ABSENCE OF LECITHIN FROM THE STROMATA OF THE RED CELLS OF CERTAIN ANIMALS (RUMINANTS), AND ITS RELATION TO VENOM HEMOLYSIS*

BY JOSEPH C. TURNER, M.D.
WITH THE TECHNICAL ASSISTANCE OF E. ANN PEARSON
(From the Department of Medicine, Columbia University College of Physicians and Surgeons, and the Presbyterian Hospital in the City of New York, New York)

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There are two mechanisms of venom hemolysis. One, which has been called indirect, implicates lysophosphatides formed by the action of venom phospholipases on extraerythrocytic sources of phospholipides; e.g., egg yolk or white blood cells (1). The second involves the direct lysis of red cells by an agent whose nature has been uncertain. The crystalline lecithinases prepared from rattlesnake (2) or cobra (3) appear to bring about lysis indirectly.

In studies of cobra venom hemolysis, Kyes found the cells of three animal species (goat, sheep, ox) to be completely insusceptible (4). Such variation among species has never been satisfactorily accounted for. Regarding the generally held view that snake venom hemolysis somehow concerns lecithinases, it has been assumed that resistance to venom reflects a difference in "availability" of cellular lecithin, and this substance, in turn, has been presumed to form part of the structure of all erythrocytes (5).

The present work was undertaken to try to clarify the nature of the direct hemolysin of the venom of the Indian cobra, *Naja naja*. On the one hand, efforts have been made to purify and isolate the active agent. While unsuccessful, they revealed that the toxin is stable to heat, a fact indicating that it might well be a phospholipase. It was decided, therefore, to approach the problem indirectly, through an analysis of the phospholipides of the red cells of a number of animals manifesting varying degrees of susceptibility to venom. This mode of attack was made possible by the recent development of a satisfactory chromatographic method of separating phospholipides on silicated papers (6), a technique already applied successfully to certain problems of venom lysis (1). The results indicate that red cells susceptible to venom invariably contain lecithin, while those resistant to venom do not. The correlation provides evidence that the direct hemolysin is indeed a lecithinase, but one whose activity is determined less by "availability" of sub-

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strate than by genetically determined variations in the phospholipide composition of erythrocytes.

**Methods**

The source of venom and the method of tests for hemolysis have been described (1). From 10 to 20 ml. of washed red cells were lysed by the addition of about 10 volumes of water. The ghosts were collected by high speed centrifugation and lyophilized. An acetone extract was discarded, and the phospholipides then extracted with chloroform-methanol (4:1). The solvent was evaporated in vacuo and the residue taken up in about 1 ml. of chloroform. Of this 20 ml was chromatographed.

The impregnation of papers with silicic acid has been described (6, 1). Experience has shown Whatman No. 3 MM to give rather better separations than No. 3, although either grade of paper can be used.

Descending chromatography was carried out in chloroform:methanol:water (160:40:5). As Lea, Rhodes, and Stoll have pointed out (6), the amount of water that it is desirable to add may vary from place to place, probably because of loss of moisture from silicated papers on storage. In New York, satisfactory separations of phospholipides were obtained during the spring and early summer, using chloroform-methanol without added water. In the fall, however, perhaps because of steam heat in the laboratory, it became necessary to add a small amount of water to the solvent. It has been found also that the time of equilibration of papers with solvent before development is begun can be of some importance. 30 to 60 minutes appear to be enough; papers allowed to equilibrate for several hours or overnight often gave more streaking and less distinct separations.

The chromatograms were run for 2 to 4 hours at room temperature; i.e., until the solvent had gone about 20 cm. The position of the front was often somewhat uncertain; two controls always applied were an extract of human red cells and synthetic 1,2-dimyristoyl lecithin (LaMotte Chemical Products Co., Baltimore).

The papers were dried in air, sprayed on both sides with 0.2 per cent ninhydrin in n-butanol, heated at 95°C. for 5 to 10 minutes, and the spots marked. After heating at 110°C for 25 minutes the papers were stained with phosphomolybdic acid according to the method of Levine and Chargaff (7). Other papers, after drying in air, were stained for plasmalogens by treatment with HgCl₂ and the application of fuchsin-sulfurous acid (8).

The number of animal subjects whose cells were analyzed chromatographically was: human, 6; ox, 2; guinea pig, 2; others 1 each.

**Results**

*Interpretation of the Chromatograms.*—While the method of chromatographing phospholipides on silicated papers is one that promises to have wide applicability, its use in separating complex natural mixtures has not yet been extensive. In the case of the red cell, however, there are available analytical data obtained by other chemical techniques, showing that the human erythrocyte contains cephalins, lecithin, and sphingomyelin (9). In the present work clear separations of these substances have been obtained, while minor differences in mobility or staining properties suggest that there may well be variations in the chemical composition of each component from species to species. Although the principal point to be made in what follows is that lecithin is absent from the red cells of some animals, it seems worth-
while to record also other chromatographic findings which may indicate additional species differences in phospholipide compositions. However, the number of these secondary observations is very few and no claim for their applicability to all members of a species is intended.

_Plasmalogen_.—Papers stained directly with Schiff's reagent showed the presence of free aldehydes close to the solvent front and in the area corresponding to the cephalin spot. If hydrolysis with HgCl₂ was first carried out, more intense staining was seen in the cephalin spot, and now also parts of the lecithin and sphingomyelin areas were also stained. This finding was common to all of the specimens examined except one, and suggests that under these experimental conditions plasmalogens usually accompany all three of the more familiar phospholipide components. The exception occurred in the goat, whose red cells appeared to contain little, if any, plasmalogen.

_Cephalins_.—Substances giving a discrete spot with a $R_f$ value corresponding to that of phosphatidylethanolamine (6), and staining with ninhydrin, were found in all cells examined. However, after ninhydrin and heat, which should destroy amino groups, the same spots took a phosphomolybdate stain. This could perhaps be due to the presence of plasmalogens, as noted above, which might contain choline.

_Lecithins_.—Discrete spots staining with phosphomolybdate and having an $R_f$ value of about 0.6 were seen with extracts of the cells of all species except the ox, the sheep, and the goat. The accompanying photograph (Fig. 1) of a chromatogram illustrates the findings. Table I sets forth details of the correlation between the presence of lecithin and susceptibility to venom hemolysis.

_Sphingomyelin_.—Spots with an $R_f$ value of about 0.45 and stained by phosphomolybdate were seen in all extracts except that of the chicken. Here,
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a slight amount of streaking behind the lecithin spot made it difficult to be certain that there was no sphingomyelin, but the amount must in any event have been very small.

Other Lipides.—In the chromatogram of the extract of sheep red cells only, after staining with ninhydrin a rather large spot was seen that had an $R_f$ value of about 0.5, and ran somewhat faster than sphingomyelin but was incompletely separated from it.

| TABLE I |
|-------------------|------------------|---------------|
| **Species**       | **Susceptibility to venom** | **Lecithin**  |
| Guinea pig        | Marked           | Present       |
| Dog               | "                | "             |
| Cat               | Moderate         | "             |
| Chicken           | "                | "             |
| Duck              | "                | "             |
| Man               | "                | "             |
| Swine             | "                | "             |
| Rabbit            | Slight           | "             |
| Ox                | None             | Absent        |
| Sheep             | "                | "             |
| Goat              | "                | "             |

Marked, hemolytic titer > 1:100,000. Moderate, titer > 1:2,000. Slight, titer 1:800. None, no hemolysis in 1 per cent venom.

DISCUSSION

The correlation between absence of lecithin from the red cells and resistance to venom hemolysin (Table I) indicates that this toxic component of cobra venom is a lecithinase. It would also appear that the enzyme is unable to attack cephalins or sphingomyelin. It is already established that the latter is unaffected by certain venom phospholipases (10). The degree of specificity might be studied directly, however, by chromatographic analysis of the products of venom action on red cell lipide extracts.

It is not yet clear what determines the degree of sensitivity of red cells to venom; i.e., why dog and guinea pig cells are so much more readily lysed than those of the rabbit. This could reflect quantitative or qualitative differences in lecithin or, perhaps, its "availability." The fact that the phospholipide pattern of the relatively insensitive rabbit cell is indistinguishable from that of the highly sensitive dog cell suggests that chemical differences in the lecithins may well be the determining factor. The direct approach to the problem of specificity, suggested above, could provide evidence on this score.

Apart from the question of the nature of the direct hemolysin of venom,
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the finding of striking species differences in the phospholipide composition of red cells would appear to be a matter of interest in certain other fields. Since the animals studied include a number of herbivores, and since the red cells of some of them contain lecithin and those of others do not, it seems clear that the difference is genetically determined. From the point of view of comparative biochemistry, it is noteworthy that the ox, sheep, and goat are classed among the Pecora, or true ruminants, and it would be of interest to study the cells of other members of the group such as the deer, giraffe, and antelope. The findings may also have relevance for investigations of in vitro hemolytic or hemagglutinative systems such as those involving bacterial phospholipases and immune reactions.

SUMMARY

Lipide extracts of the red cells of several animal species have been analyzed chromatographically. Genetically determined differences in phospholipide composition were found. Lecithin is absent from the cells of ox, sheep, and goat. Cells containing lecithin are susceptible to the direct hemolysin of cobra venom while cells not containing lecithin are resistant. The facts indicate that the direct hemolysin is a lecithinase.

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BIBLIOGRAPHY