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ACTION OF ANTIHISTAMINE DRUGS *IN VITRO*—II. ION MOVEMENTS AND PHOSPHOPROTEINS IN WHOLE CELLS

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Abstract—Slices of rat liver and suspensions of ascites cells were used to determine the effects of antihistamine drugs upon ion movements; it was shown that these drugs inhibit uptake of potassium in such systems. The relationship between phosphoproteins and these effects was also investigated, and there appeared to be a direct relationship between protein phosphorylation and water and ion movements; this relationship is not mediated by effects on ATP levels or turnover rates. The nature of the protein fraction that takes up ^{32}P and shows diminished turnover in the presence of the drugs has been established by partial hydrolysis and identification of radioactive phosphoserine by chromatography on ion-exchange columns and upon paper.

IN PREVIOUS papers[1, 2] the effect of some antihistamine drugs on water movements in mitochondria was investigated. It was shown that antihistamines inhibited both swelling and contraction of mitochondria; that is, both uptake and extrusion of water. The present communication deals with the actions of these drugs on ion and water shifts in whole cells. Most of the experiments were done with slices of rat liver, but a few experiments with Ehrlich ascites tumor cells are included. Antihistamine drugs reduce water and ion movements in these systems at concentrations that leave the energy supply unaffected. As in isolated mitochondria, protein phosphorylation appears to go hand in hand with water extrusion (or what is more conveniently measured in whole cells, the uptake of potassium), and inhibition of the one is associated with inhibition of the other. Experiments with ^{32}P-phosphate show that phosphoprotein turnover is reduced by antihistamine drugs. The nature of the phosphoproteins has been explored by isolation of radioactive phosphoserine from partial acid hydrolysates of the tissue residues.

MATERIALS AND METHODS

Sprague–Dawley male rats of 150–200 g body weight were used for all experiments with liver slices. The animals were maintained on a normal laboratory diet, supplemented by vitamin E (α-tocopherol acetate) given by mouth, 10 mg thrice weekly. In the absence of this supplement, Na extrusion or K uptake is considerably reduced,[3–5] suggesting that the standard diet is inadequate.

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The line of tetraploid Ehrlich ascites cells, obtained from Dr. J. Colter of The Wistar Institute, was maintained in random-bred Swiss mice and was passed weekly by intraperitoneal injection into fresh mice. The cells were harvested after 7 days and 3 ml of the cell suspension was diluted with 0·9% (w/v) NaCl at 0 °C to 10 ml. The cells were sedimented at 800 × *g* for 22–25 sec and then made up to 10 ml with cold 0·9% NaCl; washing in this manner was repeated until a clear supernatant fluid, free of hemoglobin, was obtained. The cells were sedimented at 1500 **g** for 1·5 min in order to determine the packed cell volume and were suspended in an equal volume of 0·9% (w/v) NaCl and held at 0°.

The medium for all experiments was a Krebs–Ringer solution[6] buffered with $NaHCO_3$ at pH 7·2; variations from this system are noted in the text. The gas phase was 95% O_2–5% CO_2 or 95% N_2–5% CO_2, as indicated. Glucose (0·02 M final concentration) was sometimes added but was usually unnecessary with ascites cells and always so with liver slices, presumably because of a sufficient endogenous supply of energy-yielding substrate for ion transport.

Acid-insoluble components of the tissue were analysed by the method of Heald[7, 8] or Schmidt and Thannhauser.[9] The latter was modified to include an extraction of nucleic acids with hot trichloroacetic acid, as described by Schneider.[10] Phosphoproteins were determined by alkaline hydrolysis and the amount of inorganic phosphate liberated was estimated by the method of Berenblum and Chain,[11] modified in the method described earlier.[1] Alternatively, the tissue residue was subjected to acid hydrolysis as described by Heald[8] and followed by chromatography of phosphoserine on Dowex-50 resin. The phosphoserine was further chromatographed on paper, as described by Kennedy and Smith.[12] The technique was confirmed by isolation of radioactive P-serine from enzymically labeled casein.[13] An excellent correlation was obtained between these techniques for phosphoprotein determination. ATP was determined by the method of Crane and Lipmann.[14]

Slices were cut with a Stadie–Riggs microtome and were about 200 mg wet weight. The cooling of slices for depletion of intracellular K was carried out by the method of McLean.[4] All results are typical of at least 6 experiments in every case.

RESULTS

Anoxia and recovery

(1) Water and ion shifts. When liver slices are placed in bicarbonate-Ringer in an anaerobic system, they gain water and lose K rapidly.[4] The extent of the changes is governed by the composition of the medium, notably the Ca^{2+} content. Table 1 shows that at a Ca^{2+} level of 10^{-3} M, there is a rapid loss of K from the slices, which is much less marked at the standard level of $2{\cdot}8 \times 10^{-3}$ M Ca^{2+}. Variation of the pH from 7·2 to 7·4 has little effect upon these changes.

Table 1 also shows the recovery of these slices when they are transferred to oxygen. It can be seen that K levels rapidly return to figures near normal, and that the systems with low Ca levels reach the same levels as do those with normal Ca, which means that their rates of K uptake are considerably higher in the initial period of maximal change.

As previously reported,[15, 16] antihistamine drugs inhibit the recovery of liver slices from anoxia. Table 2 shows that all of the drugs tested inhibit uptake of K. Variation in Ca^{2+} concentration between 10^{-3} M and $2{\cdot}8 \times 10^{-3}$ M did not affect the inhibition

nor did variation of pH between 7·1 and 7·4. Repeated attempts have been made to learn whether antihistamine drugs act also in the opposite sense namely, to prevent loss of K and gain of water and Na during the anoxic phase but without success.

The rate of recovery is of interest: after 30 min in nitrogen, liver slices gain K extremely rapidly; the gain is maximal in the first 10 min and the slices reach about 80% of their normal K content in this time. The rate then falls off, and at the end of 60 min, levels of K are 90–100% normal.

TABLE 1. LEVELS OF K IN LIVER SLICES AFTER ANOXIA AND SUBSEQUENT RECOVERY

Liver slices were of 150–200 mg wet weight. They were incubated singly in 5 ml bicarbonate-Ringer, gassed with 95 N_2–5% CO_2, and then with 95% O_2–5% CO_2, pH 7·2. At the times indicated, slices were removed from vessels, blotted on Whatman 54 paper and weighed rapidly. After drying (105 °C, 24 hr) the slices were weighed and placed in 0·1 N HNO_3, the extract being used for flame photometry. Temperature was 38 °C throughout the experiment. The rates are calculated for the first 10 min of oxygenation.

Treatment	1·0 mM Ca^{2+}	mEquiv K/kg dry wt	2·8 mM Ca^{2+}
Initial		293	
30 min N_2	128		212
10 min O_2	250		285
20 min O_2	275		305
Max. rate of K uptake, mEquiv/kg dry wt/min	12·2		7·3

TABLE 2. EFFECT OF SEVERAL DRUGS ON UPTAKE OF K DURING RECOVERY AFTER ANOXIA

The conditions of the experiment were as described in Table 1. Only the normal concentration of Ca^{2+} ions (2·8 mM) was used. The rates are calculated for the first 20 min of oxygenation.

Drug*	Conc.	Max. rate of K uptake, mEquiv/kg dry wt/min
Nil		4·4
Promethazine	10^{-4} M	2·4
Diphenhydramine	5×10^{-4} M	2·4
Trimeprazine	10^{-4} M	0·7
Tripelennamine	2×10^{-4}	3·6
Tripelennamine	5×10^{-4}	0·3
Diphenylpyraline	5×10^{-4}	3·5
N-Dimethylamino isopropylthiophenylpyridylamine	5×10^{-5} M	2·2

* All drugs were used in the form of the hydrochloride except trimeprazine, which was in the form of the tartrate salt.

The experiments shown in Table 1 cannot be compared directly with other tables and figures in this section for two reasons. One is that different animals were used: the figures for Table 1 were obtained with Sprague-Dawley rats, while the others were obtained with another strain. The second reason is that the diet of the animals was also different: that for the animals in Table 1 was supplemented with α-tocopherol, and the others were not. This probably accounts for the major part of the difference in rates of K uptake observed.[5]

The duration of anoxia is of great importance in determining the recovery after O_2 is admitted to the system. Recovery is invariable after 30 min, extremely irregular after 45 min, and never occurs after 60 min of exposure to anoxic conditions.

(2) Changes in intracellular phosphate compounds. When slices of liver are exposed to anoxia for 30 min at 38 °C there is an abrupt fall in ATP levels from about 1–2 μmoles/g original wet weight. Some of this loss is permanent in that it is never made good upon oxygenation, but, nevertheless, ATP levels rise during recovery from anoxia until they reach a point that is some 80 per cent of normal. This takes about 60 min, and after it is reached there is no further increase. The level of phosphoprotein-P falls during 30-min anoxia from 1–1·5 μmoles/g wet weight to about 0·4–0·5 μmoles/g original wet weight before anoxia. Upon aerobic recovery there is a rapid rephosphorylation which, like the ATP, reaches about 80 per cent of the initial level between 30 and 60 min from the start of oxygenation. The levels of other of the insoluble compounds to not alter appreciably during anoxia or recovery. If antihistamine drugs are present in the flasks during oxygenation, the net increase in ATP[15] is unaffected, but phosphoprotein phosphorylation is reduced approximately by the same proportion as are ion and water movement. This is shown in Fig. 1.

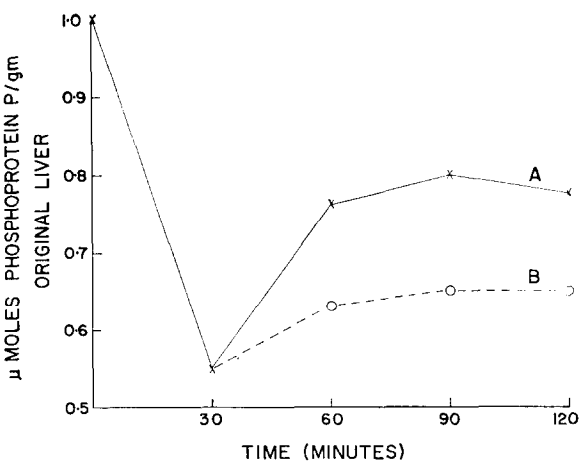

FIG. 1. Net synthesis of phosphoprotein in liver slices after anoxia. Slices about 200 mg wet weight were incubated in 5 ml Ringer-$NaHCO_3$, pH 7.2. Gas phase was 95% N_2 — 5% CO_2 during anoxia, and 95% O_2 — 5% CO_2 during recovery. The anoxic phase was the first 30 min shown in the figure. For determination of phosphoprotein-P, the slices were weighed prior to incubation, and the experiment was ended by addition of trichloroacetic acid to 5% at the times shown. Curve A represents control slices, and curve B slices in the presence of promethazine at 10^{-4} M.

The effects of the drugs are often enhanced if a 10-min contact time is allowed with the slices, which in this instance involves addition of inhibitor 10 min before the end of anoxia.

Experiments with ^{32}P in Table 3 show that the radioactivity of phosphoprotein-P is far higher than that of lipid P or RNA-P. The table also shows that two drugs,

promethazine and diphenhydramine, have no effect upon ATP radioactivity, but decrease that of the phosphoproteins. There is also no effect of the drugs upon lipid or RNA-P; indeed in the presence of diphenhydramine these are a little higher than in the controls. Thus, the experiments with tracer support the conclusions of the non-isotopic experiments.

TABLE 3. THE EFFECT OF ANTIHISTAMINE DRUGS ON RADIOACTIVITY OF CELLULAR CONSTITUENTS DURING RECOVERY FROM ANOXIA

The experiment was conducted as in Table 1. ^{32}P (4×10^6 cpm, specific activity 800,000 cpm/μmole P) was added at initiation of aerobic recovery. The slices were inactivated by tipping the entire flask contents into sufficient trichloroacetic acid to give a final concentration of 5%, after which the tissue was rapidly homogenized. Time of the experiment was 20 min. Temperature was 38 °C throughout.

Drug and conc.	Specific activity, cpm/μmole P			
	ATP	Phosphoprotein P	Lipid P	RNA—P
Control	61,000	10,600	305	366
Promethazine 10^{-4} M	58,000	5,500	340	328
Diphenhydramine 5×10^{-4} M	65,000	7,000	360	465

Recovery from cooling

(1) Water and ion shifts. McLean[4–6] showed that, upon cooling, rat liver slices lost some 70 per cent of their potassium after 35 min at 0 °C. Equivalent amounts of Na were gained, and the dry weight dropped from about 30 per cent of wet weight to about 22 per cent. These changes were reversed when the slices were warmed in Ringer's solution in the presence of O_2. McLean[4] has shown that K is taken up at a maximal rate of 4·5–6·5 mEquiv/min/kg dry wt, which is of the same order as that shown for slices recovering from anoxia.

The recovery after cooling does not begin at once, however; during the first 10 min there is virtually no gain of K. In the next 30 min K uptake is linear and maximal, and at the end of this period K levels at 70–80 per cent of normal. Thereafter K gains slowly, reaching its limit at about 60 min. This is a very convenient system for testing the effect of drugs. Fig. 2 illustrates a typical recovery from cooling, together with the action of promethazine at 10^{-4} M. A marked reduction in the rate of K uptake can be seen. Several tests with other drugs are shown in Table 4; all inhibit K uptake. Essentially similar results are obtained if Na extrusion or loss of water is determined.

The effects of drugs closely related to promethazine have been studied. Of these the sulfoxide, which is devoid of pharmacological activity, is completely without effect on ion movements.

The effects of the drugs were tested on K uptake at two levels of Ca, 10^{-3} M and $2{\cdot}8 \times 10^{-3}$ M, and also at pH 7·1 and 7·4. No variation in the results was observed.

(2) Changes in intracellular phosphate compounds. There is no change in levels of ATP and phosphoprotein-P either upon cooling or upon subsequent warming. We have therefore used ^{32}P as a tracer to study the turnover of these compounds. Fig. 3 shows the variation of the specific activity of phosphoprotein-P during recovery and illustrates the effect of diphenhydramine; a marked reduction can be seen. The turnover of ATP was also studied, and no effect of the drug was observed.

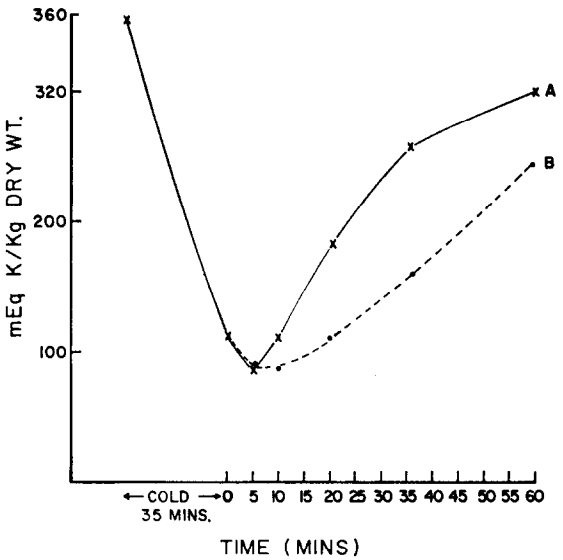

FIG. 2. Recovery of liver slices from cooling to 0 °C. The slices, about 20 mg wet weight, were cooled in ice-cold 0·9% (w/v) NaCl, for 35 min. At the end of this time samples were taken to establish potassium levels as described in Methods, and the slices transferred to Ringer-$NaHCO_3$, gassed with 95% O_2 – 5% CO_2, pH 7·2, and held at 37°, one slice being added to each flask containing 5·0 ml Ringer. Samples were withdrawn at the times shown. Curve A represents controls; curve B represents effect of promethazine at 10^{-4} M.

TABLE 4. THE EFFECT OF SEVERAL DRUGS ON UPTAKE OF K BY LIVER SLICES AFTER COOLING

The slices, 150–200 mg wet weight were cooled in 25 ml 0·154 M NaCl at 0 °C for 35 min. They were then transferred to bicarbonate-Ringer, gassed with 95% O_2–5% CO_2, pH 7·2 Each slice was placed in a separate flask containing 5·0 ml medium and which was kept at 38 °C prior to introduction of the slice. Enough slices were cut to enable samples to be taken at 10, 25, and 40 min after they had been transferred. The rates were calculated from differences in level between 10 and 40 min, the uptake being linear during this time for the controls. The slices were removed from the flasks and treated as described in Table 1 for the determination of the dry weight and K content.

Experiment 1		Experiment 2	
Drug	Max. rate of K uptake, mEquiv/kg dry wt/min	Drug	Max. rate of K uptake, mEquiv/kg dry wt/min
Nil	4·5	Nil	4·2
Diphenhydramine 5×10^{-4} M	1·9	Diphenylpyraline 5×10^{-4} M	2·6
Promethazine 10^{-4} M	1·7	N-Dimethylamino *iso*propyl-thiophenylpyridylamine 10^{-4} M	2·3
Chlorpromazine 10^{-4} M	3·5	Trimeprazine 10^{-4} M	2·3
Triprolidine 10^{-4} M	1·0	Tripelennamine 5×10^{-4}	2·7
Promethazine sulfoxide 10^{-4} M	3·8	Tripelennamine 2×10^{-4}	3·0

Identification of the phosphoproteins

It has been shown that phosphoproteins of brain[8] and of ascites cells[12] yield O-phosphoserine upon acid hydrolysis of the proteins, and that if the proteins be labeled with ^{32}P, the phosphoserine constitutes the major radioactive component of the hydrolysate.

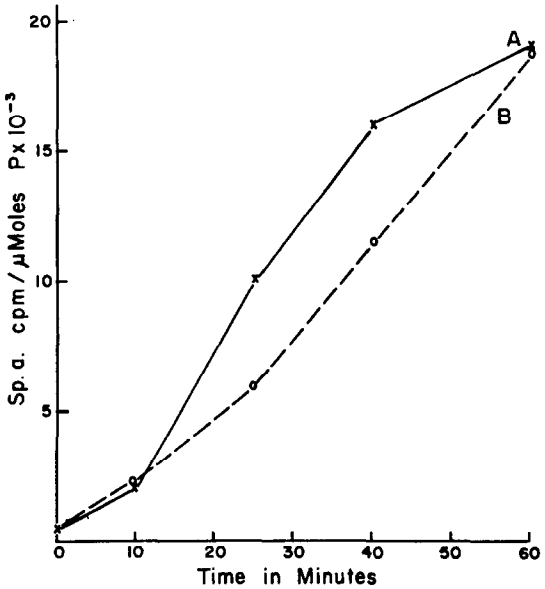

FIG. 3. Phosphoprotein turnover of liver slices during recovery from cooling. The experiment was done as described in Fig. 2 with addition of ^{32}P to the Ringer so that specific activity was approximately 1×10^6 cpm/μmole Pi. Curve A, controls; curve B, diphenhydramine at 5×10^{-4} M.

The methods described by Heald[8] have been applied to liver slices, and it can be shown that O-phosphoserine may be isolated from the phosphoprotein fraction.

For the experiments a carefully standardized method was used, the slices being of the same weight and thickness. Before acid hydrolysis, 10 μmoles of carrier phosphoserine was added to the tissue residue, which was derived from 200 mg of tissue. After chromatography on Dowex-50, the tube containing peak radioactivity and two tubes on each side of it were taken for determination of the specific activity. As found by Heald,[8] the activity of the P was the same in all of these tubes, indicating the presence of a pure compound. Paper chromatography[12] confirmed this.

In the presence of promethazine and diphenhydramine, the phosphoserine derived from the slices showed a reduction in radioactivity of the same order as that observed by the cruder method of alkaline hydrolysis and confirms the nature of the fraction inhibited (Table 5).

Experiments with Ehrlich's ascites tumor cells

Only a few experiments were performed with these cells; they were designed only to find whether ion movements in such cells were sensitive to the antihistamine drugs. The freshly washed cells were cooled in 0·9% (w/v) saline for 30 min at 0 °C when it

was found that their level of K had fallen to about 30 per cent of the initial value. Upon warming and oxygenating, a very rapid rate of recovery was seen, the rates of K uptake being of the order of 10 mEquiv/min/kg dry weight of cells. The effect of glucose on this uptake was negligible, but storage of the cells for more than 2–3 hr reduced it greatly. The experiments were therefore carried out as soon as possible

TABLE 5. IDENTIFICATION OF PHOSPHOPROTEIN FRACTION INHIBITED BY ANTIHISTAMINE DRUGS

Slices of liver (200 mg wet wt) were cooled in ice-cold 0·9% (w/v) NaCl for 35 min. They were then transferred to Ringer-$NaHCO_3$, already equilibrated with 95% O_2–5% CO_2, pH 7·2, temp. 38 °C. At 32·5 min the slices were removed and rapidly homogenized in 5% trichloroacetic acid. The separation of phosphoserine was carried out as described in the text. K analysis was performed on duplicate liver slices.

Drug and conc.	Phosphoserine spec. act. cpm/μmole P	K uptake mEquiv/min/kg dry wt
Control	650	5·6
Promethazine 10^{-4} M	360	3·4
Diphenhydramine 5×10^{-4} M	135	2·8

after the cells were withdrawn. It can be seen from Table 6 that promethazine and diphenhydramine cause very powerful inhibition of K uptake, and that the ascites cells are far more sensitive to the drugs than are liver cells. Thus, promethazine at 10^{-4} M inhibits uptake of K by liver cells by some 50 per cent, whereas K uptake by the ascites cells is inhibited by almost 50 per cent at 10^{-5} M of the drug.

This result is in keeping with our other experiments with human red cells in which it was shown that promethazine inhibited both uptake of K and protein phosphorylation.[2] It may be concluded that the effects of the drugs are not restricted to liver cells.

TABLE 6. EFFECT OF TWO DRUGS UPON UPTAKE OF K BY COOLED EHRLICH'S ASCITES CELLS

The experiment was conducted in 5·0 ml of bicarbonate-Ringer, pH 7·2, with a gas phase of 95% O_2–5% CO_2, temp. 38°C, time 20 min. Cells, equivalent to 0·1 ml of packed cells, were added. The experiment was ended by transferring flask contents to centrifuge tubes and centrifuging off the cells rapidly. The tubes were drained and wiped, the packed cells were treated with 5% (w/v) trichloroacetic acid, and centrifuged again. Portions of this extract were used for K estimation by flame photometry. The cells were depleted of K by cooling at 0 °C for 30 min.

Drug and conc.	Max. rate of K uptake, mEquiv/L cells/min
Nil	2·2
Promethazine 10^{-4} M	0·0
$3·3 \times 10^{-5}$ M	0·8
10^{-5} M	1·3
Diphenhydramine 5×10^{-4} M	0·4

DISCUSSION

As with isolated mitochondria,[1, 2] the antihistamine drugs inhibit water extrusion by liver slices of rat in suitable systems. These changes are best observed by measuring uptake of potassium. In contradistinction to isolated mitochondria, no effect of the drugs upon water and sodium entry could be shown. Numerous attempts were made

to inhibit water uptake during cooling, during anoxia and in the presence of DNP, but without success. It has not been possible, therefore, to show the double action of the drugs, which is a feature of the mitochondrial systems.

The relationship between water and ion movement and phosphoproteins, already described for isolated mitochondria, appears to hold good for slices. It seems probable that the phosphoproteins involved include not only the mitochondrial fraction but other cellular structures also. This is inferred from the magnitude of the net changes observed during recovery from anoxia, which are too large to be accounted for solely on the basis of mitochondrial phosphoprotein-P. This has been confirmed in other work (Ahmed and Judah,[27]) Heald[8] has identified in brain slices a phosphoprotein fraction that responds to electrical stimulation and which is apparently non-mitochondrial in distribution. In this connection it is of interest that partial hydrolysis and chromatography of radioactive phosphoserine have enabled positive identification of the liver phosphoproteins. This brings these compounds into accord with those of ascites cells[12] and of brain.[8] In other work (Ahmed and Judah,[28]) the phosphoprotein fraction in mitochondria that is inhibited by antishistamines and other drugs has been identified in like manner.

This brings us to the work of Heald,[7, 8, 17] who has demonstrated an increased turnover of cerebral phosphoprotein after electrical stimulation of slices of cortex. It may therefore be inferred that ion movements and phosphoproteins are related in brain as well as in liver slices. Further work in support of this idea soon will be published (Ahmed and Judah.[27]) The results of Hokin and Hokin[18, 19] are also of interest in this discussion. These authors have shown that phosphatidic acids become extremely radioactive in slices of salt glands of certain sea birds, stimulated by acetylcholine; they also have brought evidence to support the view that phosphatidic acids may be Na carriers in these cells and in erythrocytes. Two points are worth making. One is that, although increases in radioactivity have been shown, there is no evidence of increased turnover; the other is that, whereas salt glands may secrete Na in response to acetylcholine, such a response has not been found for most mammalian tissues. Since it appears likely that most mammalian cells have to work continuously to exclude Na and retain K, it seems probable that Na carriers would display constant high rates of turnover, and it is to be noted that extremely high rates of turnover have been reported for phosphoproteins in all cells studied. For the moment it is sufficient to say that, under certain conditions, the movement of ions and the turnover of phosphoproteins go hand in hand.

The next point to be considered is the protective action of the antihistamine drugs against cellular injury. Much work has now been done to show that such drugs prevent injury caused by chemicals,[20, 21] by nutritional deficiency,[3–5] and by virus attack.[22–24] The effects are not specific to liver cells. Mouse macrophages and embryonic bones in tissue culture (Biggers, Gwatkin and Judah, unpublished) also may be protected from injury, as may human erythrocytes against osmotic shock. The question arises as to whether these protective effects are related in any way to the actions on ion movement here described. It probably would be most profitable to consider that these phenomena are related, and to try to bring the findings into one theory. The connecting link appears to be in the phosphoproteins; it may be suggested that these form an integral part of many cellular and intracellular membranes, and that drugs affecting their stability are likely to affect the response of cells to damage. The relationship

of the drugs to the proteins may be indirect, since it seems unlikely that the great variety of agents that alter mitochondrial response to swelling agents, and which in many cases protect cells against adverse conditions, can have identical sites of action. A paper of Shanes[25] may be of importance here. He suggests that local anesthetics and possibly antihistamines might exert direct effects upon membranes by penetration and subsequent molecular crowding. It is conceivable that some of the effects described in this paper are caused by direct interactions of the drugs and components of the membranes. Further work in progress may lead to answers to some of these questions. For further discussions of these aspects, see Rees[26] and Judah,[2] where the relationships between antihistamine activity of drugs and their protective actions are considered. It is certain, however, that protective drugs do not have to show antihistamine activity.

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