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The Effects of 3-Methylindole on Hemolysis, Transport of $^{22}Na^+$, and ATPase Activities of Bovine Erythrocytes[1] (38651)

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3-Methylindole (3MI) has been implicated as the causative agent in tryptophan-induced pulmonary edema and emphysema in cattle and it also induced similar pulmonary lesions in goats (1). The clinical signs and pulmonary lesions of this experimentally induced disease resemble those in naturally occurring acute pulmonary edema and emphysema in cattle (1, 2). Studies have shown that 3MI adversely affects biological membranes (3, 4) and interacts with membrane components (5, 6). The biochemical effects of 3MI on cellular membranes have not been sufficiently investigated to evaluate the mechanism by which 3MI causes tissue damage. The purpose of this study was (a) to investigate the hemolysis of erythrocytes induced by 3MI, (b) to determine the effect of 3MI on passive and active transport of $^{22}Na^+$ on resealed erythrocyte ghosts, and (c) to examine the effects of various concentrations of 3MI on total and Na^+-K^+-dependent ATPase activities in erythrocyte membranes.

Materials and Methods. Examination of hemolysis. Fresh saline washed bovine erythrocytes were resuspended in 0.17 *M* NaCl to give a 50% cell suspension. Sixty mg of 3MI were evenly coated on the sides of a 100 ml round bottom flask by dissolving it in 1 ml of acetone and then drying it on a rotary evaporator. Thirty ml of 50% RBC suspension were transferred into the 3MI coated flask and incubated at 37° in a shaking water bath. The control cells were incubated under the same conditions without 3MI. At times of 0, 5, 15, 30 min and 1, 2, 3, 4, 5.5, 7, 10, 12 and 24 hr incubation, 2 ml aliquots were pipetted into 10 ml of ice cold isotonic NaCl solution buffered with Tris (pH 7.4) to stop hemolysis. The cells were then packed by centrifuging at 34,500 *g* for 5 min. The absorbance of the hemoglobin released in the supernatant was determined at 500 nm. The 3MI in the packed cells was extracted and quantitated using gas-liquid chromatography (7).

Determination of passive and active transport of $^{22}Na^+$ in resealed erythrocyte ghosts. The erythrocytes were washed three times in isotonic NaCl and resuspended in isotonic Tris buffer (pH 7.4) to give a 50% cell suspension. The method used for determination of passive and active transport of $^{22}Na^+$ in resealed ghosts was modified from Bodemann and Passow (8) and Passow (9). For passive transport, 3 ml of this suspension were hemolyzed at 0° by mixing with hemolyzing fluid which contained 2 ml $^{22}Na^+$ (5 μCi/ml), 1.5 ml ^{14}C-sucrose (20 μCi/ml) and 21.75 ml $MgSO_4$ (2 m*M*) in 2 m*M* Tris buffer (pH 7.4). Carrier-free $^{22}Na^+$ ($^{22}NaCl$, 10 mCi/mg) and crystalline ^{14}C-sucrose (^{14}C[U]-sucrose, 4.87 mCi/m mole) were purchased from New England Nuclear Corp. For active transport, 4 m*M* of ATP was included in the hemolyzing fluid as an energy source. Five min after hemolysis the isotonicity was restored by the addition of 1.75 ml of 3.32 *M* NaCl. The suspensions were then incubated at 37° for 45 min. Three ml aliquots were taken at the end of the resealing period for the 0 time sample. The resealed ghosts containing $^{22}Na^+$ and ^{14}C-sucrose were collected by centrifuging at 34,500 *g* for 10 min. For passive transport, the sedimented ghosts were resuspended in 27 ml of isotonic KCl solution buffered with Tris pH 7.4, containing 500 μg/ml of 3MI. For active transport, the sedimented ghosts were resuspended in 27 ml of isotonic solution containing KCl (20 m*M*), NaCl (40 m*M*), choline chloride (95 m*M*), Tris (5 m*M*), and 500 μg/ml 3MI. The control cells were

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handled in the same way. At times of 2, 5, 15, 30, 60 and 120 min, 3 ml of suspension were transferred into 10 ml ice cold isotonic KCl solution and centrifuged at 34,500 *g* for 10 min. The intracellular $^{22}Na^+$ in the packed ghosts was counted using a γ-ray counter (1185 series, Nuclear-Chicago, IL), and ^{14}C-sucrose was determined by using a liquid scintillation counter (720 Series, Nuclear-Chicago, IL). The data were analyzed by computer program LINLOG to fit the least square line $Y = A^{bx}$. A Student's *t* test was used to compare the slopes of curves between treatments at the level of 5% probability (10).

Assay of total and Na^+-K^+-dependent ATPase activities. The ATPase assay was modified after Bonting and Caravaggio (11). To measure total activity, 0.2 ml of packed erythrocyte membranes prepared by the method of Dodge *et al.* (12) were incubated in 2.8 ml of solution containing 2 mM $MgCl_2$, 5 m*M* KCl, 60 m*M* NaCl, 0.1 m*M* EDTA, 92 m*M* Tris–HCl and 3 m*M* ATP. Mg^{2+}-dependent ATPase activity was measured by incubating an identical aliquot of membranes with the same solution except that NaCl and KCl were deleted; the Na^+-K^+-dependent ATPase activity was taken to be the difference between the total and Mg^{2+}-ATPase activities. The effects of 3MI on ATPase activities were examined by adding 25, 50, 100, 200, 300, 400 and 500 μg/ml of 3MI in the assay mixture. The membranes were incubated at 37° for 1 hr. At the end of the incubation period, 0.2 ml (5%) deoxycholate and 0.8 ml (6%) $HClO_4$ were added. The precipitated membranes were centrifuged and 1 ml of the supernatant solution was taken for phosphate analysis by the Fiske and Subbarow method (13).

Results and Discussion. Hemolysis of erythrocytes. The time course for the hemolysis of erythrocytes incubated with 3MI, and the 3MI uptake by erythrocytes is shown in Fig. 1. When the erythrocyte suspension was treated with 3MI, the percent of hemolysis as a function of 3MI incubation time was sigmoidal. There was no hemolysis at concentrations of less than 500 μg 3MI/ml of packed erythrocytes. Seventy-five percent of the hemoglobin was released from 2 to

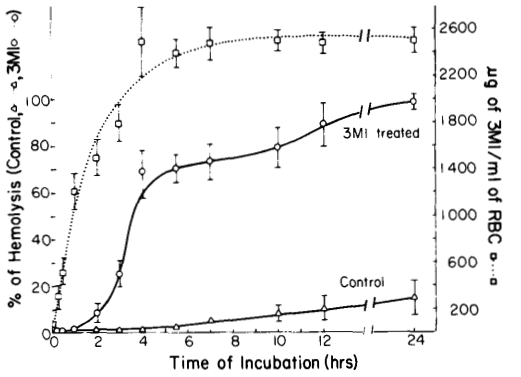

FIG. 1. The time course for the hemolysis of erythrocytes incubated with 3MI, and the 3MI uptake by erythrocytes. Each point indicates the mean from four experiments. The standard errors of the means are represented by the vertical lines.

4 hr of incubation during which the concentration of 3MI in the cells reached a plateau of 2500 μg 3MI/ml of packed erythrocytes. More than 20 hr of incubation were required for the remaining 25% of hemoglobin to be released. The control cells hemolyzed 15% after 24 hr of incubation. It is suggested that the occurrence of hemolysis depends on the total 3MI uptake by the cell. Azuma and Yoshizawa (14) observed a sigmoidal hemolysis curve when erythrocytes were incubated with all-trans-retinal and the percentage of hemolysis was dependent upon the number of retinal molecules per erythrocyte. These authors suggested that the sigmoidal shape of the hemolysis curve may have resulted from an increase in the nonpolar character of the erythrocyte membrane following the initial binding of nonpolar compounds to the membrane. It is also possible that time of incubation affects the rate of hemolysis.

The solubility of 3MI in an aqueous solution is 500 μg/ml, but the final concentration of 3MI within an erythrocyte membrane depends on (a) the ratio of 3MI to membrane surface area, and (b) any factor(s) affecting the transfer of 3MI from the glass surface to the membrane such as its lipid: aqueous solubility. In the work of Rogers *et al.* (15), the natural logarithm of the partition coefficient of 3MI, as determined from the ratio of 3MI in *n*-octanol:aqueous sodium phosphate buffer, was 5.984, indi-

cating that 3MI partitioned into the lipid phase.

$^{22}Na^+$ transport. 3MI may affect the erythrocyte membranes at subhemolytic concentrations (500 μg/ml). Since the cation transport system of erythrocytes is an important membrane function associated with its integrity, erythrocyte ghosts loaded with $^{22}Na^+$ were used as a model system to study the passive and active transport. If the effect of 3MI on permeability is to be studied, it is necessary to ascertain that the results are not invalidated by lysis of the ghosts. Erythrocyte ghosts are impermeable to sucrose. Lysis can be followed by preloading the ghosts with ^{14}C-sucrose (9). The time course of $^{22}Na^+$ and ^{14}C-sucrose diffusion of control and 3MI treated resealed ghosts is shown in Fig. 2. The $^{22}Na^+$ diffusion into the medium appears to be biphasic. Initially, $^{22}Na^+$ and ^{14}C-sucrose leakage are rapid in both control and 3MI treated ghosts. After about 5 min and before reaching diffusion equilibrium, $^{22}Na^+$ and ^{14}C-sucrose leakage decreases and continues at a lower rate. The observed kinetics represent the average behavior of the population. One possible explanation would be that the rapid phase of cation exit is due to leakage from ghosts which had not resealed (Type III ghosts). At the end of the rapid phase, the resealed ghosts (Type II ghosts) still contain $^{22}Na^+$ which continues to escape slowly into the medium until it reaches equilibrium (8). The leakage of ^{14}C-sucrose from these same resealed ghosts lends support to this interpretation (Fig. 2). The differences between the $^{22}Na^+$ kinetics of control and 3MI treated ghosts were not statistically significant. However, the $^{22}Na^+$ diffusion in 3MI treated ghosts continued to diffuse into the medium after the rapid phase. The ^{14}C-sucrose in 3MI treated ghosts decreased, suggesting that the effect of 3MI on erythrocytes was to lyse the cells with little effect on $^{22}Na^+$ diffusion. The effects of 3MI on the $^{22}Na^+$-pump were studied by incorporating ATP into the ghosts as an energy source for active transport of Na^+ and by adding extracellular Na^+ (40 m*M*) and K^+ (20 m*M*) for movement against a concentration gradient. The $^{22}Na^+$ and the intracellular ^{14}C-sucrose for the control and the 3MI treated ghosts are shown in Fig. 3. The population of the resealed ghosts was similar to that seen in the diffusion study. The observed curves represent the average behavior of Type II and Type III ghosts. Although the 3MI treated ghosts continued to pump $^{22}Na^+$ into the medium after the rapid phase, the difference between the control and the 3MI treated ghosts was not statistically significant. The lack of influence of 3MI on Na^+ active transport may be due to the fact that the 3MI was not incorporated inside the cell where the Na^+ binding site of ATPase is located. The ^{14}C-sucrose continued to decrease after the rapid phase in 3MI treated resealed ghosts, but not in the controls in both passive and active transport studies. These results also suggest that 3MI lyses the cells. The system used (8) to examine Na^+ transport may not be suitable for studying the effect of 3MI since this compound lyses the cells and may prohibit accurate measurement of transport rates.

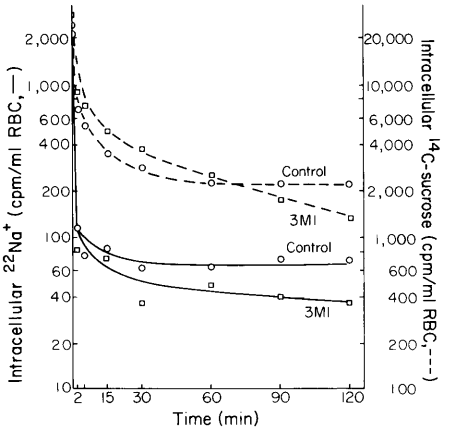

FIG. 2. The time course of $^{22}Na^+$ and ^{14}C-sucrose diffusion of control and 3MI treated resealed ghosts. Each point indicates the mean of four experiments. The data fit the least square line $Y = A^{bx}$ with a coefficient of correlation $\geq 0.88 \pm 0.071$.

ATPase activities of erythrocyte membranes. 3MI increased the total and Mg^{2+}-dependent ATPase activities of erythrocyte membranes after 1 hr incubation with 3MI at various concentrations. The effect of 3MI on the ATPase activities is shown in Fig. 4. The total ATPase activity in control erythrocyte membranes was 2.02 μmoles/ml

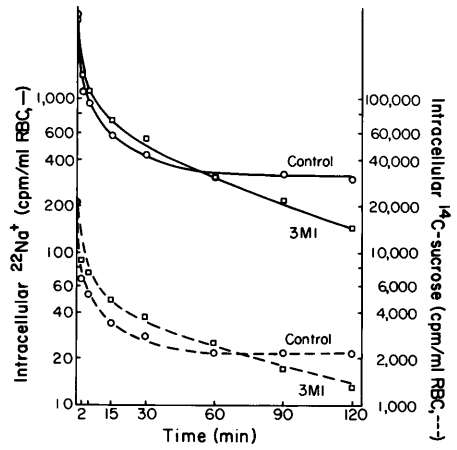

FIG. 3. The active efflux of the $^{22}Na^+$ and ^{14}C-sucrose of control and 3MI treated resealed ghosts. Each point indicates the mean of four experiments. The data fit the least square line $Y = A^{bx}$ with a coefficient of correlation $\geq 0.89 \pm 0.038$.

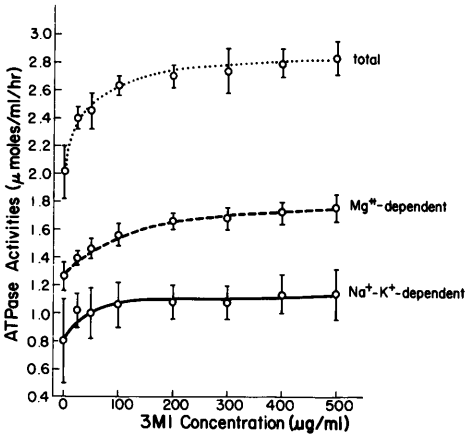

FIG. 4. The effect of 3MI on ATPase activities in erythrocyte membranes. Each point indicates the mean of four experiments and the vertical lines show the standard errors of the means.

ghosts/hr. Upon the addition of 3MI, the total activity was significantly increased at the concentrations of 100, 200, 300, 400 and 500 μg 3MI/ml incubation medium ($P \leq 0.02$). The Mg^{2+}-dependent ATPase activity also increased significantly upon the addition of 3MI at the levels of 100, 200, 300, 400 and 500 μg 3MI/ml of incubation medium ($P \leq 0.02$). The membrane Na^+-K^+-dependent ATPase activity which was obtained from the difference between the total and Mg^{2+}-dependent ATPase increased slightly but not significantly under the influence of 3MI. The effect of 3MI on ATPase activities may be due to its lipophilic properties which presumably increases fragmentation of the membrane or loosens up the phospholipid structure around the enzyme and thereby increases access of the substrate to enzyme. Similar increases in ATPase activity have been observed when cellular membranes were fragmented with deoxycholate for 5–7 days at 0° (17). It indicated that detergents such as deoxycholate increase the enzyme activity by unfolding membrane fragments and increasing the access of enzyme to substrate.

Summary. Biochemical effects of 3MI on cellular membranes were investigated. This study was conducted to examine the effects of 3MI on the hemolysis of erythrocytes, the transport of $^{22}Na^+$ in resealed erythrocyte ghosts, and on the ATPase activities of erythrocyte membranes. The percent of hemolysis as a function of 3MI incubation time was sigmoidal. Seventy-five percent of the hemoglobin was released with the second 2 hr of incubation during which the concentration of 3MI in the cells reached a plateau of 2500 μg/ml of packed RBC. The effect of 3MI at a subhemolytic concentration on passive and active $^{22}Na^+$ transport were not significant. The total and Mg^{2+}-dependent ATPase activities in the membranes were significantly increased after 1 hr of incubation with 3MI at concentrations of 100, 200, 300, 400 and 500 μg/ml ($P \leq 0.02$).

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