ing lymph formation in the liver appears to be major reasons for the greater stimulation of thoracic duct lymph flow by epinephrine as compared to norepinephrine.

Summary. The effect of 2 pressor amines on rate of thoracic duct lymph flow was studied in conscious dogs. Intravenous epinephrine and norepinephrine both produced a significant increase in lymph flow. The response was significantly greater for epinephrine.

1. Gesler, R. M., Matsuba, C. A., Dragstedt, C. A., *J. Pharm. Exp. Therap.*, 1956, v116, 356.

2. Doemling, D. B., Steggerda, F. R., *Physiologist*, 1957, v1, 21.

3. ———, *J. Appl. Physiol.*, 1960, v15, 745.

4. Baez, S., *Shock and Circulatory Homeostasis*, edited by Green, H. D. Trans. 4th Conf., New York, Josiah Macy, Jr. Foundation, 1955, p112.

5. Ahlquist, R. P., Taylor, J. P., Rawson, C. W., Sydow, J. L., *J. Pharm. Exp. Therap.*, 1954, v110, 352.

6. Grayson, J., Johnson, D. H., *J. Physiol.*, 1953, v120, 73.

Received May 7, 1962. P.S.E.B.M., 1962, v110.

Relationship between Adenosine Triphosphate and Cation Transport in the Human Red Cell.* (27660)

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Demonstration that the red cell membrane was permeable to Na^+ and K^+(1,2) led to the conclusion that gradients of these ions across the red cell membrane must be maintained by active processes. Since the mammalian red cell derives virtually all its energy from glycolysis and stores much of this energy as ATP it was not surprising that obligatory relationships between cation transport and ATP utilization of metabolic activity should have been shown by Danowski(3), Harris(4), Eckel(5) and Maizels(6,7). Schatzman(8) discovered that strophanthin abolished the red cell's ability to concentrate K^+ or eject Na^+ but the mechanism could have been production of "leaky" cells or interference with active transport mechanisms in the cell membrane. Whittam(9) correlated K^+ movement with ATP concentration and noted that digoxin or ouabain allowed K^+ leakage without affecting ATP levels, thus suggesting interference with ATP utilization for cation transport. Subsequently(10) he showed that glycolysis *per se* was not required for active K^+ uptake in stored cells but that shunt activity from added nucleosides could also supply the energy needed. Straub(11) "lysed" red cells and after "reconstituting" them showed that the inward movement of K^+ was linked to lactic acid production. In the presence of arsenate, lactate production continued but K^+ inflow ceased. Gardos(12) was able to force ATP into these "lysed" cells at pH 2.3 and when the cells were "reconstituted", K^+ accumulated indicating the need for ATP only. Logically an ATPase would couple ATP breakdown to cation transport and Skou(13) found in crab nerve an ATPase activated by the simultaneous presence of Na^+ and K^+. Post, *et al.*(14) demonstrated a similar ATPase in red cell ghosts. Since so many of the preceding studies were performed on enzymes, pieces of membrane, on non-intact red cells it seemed necessary to restudy some of the relationships in normally functioning human red cells in their natural environment. The development in this laboratory of methods for maintaining and assessing viability of incubated human red cells(15,16) has allowed the following studies on the precise relationships among Na^+ and K^+ movements, ATP

* Supported in part by grants from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., Bethesda, Md., and Western New York Chapter, Arthritis and Rheumatism Foundation. The first author was a summer fellow of Nat. Science Foundation.

(and other nucleotide) levels, and rates of glycolysis in the cell. Although these studies may lack the ultimate precision of studies on isolated enzymes or on isolated red cell membranes, they offer the one unique advantage of mirroring physiological events at their site of origin, the intact red cell.

Experimental. Approximately 500 ml of human blod was collected in a standard vacuum blood bottle containing 10,000 units of sodium heparin solution. Aliquots of approximately 125 ml were transferred to 4 water-jacketed spinner flasks.† In the first experiment, SF-20, 406 mg of glucose was added to one flask, 635 mg of deoxyglucose was added to another (final concentration 3×10^{-2}M), and 15 mg of iodoacetic acid was added to another flask (final concentration 6×10^{-4}M). One flask acted as a control and contained only heparinized blood. The circulating water was kept at 37°C and under these conditions, 24 hours of incubation are equivalent to approximately 19 days at 4°C (15), if one wishes to extrapolate incubation studies to blood storage conditions. Samples were drawn aseptically from the 4 flasks at different time intervals for measurement of pH by the Astrup apparatus,‡ glucose by the Glucostat method,§ hematocrit, plasma and hemolysate sodium and potassium concentration using the Advanced‖ flame photometer. Hemolysates were prepared by freezing and thawing the blood twice in Dry Ice - alcohol slurries. Trichloroacetic acid extracts were prepared for subsequent nucleotide fractionation on Dowex-1-formate columns(16).

In the second experiment, SF-24, blood was collected in heparin and glucose, the latter being added to bring total glucose concentration to about 500 mg%. Aliquots were transferred to 3 spinner flasks. Disodium arsenate was added to 2 flasks to bring the arsenate concentration to 10^{-4} and 10^{-2}M while one flask served as control with no arsenate. Although disodium arsenate is alkaline, the amounts added did not affect the pH of the blood samples.

In a third experiment, SF-25, aliquots of heparin-glucose blood were transferred to spinner flasks and ouabain added to give concentrations of 0, 10^{-7}, 10^{-5}, and 10^{-3}M.

Intraerythrocytic K^+ and Na^+ concentrations were calculated from cation concentrations in the plasma and in the hemolysate by the following simple equations:

$$[Na^+]_cH + [Na^+]_p(1\text{-}H) = [Na^+]_h$$
$$[K^+]_cH + [K^+]_p(1\text{-}H) = [K^+]_h$$

Where the subscripts c, p, and h refer to cell, plasma, and hemolysate, and H is the hematocrit on the basis of 1 for whole blood. This calculation ignores any contributions by leukocytes. All donors were healthy young males and the amount of leukocytes appeared negligible by comparison to the red cell mass.

Results. The results of all experiments are given in Fig. 1-3. In an effort to bring the different kinds of data into an understandable framework, certain conventions were adopted in the graphing. For the cation distribution changes, intracellular K^+ was plotted above a line and intracellular Na^+ was plotted directly below the same line, in the corresponding position, but with *increasing concentrations downward,* in order to emphasize that as intracellular K^+ decreased, intracellular Na^+ increased, and usually in about the same amount. Thus non-maintenance of the usual intracellular-extracellular K^+ and Na^+ gradients would be signalled by downward displacement of the combined $K^+ - Na^+$ rectangle with respect to the reference line.

A similar convention was adopted for the blood nucleotides. The sum of ADP and ATP was plotted above the reference line since these 2 compounds represented stored high energy phosphate in the cell. The sum of AMP, IMP, inosine, and hypoxanthine was plotted on a *reversed scale* below the reference line. Since the latter group of compounds has been shown to constitute the only degradation products of the high energy adenine nucleotides in human blood(17), again the loss of high energy phosphate with-

† Bellco Glass Co., Vineland, N. J. In addition to the water jackets for temperature control these flasks has ports for gassing and a magnetic stirrer driven externally.

‡ Radiometer Instruments, The London Co., Cleveland, Ohio.

§ Worthington Biochemical Corp., Freehold, N. J.

‖ Advanced Instruments, Inc., Newton Highlands, Mass.

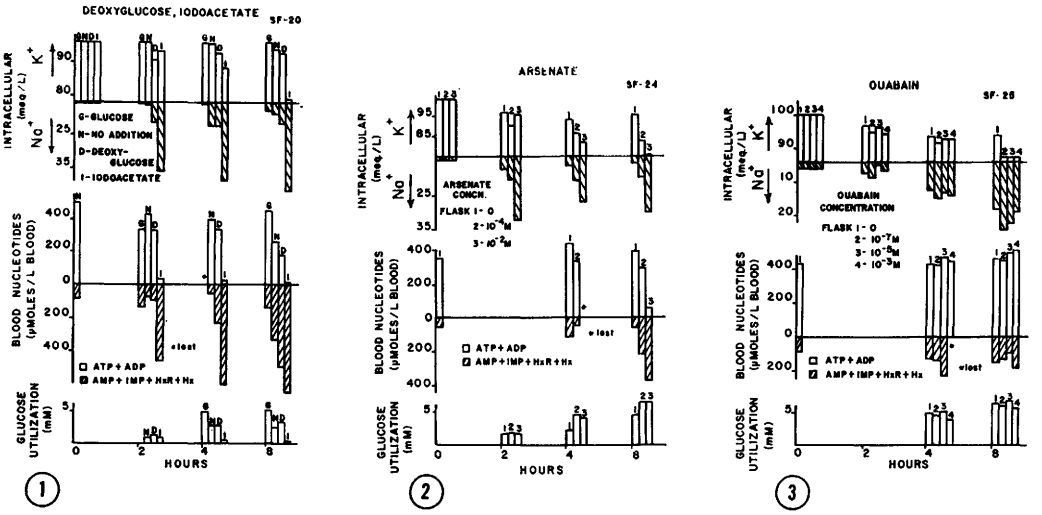

FIG. 1. Intracellular K^+ and Na^+, blood nucleotides, and glucose utilization in human red blood cells incubated at 37° without and with glucose and with the glycolytic inhibitors, iodoacetate (6×10^{-4}M), and 2-deoxyglucose (3×10^{-2}M).

FIG. 2. Same determinations in human red cells incubated with arsenate.

FIG. 3. Same determinations in human red cells incubated with ouabain.

in the cells would be signalled by downward displacement of the combined rectangle with respect to the reference line. The object of plotting intracellular $K^+ - Na^+$ changes and cellular energy stores in the present framework is that similar changes can be followed easily. Glucose utilization has been plotted on a conventional upward scale since its decreased utilization is commonly associated with decreased cation gradients and lowered cellular energy stores.

Discussion. From the results of Experiment SF-20 (Fig. 1) it can be seen that after 2 hours incubation with iodoacetate, the high energy stores of the cells were essentially depleted and concomitantly considerable Na^+ had leaked into the cells. At 4 hours these changes were more marked for both K^+ and Na^+, and high energy phosphate had all but disappeared. The same trend persisted through the 8 hours of incubation. The effectiveness of blocking of glycolysis by iodoacetate was shown not only by the failure to maintain ATP and ADP levels but also by the very low glucose utilization as depicted on the bottom line of Fig. 1. The influence of 2-deoxyglucose on cation distribution and high energy phosphate was similar to that of iodoacetate but not so drastic at the concentrations used. In fact, the effects were just becoming marked at the 8-hour period. For the sample with no addition, all the glucose it contained was used up by 4 hours. Thus between 4 and 8 hours it could not carry on glycolysis and in consequence the ATP and ADP decreased. Likewise the K^+ and Na^+ gradients decreased slightly. On the other hand, the aliquot of blood to which glucose had been added carried on glycolysis at a good rate and at 8 hours the high-energy phosphate stores and intracellular K^+ and Na^+ concentrations were close to what they had been at the beginning of the incubation.

There has been much discussion concerning the exact relationship between Na^+ efflux and K^+ influx. The foregoing experiment indicates that they are correlated but the simple methods employed do not allow a more detailed analysis. The object, instead, was to demonstrate in an essentially *in vivo* situation the dependence of K^+ and Na^+ transport on high energy stores which themselves depend upon active glycolysis in this simple metabolic system.

In the second experiment (SF-24), the objective was to allow glycolysis to proceed, but to dissociate it from storage of the generated high energy phosphate. This was done by adding arsenate at low levels of effect. The arsenate at either 10^{-4} or 10^{-2}M did not slow

glucose utilization, but seemed to enhance glycolysis, as one would expect if ADP is a stimulator of glycolysis (Fig. 2, bottom line). The effect of arsenate on the high energy stores was not apparent at 4 hours because one sample was lost. It was marked at 8 hours, however. Concomitantly, K^+ was leaking out of the arsenate-poisoned red cells and Na^+ was leaking in. The difference between 10^{-4} and 10^{-2}M arsenate was easily seen in both the high energy phosphate stores and intracellular cation concentrations.

The experiments thus far demonstrated first, that in presence of glycolytic inhibitors, high energy phosphate stores were lost and intracellular cation gradients began to decrease. Second, in presence of arsenate, glycolysis could continue but without effective trapping of the energy generated, and this situation also would not maintain cation gradients across the cell membrane. A third possibility was to maintain glycolysis and high energy phosphate stores but to dissociate the utilization of ATP energy from the process of active cation transport. The coupling of ATP breakdown with cation transport is postulated to take place on an ATPase and according to Post *et al.*(14) and Whittam(9), among others, this enzyme is inhibited by ouabain or digoxin. In the last experiment (SF-25) ouabain was added at 3 different concentrations to aliquots of fresh heparin-glucose blood, and the same constituents determined as before. Ouabain at these concentrations had no effect on either glucose utilization or high energy phosphate stores but by eight hours it did lessen intracellular K^+ and increase intracellular Na^+ (Fig. 3). Ouabain acted slowly in this system, regardless of its concentration. Since most other reported studies have used ouabain in enzyme or membrane systems one might suspect that in our system there was a rate-limiting step in the ability of the ouabain to penetrate the intact, viable red cell.

Thus it has been shown that high energy phosphate utilization is coupled to maintenance of K^+ and Na^+ gradients across the viable red cell membrane. Similar conclusions are implicit in the literature previously cited but are here clearly demonstrated and the events correlated under essentially *in vivo* conditions. A practical implication of the present findings is that stored human red cells will not leak appreciable amounts of K^+ into the plasma or medium as long as normal glycolysis and high energy phosphate stores can be maintained and utilized and as long as the transport mechanism in the membrane remains operable.

Summary. Fresh human blood collected in heparin and glucose was treated with glycolytic poisons such as iodoacetate and 2-deoxyglucose to retard glycolysis and reduce the high energy phosphate stores of the red cell. Under these circumstances K^+ began to leak out of the cells and Na^+ began to leak in. In the presence of arsenate, glycolysis proceeded but high energy phosphate stores were not maintained and cation leakage also occurred. In a final experiment, both glycolysis and high energy phosphate stores were continued normally but ouabain was added to inhibit the transport ATPase. This prevented the coupling of ATP breakdown to active cation transport and allowed K^+ to leak out of the red cell and Na^+ to leak in.

The authors are happy to acknowledge the invaluable technical assistance of Miss Ann Dutton and Miss Jeanette LaDuca, the latter of whom also drew the graphs.

1. Cohn, W. E., Cohn, E. T., PROC. SOC. EXP. BIOL. AND MED., 1939, v41, 445.

2. Mullins, L. J., Fenn, W. O., Noonan, T. R., Haege, L., *Am. J. Physiol.,* 1941, v135, 93.

3. Danowski, T. S., *J. Biol. Chem.,* 1941, v139, 693.

4. Harris, J. E., *ibid.,* 1941, v141, 579.

5. Eckel, R. E., *Am. J. Physiol.,* 1954, v179, 632.

6. Maizels, M., *J. Physiol.,* 1951, v112, 59.

7. ———, *Symp. Soc. Exp. Biol.,* 1954, vVIII, 202.

8. Schatzman, H. J., *Helv. Physiol. Acta,* 1953, v11, 346.

9. Whittam, R., *J. Physiol.,* 1958, v140, 479.

10. ———, *ibid.,* 1960, v154, 608.

11. Straub, F. B., *Acta Physiol. Hung.,* 1954, v4, 235.

12. Gardos, G., *ibid.,* 1954, v6, 191.

13. Skou, J. C., in *Membrane Transport and Metabolism,* Kleinzeller, A., Kotyk, A., editors, Academic Press, New York, 1961, p228.

14. Post, R. L., Merrit, C. R., Kinsolving, C. R., Albright, C. D., *J. Biol. Chem.*, 1960, v235, 1796.
15. Bishop, C., *Transfusion*, 1962, v2, 256.
16. Bishop, C., Rankine, D. M., Talbott, J. H., *J. Biol. Chem.*, 1959, v234, 1233.
17. Bishop, C., *J. Biol. Chem.*, 1960, v235, 3228.

Received May 14, 1962. P.S.E.B.M., 1962, v256.

The Path of Cobalt-60, Ionic or Chelated, in Albino Rats after Intravenous Administration.*† (27661)

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Previous investigation in this laboratory has shown that simultaneous administration of chelating agents can considerably alter the tissue distribution of cobalt-60 in albino rats (1). While nearly 80% of the radioactive cobalt, injected alone, is excreted within 24 hours, mainly through urine, most of the chelating agents cause decreased excretion and preferential localization in various organs. Cysteine and ethylenediaminetetraacetic acid (EDTA) considerably enhance excretion of the radioisotope, excretion with EDTA amounting to 96.3%; with cysteine, the excretion is 88.1%. As a first step towards understanding the mechanism of these changes, the exact state in which the cobalt-chelate exists in the blood after intravenous administration has been studied(2). The results suggest that cobalt-60, injected alone or with any one of the chelating agents, is partly bound to serum proteins (mostly to albumin and α-globulin) and partly free, except that the EDTA-cobalt chelate exists as a separate soluble entity in the blood, being free and completely dialysable. The 1-nitroso 2-naphthol chelate in the blood has been found to be completely nondialysable, probably colloidal, but not completely protein-bound. Continuing these experiments, the movement of the tracer, ionic or chelated, after it leaves the vascular compartment has been investigated through time-distribution studies. The results are presented here. While the distribution of radioactive cobalt has been studied extensively in a number of species, most of the work relates to orally-administered cobalt over a period of days(3,4,5). Greenberg and coworkers observed that parenterally-administered cobalt is excreted mainly through urine(6).

Materials and methods. Cobalt-60, obtained from the Radiochemical Centre, Amersham, U.K., was as an aqueous solution of cobaltous chloride, containing about 35 μg cobalt per mc. The solution was suitably diluted with isotonic saline prior to injection. The chelating agents used, 1-nitroso 2-naphthol, sodium diethyldithiocarbamate (SDDC), rubeanic acid, 2:3 dimercapto propanol-1 (British Antilewisite, BAL), EDTA as its disodium salt, and L-cysteine HCl · H_2O, were all of high grade purity.

The tissue distribution of cobalt-60, injected alone or with a chelating agent, at various intervals after administration was studied. Four hundred adult male albino rats, weighing 150-250 g, divided into groups of 5, were used. The experimental procedure for administration of the isotope in the ionic or chelated form and for subsequent assay of radioactivity in the various tissues is the same as described earlier for studying the 24-hour distribution(1). In the present investigation, in addition to the 24 hours, the distributions at various other times from 5 minutes to 72 hours, were also determined by sacrificing the

* Supported by a grant from Dept. of Atomic Energy, Government of India.

† Paper No. 3 in the series "The effects of chelating agents on the biological distribution of radioisotopes." Presented in part at the Indian Science Congress, Jan. 1961.

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