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ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *School of Pharmacy, Federal Institute of Technology, Zurich, Switzerland.*

Accepted for publication December 2, 1969.

Abstracted from the dissertations submitted by S. C. Khanna and T. Jecklin to the Swiss Federal Institute of Technology in fulfillment of Doctor of Natural Sciences degree requirements.

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Mechanism of Dimethyl Sulfoxide-Induced Hemolysis

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Abstract □ A study has been made of the possible mechanism by which dimethyl sulfoxide induces hemolysis of rabbit erythrocytes *in vitro*. Erythrocytes were shown by spectral analysis to remove dimethyl sulfoxide from aqueous solution and to resist the agent's release upon washing. Electron microscopy revealed the increased formation of lesions in the erythrocyte membrane with increasing concentrations of dimethyl sulfoxide. Dimethyl sulfoxide was also shown to be increasingly capable of removing fatty acids from the erythrocyte membrane with an increase in concentration. Results of the study indicate that dimethyl sulfoxide is capable of causing the hemolysis of erythrocytes by virtue of its affinity for the erythrocyte membrane and the disruption of its integrity, in part due to its lipid solvency action.

Keyphrases □ Erythrocyte hemolysis—dimethyl sulfoxide induced □ Mechanism—dimethyl sulfoxide-induced hemolysis □ Dimethyl sulfoxide retention—erythrocytes □ Fatty acid removal, erythrocytes—dimethyl sulfoxide □ Electron microscopy—erythrocyte lesion determination □ UV spectrophotometry—analysis

Although dimethyl sulfoxide (DMSO) was first synthesized in 1867 (1), it has only recently become the object of intensive scientific investigation. Used for years by the chemical industry as a solvent, the compound became of special interest to biologists after it was found to have a wide range of solvent action for chemicals employed in various laboratory procedures (2–7). DMSO was subsequently utilized investigationally as the solvent for poorly soluble drugs to be employed parenterally in the clinical treatment of cancer and leprosy (8, 9).

Much of the recent flourish of investigation activity centered about DMSO has been prompted by reports of the agent's apparent great ability to traverse biologic membranes and exert its pharmacologic activity (10–14), as well as to increase the degree and rate of penetration of other drugs across biologic membranes (15–17).

DMSO has also received considerable attention concerning its ability to serve effectively as a cryophylactic agent in the preservation of various body organs, tissues, and cells (7, 17–35), including red blood cells (31–35).

One difficulty concerning the use of DMSO as a cryophylactic agent in the preservation of blood for transfusion has been its nature to permeate the erythrocyte along with the subsequent difficulty experienced in its removal from the blood preparative to transfusion (32, 34, 35). Cellular destruction, resulting in hemolysis, commonly accompanies attempts to rid the cells of DMSO by washing (25, 36).

The hemolytic activity of DMSO, *in vitro* and *in vivo*, has been noted in reports from this (36) and from other laboratories (25, 37, 38). It was the purpose of the present work to investigate the mechanism by which DMSO exerts its hemolytic effects against the erythrocyte.

EXPERIMENTAL

Materials—The DMSO employed in this investigation was reagent grade and was obtained commercially.

Blood Samples—In previously reported studies the hemolytic activity of DMSO against human and rabbit erythrocytes was quite comparable (36, 37). This was verified in preliminary experiments to the present work using blood obtained from the forearm veins of several Caucasian donors, 20–25 years of age, and from rabbits by cardiac puncture. For convenience, rabbit blood was employed throughout this study. Fresh blood was obtained immediately prior to each experiment and defibrinated by gentle swirling with glass beads for approximately 5 min. The defibrinated blood was then transferred by decantation to a clean container and employed in the following procedures.

Absorption of DMSO by Red Blood Cells—To assess the capabilities of DMSO to enter and/or bind with the erythrocyte, red blood cells were incubated with solutions of DMSO, and any alteration in the absorbance of the original DMSO test solution was determined spectrophotometrically.

Erythrocytes were separated from the defibrinated blood sample by centrifugation and decantation. Then the cells were washed five times with normal saline solution and reconstituted to the original blood volume. This cell suspension was then employed to prepare test samples containing volumes of erythrocytes ranging in concentration from 0.06 to 0.48%, with 0.004% DMSO in normal saline. The suspensions were allowed to incubate for 30 min. at 37°, after which time the cells were settled by centrifugation at 13,500×g for 15 min. The supernatant was then examined spectrophotometrically with a Beckman DU for its DMSO content, using a wavelength of 208 mμ. The amounts found were compared to the amounts originally present in the test solutions, and the amounts absorbed determined by difference.

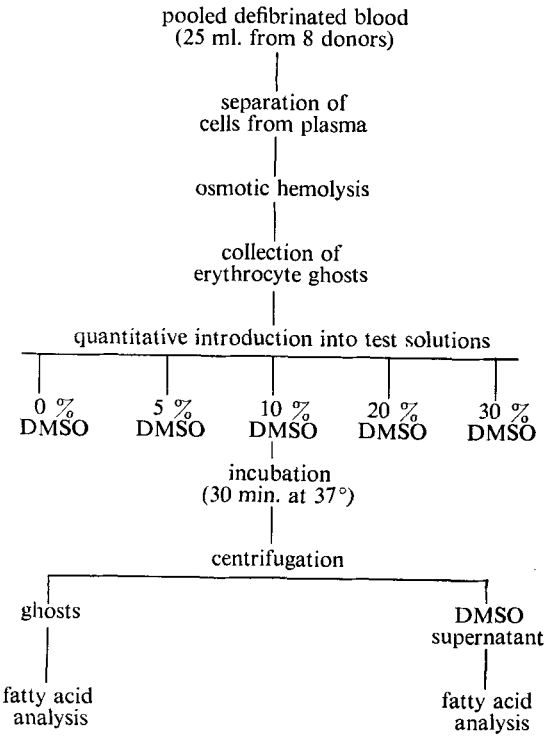
Preparation of Erythrocyte Ghosts for Electron Microscopy—One percent suspensions of defibrinated blood were prepared in 0.9% sodium chloride and varying proportions of DMSO, and incubated at 37° for 45 min. Control suspensions of blood in 0.9% sodium chloride were prepared and concurrently processed with the DMSO-containing suspensions.

After the incubation period, erythrocyte ghosts were prepared essentially by the procedure used by Hillier and Hoffman (39) in their study of the ultrastructure of the human and animal erythrocyte membrane. By this method, hemoglobin-free ghosts are prepared by subjecting the cells to a series of osmotic shocks by washing successively in sodium chloride solutions of decreasing tonicity. The present method differed from that of Hillier and Hoffman only in that the final wash was conducted with distilled water in the present instance rather than with saline of extremely low tonicity. Following each saline washing the cells were resuspended in a solution of lower tonicity, centrifuged at 3500×g for 5 min., until finally they were triple-washed with distilled water and resuspended to one-fourth of the volume of the original suspension of defibrinated blood.

In mounting the ghosts for examination by electron microscopy, one drop of the aqueous suspension was placed on each of several collodion-coated copper grids of 200 mesh and allowed to remain undisturbed for approximately 5 min. Excess liquid was then drawn off the grid using absorbant paper, and the grid was allowed to dry overnight.

It was found unnecessary to employ staining or shadowing procedures. The grids were examined in an RCA EMU 2 electron microscope operating at a voltage of 50 kw. Micrographs were photographically enlarged as noted.

Fatty Acid Analysis—To determine the solvent characteristics of the various DMSO solutions on the erythrocyte membrane, an analysis was performed to establish the degree of fatty acid removal from the membrane. Ghosts were prepared from 25 ml. of pooled defibrinated blood (Scheme I) by successive exposure to solutions of decreasing tonicity and resuspended in 25 ml. of distilled water. One milliliter of the ghost suspension was introduced into 24 ml. of each of the various DMSO test solutions. The samples were incubated 30 min. at 37°, then centrifuged at 20,000×g for 20 min. to settle the ghosts. The supernatant was decanted and the ghosts were resuspended in 10 ml. of 50% ethanol to inhibit enzyme activity Ethanol was also added to the supernatant to a concentration of 50%. The samples were saponified with 2 *M* KOH and the total fatty acids isolated with chloroform. The fatty acids were converted to methyl esters using BF_3-reagent and procedures described by Metcalfe and Schmitz (40). Quantitation of total fatty acids was based on addition of behenic acid (22:0) as an internal standard and gas-liquid chromatography analysis of the methyl esters using a Packard model 845 instrument equipped with dual hydrogen flame detectors and techniques previously described (41).



Scheme I—Preparation of erythrocytes for fatty acid analysis

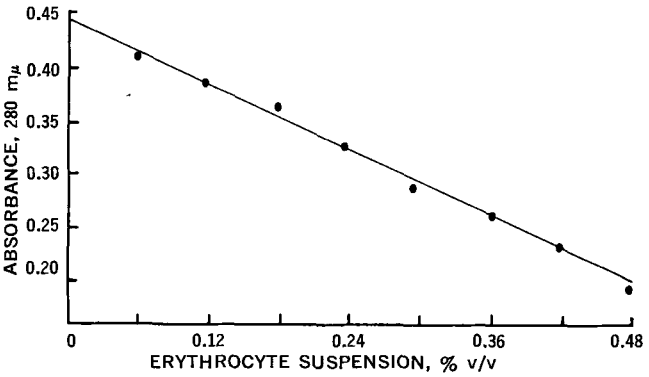


Figure 1—*Spectral absorbance of DMSO (0.004%) in the presence of washed rabbit erythrocytes in 0.9% NaCl. Data are the averages of three samples.*

RESULTS AND DISCUSSION

Absorption of DMSO by Red Blood Cells—Ansel and Leake (36), reporting on the *in vitro* hemolysis of erythrocytes in the presence of DMSO, noted that without the presence of added saline, DMSO is incapable of maintaining the integrity of the erythrocytes, and hemolysis is total at all DMSO concentrations. To prevent osmotic hemolysis, the presence of an extracellular material to which the red blood cell membrane is impermeable is required. In the presence of 0 6% sodium chloride the red blood cells are protected from hemolysis until the DMSO concentration is raised to above 25%. At DMSO concentrations above 30% in the absence of saline and above 45% in the presence of saline, the blood was denatured as observed by brown discoloration of blood cells and released hemoglobin and the flocculation of the cell components.

The ability of erythrocytes to hold DMSO was examined in the present study by incubating previously washed erythrocytes in 0.9% saline solutions containing a fixed quantity of DMSO, and comparing the spectrophotometric absorbance for DMSO in the control sample (without cells) and in the supernatant of the various cell samples. In each of the experiments conducted, the spectral absorbance of extracellular DMSO decreased with increased concentration of cells present. Figure 1 represents one experiment which typifies the data obtained in this series. The data indicate that DMSO is removed from the extracellular medium and held by the erythrocytes. This would tend to support the experiences of others (25, 36) who have found the removal of DMSO from blood cells prior to transfusion so difficult.

The hygroscopic nature of DMSO undoubtedly plays a role in its ability to traverse biologic membranes and perhaps to affect the configuration of membrane proteins. DMSO has a strong affinity for water molecules, with a maximum hydration of DMSO in aqueous solution being three water molecules per DMSO molecule (42). It has been suggested that the hydrogen bonds which exist between water and DMSO are stronger than the hydrogen bonds which exist between water molecules (43). Because of its comparatively small size, the DMSO molecule is thought to be capable of penetrating regions on certain protein subunit interfaces more readily than other bulkier solvents (44).

Rammler and Zaffaroni (44) suggest that the ability of DMSO in high concentrations to cross rapidly the dermal protein barrier, whose conformational integrity is dependent upon bound water, is the result of reversible configuration changes of these proteins due to water substitution by DMSO.

Since the principal component of the living cell is water, and the native form of biopolymers such as proteins, polysaccharides, and nucleic acids is surrounded by arrays of water molecules (the hydration sheath), these seem to suggest the manner in which DMSO gains entrance to the cell and is bound within (44). Substitution or removal of the hydration sheath would be expected to alter the configuration of the biopolymers and consequently the cell or tissue. Since in many instances it has been found that the cells apparently so affected are not permanently altered, the reversible process could be envisaged as occurring either as diffusion or active transport of the major amount of DMSO from the tissue and the gradual removal of the protein-bound DMSO by competitive bonding with cellular water (44).

Figure 2—*Erythrocyte ghosts (× 6900) hemolyzed by osmotic pressure.*

In studying the metabolism and distribution of DMSO in man and in animals, Gerhards and Gibian (45) found that in human blood about 30% of DMSO is bound to plasma protein and 25% to the formed elements, the remainder being free. Denko *et al.* (46), using ^{35}S-labeled DMSO and rat blood, found that DMSO radioactivity is associated predominantly with the serum albumin. They reported that the biologic half-life of DMSO-^{35}S is prolonged by 25% in hard tissues and thus may indicate some tissue binding.

Huggins (31) reported on his observations of the interaction between DMSO and human plasma proteins, noting that true denaturation of plasma protein seems to occur at DMSO concentrations approximating 50%. He stated that precipitation of protein by DMSO occurred with individual plasma fractions—albumin, fibrinogen, and gamma globulin—and appears to be a general effect rather than denaturation of a specific plasma component. DMSO has also been reported to be a good solvent for a number of proteins (47).

DiStefano and Klahn (38) concluded that DMSO probably exerts its hemolytic effect by direct action on the blood cells, although they hesitated to suggest a mechanism for this action.

It is likely that part of the means by which DMSO is capable of acting as a hemolytic agent is its initial ability to gain entrance into the red blood cell (due to its hygroscopicity and small size) and its affinity for proteins, resulting in their disfiguration, denaturation, or dissolution, and thus a loss of cellular integrity.

Electron Microscopy—An ultrastructural examination of the erythrocyte membrane was made following exposure to various DMSO concentrations. It is important to note that the particular cells photographed in each instance are representative of the general appearance of the field of cells examined.

Figure 2 shows the control erythrocytes which were osmotically hemolyzed in the presence of hypotonic saline and then mounted on a collodion-coated copper grid. These cells are intact, with a fine granular appearance. This type of granularity has been noted by other investigators (39, 48, 49). Hillier and Hoffman (39) observed erythrocyte membranes at much higher magnification (× 100,000 to × 200,000) and noted that the granularity is due to closely packed "cylinders" approximately 30 Å thick with a diameter of 100 to 500 Å. They termed these structures "plaques."

Figure 3 shows the appearance of erythrocytes exposed to 5% DMSO and then osmotically hemolyzed. The membranes are intact and have the same general appearance as the osmotically hemolyzed control cells. Some of the cells show the presence of

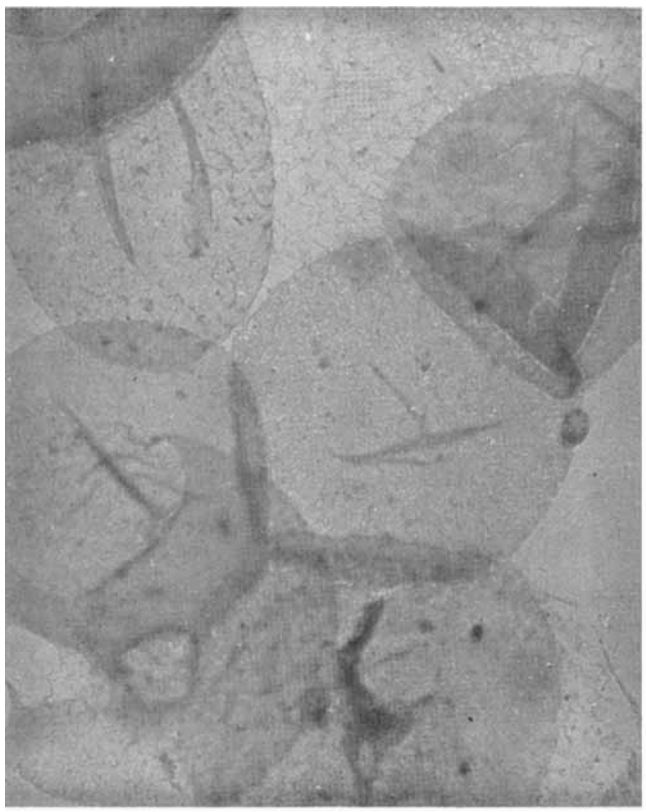


Figure 3—*Erythrocyte ghosts (× 7350) exposed to 5% DMSO in 0.9% NaCl for 45 min. at 37°.*

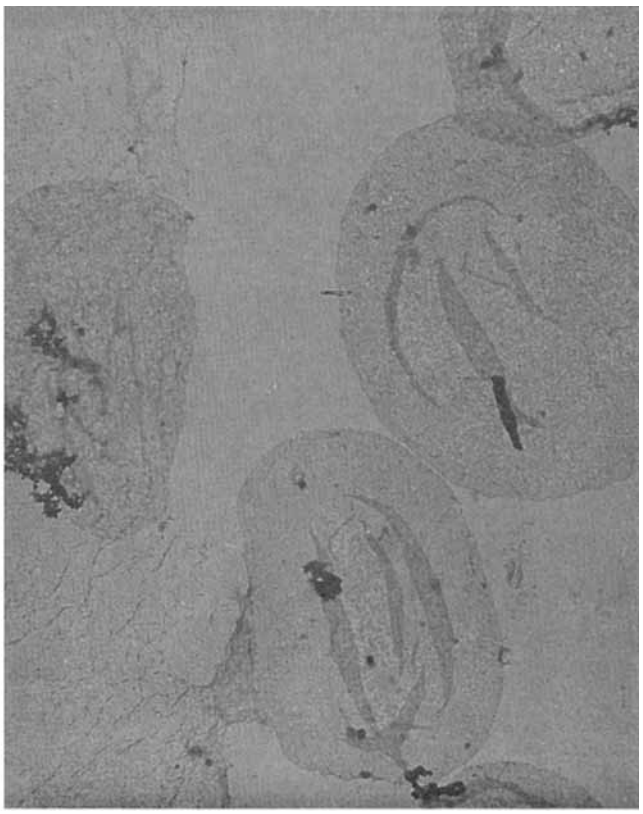


Figure 4—*Erythrocyte ghosts (× 6750) exposed to 20% DMSO in 0.9% NaCl for 45 min. at 37°.*

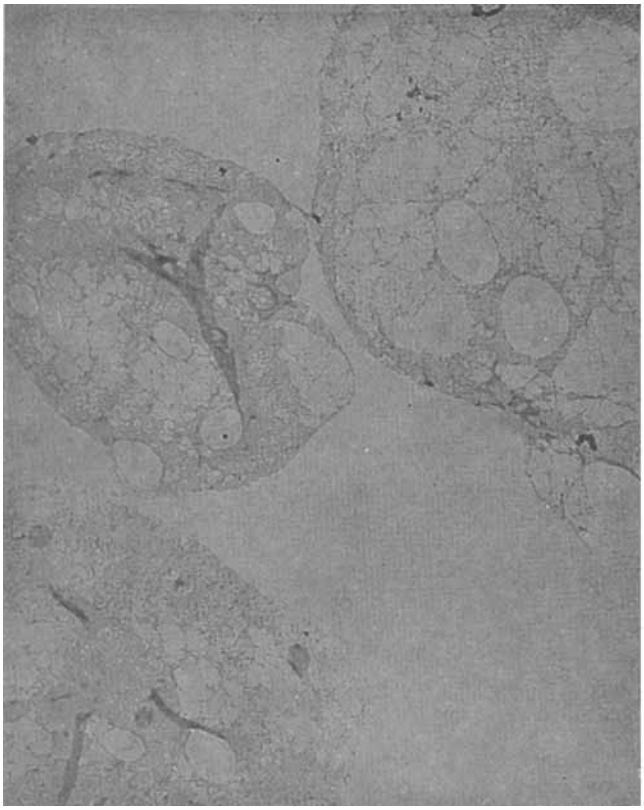


Figure 5—*Erythrocyte ghosts (× 6400) exposed to 30% DMSO in 0.9% NaCl for 45 min. at 37°.*

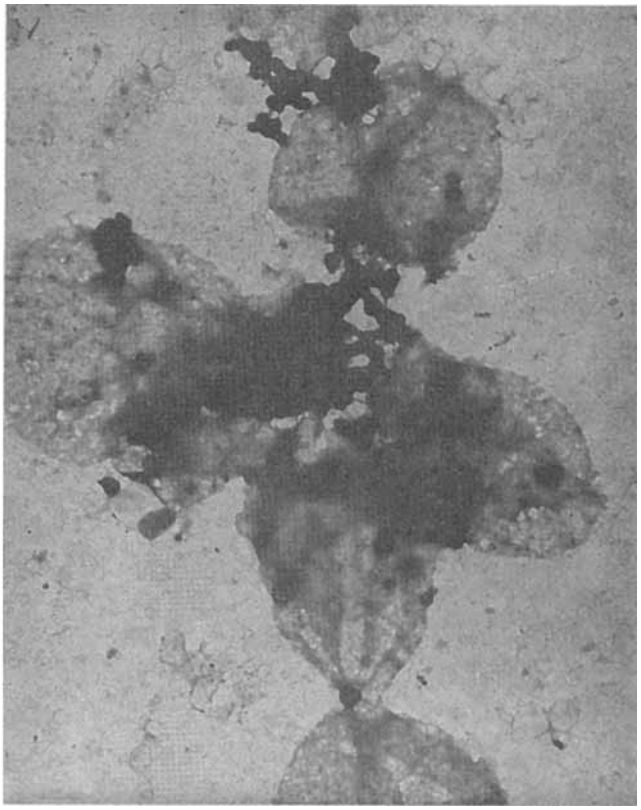


Figure 6—*Erythrocyte ghosts (× 7200) exposed to 40% DMSO in 0.9% NaCl for 45 min. at 37°.*

folds, which probably occurred as the saclike membane collapsed upon itself in attaching to the surface of the collodion membrane.

Figure 4 shows cells after treatment with 20% DMSO, followed by osmotic hemolysis. Definite lesions in the membrane are visible, and more cell fragments are present than in the previous samples. The particles seen on the collodion membrane surrounding the ghosts are interesting. A possible explanation is that these particles are cellular plaques which have been removed from the membrane surface by the action of the DMSO. Hillier and Hoffman (39) noted a similar phenomenon when treating cells with a variety of lipid solvents such as alcohol–ether–chloroform. They suggested that the plaques themselves may be insoluble in the solvent, but are attached to an underlying fibrous membrane by some type of lipid. A lipid solvent may then remove the lipid component, releasing the plaques and thereby cause lesions in the membrane surface. Since the total lipid of the red cell lies mainly within the membrane (50), the solvency of DMSO toward lipoidal and nonlipoidal materials (51) and the concepts of Hillier and Hoffman would seem to support the suggestion that the loss of the erythrocyte's integrity in the presence of DMSO is due to the dissolution of one or more of the membrane components. Another possible mechanism for the disruptive influence of DMSO on the cell membrane has been hypothesized by Puig Muset and Martin-Esteve (52). They propose that DMSO might produce some change in the isomeric conformation of cellular fatty acids, as oleic acid and linoleic acid, with a resulting alteration in the permeability barriers of the cell.

Table I—Fatty Acid Content of Erythrocyte Ghosts Following Incubation with DMSO[a]

% DMSO	Fatty Acid Content	
	mcg.	% of Total (Control)
0 (Control)	646	100 (Standard)
5	562	87.0
10	478	74.0
30	382	59.2

[a] Each sample was incubated for 30 min. with the stated concentration of DMSO.

Figure 5 shows erythrocytes after 30% DMSO treatment, followed by hemolysis. Gross disruption of the membrane has resulted. As could be expected, these cells were partially hemolyzed after incubation with the DMSO, even though the surrounding medium was isotonic with sodium chloride (36).

When cells were treated with 40% and higher concentrations of DMSO, a brown flocculation was observed in the test tubes. The appearance of the cells is shown in Fig. 6. Hemoglobin could not be removed by introduction of the cells into solutions of decreasing tonicity, and in the micrograph appears to be outside the cells but coagulated. The diameter of the ghosts is smaller than the osmotic controls, and the general appearance is that of coagulation of membrane components, possibly due to an alteration in protein configuration.

Fatty Acid Analysis—The lipid studies in this work were limited to the determination of total fatty acid removal from erythrocyte ghosts following incubation for 30 min. in various concentrations of DMSO. As can be seen in Table I, increasing amounts of fatty acids were removed from erythrocyte ghosts with increasing concentrations of DMSO. Work now in progress will attempt to identify the specific fatty acids removed from the erythrocyte by DMSO and to quantitate the rate and extent of such removal.

In conclusion, it appears as though the affinity of DMSO for the erythrocyte membrane and its lipid solvency contribute to the disruption of the integrity of the erythrocyte, resulting in hemolysis, and at high concentrations, in denaturation.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 6, 1969, from the *Department of Pharmacy, School of Pharmacy, University of Georgia, Athens, GA 30601*
Accepted for publication December 8, 1969.
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Constituents from *Gymnema sylvestre* Leaves V: Isolation and Preliminary Characterization of the Gymnemic Acids

JOSEPH E. SINSHEIMER, G. SUBBA RAO*, and HUGH M. McILHENNY†

Abstract □ The objectives of this investigation were to isolate and characterize the constituents of gymnemic acid, the antisweet principle of *Gymnema sylvestre* leaves, and to make them available for further biological testing. Gymnemic acid was found to be a complex mixture of at least nine closely related acidic glycosides. Solvent extraction and chromatography of gymnemic acid resulted in the isolation of gymnemic acids A–D (the major constituents) and V in crystalline form. Acids A–D are glycosides which yield glucuronic acid on hydrolysis while acids C and D also yield glucose. The gymnemic acids isolated in this study are compared to those described in the literature.

Keyphrases □ *Gymnema sylvestre* leaves—constituents □ Gymnemic acids—isolation characterization □ Column chromatography—separation □ TLC—separation □ Reverse phase chromatography—separation □ IR spectrophotometry—glycosidic structure □ UV spectrophotometry—glycosidic structure □ NMR spectroscopy—glycosidic structure

While the unique property of the leaves of *Gymnema sylvestre* R. Br. (*Asclepiadaceae*) to inhibit temporarily the ability to taste sweet substances has been known in India since antiquity (1, 2), the first such report to be published in the Western literature appears to be that of Falconer (3) in 1847. Chemical investigations were initiated by Hooper (4, 5) who isolated the antisweet principle as an amorphous monobasic acid, $C_{32}H_{55}O_{12}$, which he named gymnemic acid. Further, Hooper described gymnemic acid as a glycoside since it reduced Fehling's solution after treatment with dilute hydrochloric acid. In 1892, Shore (6) reported a modified procedure to isolate gymnemic acid in white crystalline form and suggested the acid to be a derivative of anthracene. Several years later, Power and Tutin (7) isolated racemic glucose as its osazone from the leaves but were unable to detect any sugar after acidic hydrolysis of gymnemic acid. Upon potassium hydroxide fusion of gymnemic acid, Power and Tutin obtained acetic acid and a mixture of protocatechuic and *p*-hydroxybenzoic acids, while alkaline potassium permanganate oxidation afforded formic acid.