemagglutinin-treated lymphocytes (28, 37), the insulin-induced stimulation of glucose transport (36), and the serum-induced changes in sodium pump density of Hela cells (20) might be explained in a similar fashion to those presented here. In line with suggestions by Holley (16), one might further speculate that malignant cells which do not demonstrate contact or density-dependent inhibition might be permanently uncoupled so that transport occurs maximally at all times. Indeed, concanavalin A binding sites seem to move more freely in the membranes of transformed as opposed to normal cells (29).

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