There was a decrease in the number of deaths (Table 1) when the mice were starved for 6 hours prior to injection. Conversely the number of deaths rose sharply after a meal of peanut butter. These data indicated that the time of feeding and possibly the diet played an important role in the toxicity of bee venom to white mice and formed the basis for further experimentation.

To eliminate the possibility that an intake of any food might cause the same effect, mice which had been maintained on a normal protein diet were starved for 18.5 hours and then allowed to feed for 20 to 30 minutes on diets which varied in protein, carbohydrate, and fat content (*3*). The mice were injected within a 15-minute period after a feeding. The data are shown in Table 2.

The number of mice killed by venom when fed a high-protein diet was twice as great as when they were fed the other diets; therefore, protein intake directly or indirectly affects the tolerance of mice to bee venom. In the aforementioned experiment white mice were fed diets, which varied in the percentage of protein, for 3 days (Table 2). The mice were injected at 3:00 P.M. with no starvation period preceding the administration of the bee venom. In this experiment not only was there a substantial increase in the number of deaths among the mice fed a high-protein diet, but the number of deaths was also reduced by two-thirds among those mice fed on a diet which contained no protein.

Mice that were 14 weeks old were twice as susceptible to bee venom as 8-week-old mice when all were kept on a normal daily routine with a normal protein diet. Ten mice out of 15 died in the 14-week-old group whereas only an average of 5 mice out of 15 in the 8-week-old group died when both groups were injected with a dose calculated to kill 5 out of 15. It also was determined that mice injected subcutaneously with 75 μg of venom per gram of body weight did not die. This is ten times the amount required to produce 100 percent killing when injected into the peritoneal cavity. In fact, the animals completely recovered in 6 to 12 hours.

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Sulfate Transport in Human Red Cells: Inhibition by Some Uncouplers of Oxidative Phosphorylation

Abstract. *Release of inorganic sulfate from human erythrocytes is depressed in the presence of 2,4-dinitrophenol (5×10^{-4}M) or dicumarol (5×10^{-4}M). This effect cannot be readily attributed to uncoupling of phosphorylation from respiration, since the study was conducted with cells that metabolize principally by anaerobic means and since the effect was not influenced by iodoacetic acid. A more reasonable explanation may be that permeability of the erythrocyte membrane to anions may be reduced by these agents.*

Uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol (DNP), are often used in studies of cell physiology to evaluate the relation between metabolism and function. The best known action of these agents is that they prevent formation of adenosine triphosphate (ATP) in mitochondria by dissociating reactions concerned with phosphorylation from those concerned with electron transfer (*1*). A change in function induced by uncoupling agents in whole, intact cells, however, cannot be ascribed simply to depressed mitochondrial phosphorylation because these inhibitors could conceivably affect some other process in the more complex, intact cell system. Thus, DNP depresses the resting membrane potential in certain excitable tissues (*2, 3*), but this change cannot be readily attributed to reduced synthesis of high-energy phosphate in the absence of more direct evidence, such as a reduction in concentration of ATP in the membrane. My report concerns an action of certain uncoupling agents on the human erythrocyte which cannot be readily explained by disruption of oxidative phosphorylation. This being the case, the question arises as to whether the effect of these inhibitors on other intact mammalian cells could also be caused by a less familiar action, such as the inhibition of ion transport observed in this investigation.

Blood collected in the usual mixture of acid, citrate, and dextrose (for blood storage) was centrifuged at 800*g* and 5°C for 5 minutes, and the buffy coat and plasma were removed. The cells were washed four times with a modified, calcium-free Ringer-Locke solution, volumes used being equal to that of the plasma removed. The salt solution was adjusted to *p*H 7.4 at 37.5°C with tris(hydroxymethyl)aminomethane buffer. After the separation and washing procedures, the cells were labeled by incubating them (*4*) for 1 to 2 hours at 37°C in calcium-containing Ringer-Locke medium in the presence of 0.5 μc of radioactive inorganic sulfate ($S^{35}O_4^{--}$) per milliliter of cells and 1m*M* sulfate. The labeled cells were then washed twice and resuspended in nonradioactive medium in order to study the release of sulfate ion.

Samples of the labeled blood suspension (3.5 ml) with a hematocrit value of 20 percent were placed in 25-ml erlenmeyer flasks to facilitate subsequent sampling, and the flasks were incubated. Portions (0.3 ml) of the blood suspension were removed after 10, 70, and 130 minutes, and the radioactivity was determined by plating the samples and the medium alone on concentrically ringed aluminum planchets; the samples were air-dried and counted with an end-window Geiger-Mueller counter. After correcting the counts for mass absorption, the results were expressed in terms of the ratio, medium $S^{35}O_4^{--}$: total $S^{35}O_4^{--}$.

The *p*H was monitored with a Beckman model G meter through external glass and reference electrodes that were placed in the incubator along with the blood samples. Decrease in *p*H was generally less than 0.05 *p*H units in 2 hours, and no significant difference was noted between control samples and samples containing an uncoupling agent. Hematocrit measurements (*5*) showed no detectable differences. Besides DNP (Eastman or Fisher), other inhibitors used were

dicumarol and gramicidin (Nutritional Biochemicals).

Significant inhibition of $S^{35}O_4^{--}$ release was noted in the presence of $5 \times 10^{-4}M$ and $10^{-3}M$ DNP (Fig. 1*A*). If red cells were first incubated with $10^{-3}M$ DNP and then resuspended in DNP-free solution, the release of $S^{35}O_4^{--}$ was indistinguishable from that obtained with untreated, control erythrocytes. Inhibition, therefore, is readily reversible. In other experiments, dicumarol caused changes of a similar nature: very little change was seen with $10^{-5}M$ dicumarol, and increasingly greater responses were noted with $5 \times 10^{-5}M$ and $10^{-4}M$ dicumarol. With either DNP or dicumarol, it could not be ascertained whether maximum effects were obtained because further increases in concentration approached the solubility limit of these agents. In contrast to the foregoing results, $10^{-6}M$ gramicidin had no effect on $S^{35}O_4^{--}$ transport up to the point where hemolysis occurred. The hemolytic action of gramicidin had been noted previously (*6*).

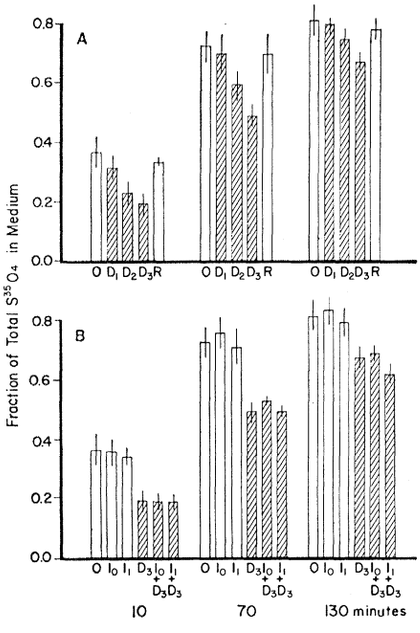

Fig. 1. *A*, Release of $S^{35}O_4^{--}$ from human erythrocytes as a function of DNP concentration. That fraction of the $S^{35}O_4^{--}$ in the total suspension which is present in the medium is plotted against time. The vertical bars represent twice the standard deviation of the mean. The symbols *O*, D_1, D_2, and D_3 represent 0, 10^{-4}, 5×10^{4}, and $10^{-3}M$ DNP, respectively; *R* refers to cells that were first incubated with $10^{-3}M$ DNP for 1 hour and then resuspended in control solution. Evaluation of significance by the paired *t*-test indicates that the mean difference (four experiments) between the control and experimental were significant at the 1 percent level in all cases except the t_{70} and t_{120} values with $10^{-4}M$ DNP. *B*, Influence of $10^{-3}M$ DNP on $S^{35}O_4^{--}$ release from erythrocytes poisoned with $5 \times 10^{-4}M$ iodoacetic acid. I_0 represents incubation in the presence of iodoacetic acid, and I_1 represents additional prior incubation with iodoacetic acid for 1 hour; *O* and D_3 are 0 and $10^{-3}M$ DNP, respectively. The mean differences (three experiments) between DNP-free and DNP-containing cells in the presence of iodoacetic acid were statistically significant at the 1 percent level.

Since the dominant action of uncoupling agents is on high-energy phosphate synthesis under aerobic conditions, the effect of these agents on the human erythrocyte, which metabolizes principally anaerobically, was surprising. Most of the biochemical apparatus associated with oxidative phosphorylation, such as the enzymes of the tricarboxylic acid cycle and the cytochromes, is almost completely lacking in the anucleate red cell (*7*). Inhibition of sulfate transport in the human red cell, therefore, strongly indicates that these agents may act on a process other than oxidative phosphorylation in the intact cell. In accordance with this view is Conway's observation that the presence of DNP results in increased glycolysis and sodium excretion in the cyanide-inhibited frog sartorius (*8*). One implication of this work on frog muscle is that DNP acts by influencing a glycolytic reaction. In my studies with human red cells, however, the DNP effect (Fig. 1*B*) was virtually the same in the presence as in the absence of the glycolytic inhibitor, iodoacetic acid, indicating that the action of the uncoupling agents in the red cell is independent of normal glycolytic rates. The iodoacetic acid concentration that I employed inhibits lactate production and P^{32} incorporation into ATP in human erythrocytes (*9*) and also reduces the ATP concentration in red cells to low values within an hour (*10*). The lack of influence of iodoacetic acid on the DNP effect also supports the view that the action of the uncoupling agent is independent of oxidative phosphorylation in my study, because glycolysis is a major source of substrate for oxidative phosphorylation.

A more reasonable explanation of my results may be that DNP and dicumarol can alter membrane structure and, thereby, permeability. Sulfate ion is believed to be passively transported across the mammalian red cell membrane (*11*), and the lack of effect of iodoacetic acid in my study supports this view. Decrease in $S^{35}O_4^{--}$ transport may, therefore, indicate decreased anion permeability of the cell membrane and also indicate that these agents act at this site. I have found in similar studies that the release of radioactive inorganic phosphate is also depressed by DNP and dicumarol but not by gramicidin so that these inhibitors appear to act on anion transport in a nonspecific manner. In other studies (*2*) it has been suggested that the rapid depolarization of the resting potential produced by DNP in the myometrial cell may reflect a nonspecific change in the passive permeability properties of the cell membrane, because changes in cation concentration associated with active sodium transport take place too slowly to account for this change.

Insight into the molecular interactions between DNP or dicumarol and the human red cell may be derived by comparing my results, obtained with cells that do not undergo oxidative phosphorylation, with those obtained in mitochondria when these organelles are suspended in a medium that does not support oxidative phosphorylation. In both cases, DNP and dicumarol inhibit phenomena which appear to be intimately related to membrane structure: anion transport in the erythrocyte and swelling in the nonphosphorylating mitochondria (*12*). Thus, there may be some similarity in the biochemical processes affected by these agents in the two systems (*13*).

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