

## 76. Yo Ueda and Eiji Kimoto : Studies on the Relation between Hemolytic Activity and Film Expanding Power of Saponins.

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It is a well-known fact that the saponins have generally a strong hemolytic action. In 1937, Schulman and Rideal<sup>1)</sup> initiated the surface chemical study on the mechanism of hemolytic action of saponins. By measuring the change in surface pressure of various kinds of monolayers at the air-water interface due to the injection of saponin, they found that saponin rapidly penetrated into the film of cholesterol, remarkably increasing surface pressure of the film, while in the case of protein monolayer, saponin adsorbed only below the film, causing little change in the surface pressure. From this result they anticipated that saponin would exercise its lytic activity primarily on the lipid portion of the cell membrane and only secondarily on the protein constituent.

Ruyssen and Loos<sup>2)</sup> classified the saponins into two main groups so far as their hemolytic properties are concerned; these are the steroidal saponins (digitonin) and the acid saponins (sapoalbin). They found that the lysis by the acid saponins was highly dependent on the pH, that these substances produced great increase in the pressure of cholesterol film, and that the power to penetrate into such film ran parallel to the hemolytic activity of the saponin.

Recently, some pure and crystalline saponins were extracted in Japan. Consequently about six kinds of these saponins including digitonin, dioscin, camellia-saponin, thea-saponin, panax-saponin, and potassium glycyrrhate, were studied on a relationship between their power to expand cholesterol film and their hemolytic activity, in the medium of various hydrogen ion concentrations. Moreover, examinations were also made on whether or not the power for the expansion of cholesterol film was parallel with that for the film of total lipid fraction of red cell membranes, i.e. whether or not the film expansion connected with hemolytic activity appeared preferentially in cholesterol alone in many kinds of lipids in erythrocyte cell membrane.

### Experimental

**Materials**—The six saponins used in this experiment were as follows :

Digitonin "Merck"—digitogenin, 2 glucose, 2 galactose, xylose<sup>3)</sup>

Dioscin (*Dioscorea nipponica* Makino)—Diosgenin,  $\alpha$  glucose,  $\alpha$  rhamnose<sup>4)</sup>

Camellia-saponin (*Camellia japonica* L.)—Camellia-sapogenol, galactose, glucose, arabinose, uronic acid, tiglic acid<sup>5)</sup>

Thea-saponin (*Thea sinensis* L.)—Thea-sapogenol, galactose, xylose, arabinose, glucuronic acid, angelic acid<sup>6)</sup>

Panax-sapoin\*\* (*Panax repens* Maxim.)—Oleanolic acid, glucose, arabinose, glucuronic acid<sup>7)</sup>

Potassium glycyrrhate (*Glycyrrhiza glabra*)—Glycyrrhetic acid, 2 glucuronic acid<sup>8)</sup>

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\*\* Amorphous powder

1) J. H. Schulman, E. K. Rideal : Proc. Roy. Soc., B **122**, 29, 46(1937).

2) R. Ruyssen, R. Loos : Mededeelingen van de Koninklijke Vlaamsche Acad. v. Wetenschappen, **8**, 5 (1946); J. Coll. Sci., **2**, 429(1947).

3) L. F. Fieser, M. Fieser : "Natural Products Related to Phenanthrene," Reinhold Publ. Corp., 3rd Ed.(1949).

4) T. Tsukamoto, T. Kawasaki, et al.: J. Pharm. Soc. Japan, **74**, 984(1954).

5) M. Ishidate, K. Takamura : *Ibid.*, **73**, 347(1953).

6) M. Ishidate, Y. Ueda : *Ibid.*, **72**, 1523(1952).

7) S. Aoyama : *Ibid.*, **49**, 678(1929).

8) B. Lythgoe, S. Trippett : J. Chem. Soc., **1950**, 1983.

The first two are steroidal saponins and the others are acid saponins. Since it is generally recognized that the impure saponin differs from pure saponin in hemolytic activity, the commercial impure thea-saponin (Theaponin) was also used in order to compare it with purified thea-saponin. The stock solution of each saponin was prepared by dissolving one portion of each saponin in 5,000 portions of distilled water containing 10% MeOH\*. However, as the solubility of dioscin in water was very low, it was dissolved in water containing 40% MeOH in the proportion of 1:10,000. These stock solutions were used for the experiments of hemolysis and of film expansion.

**Hemolysis Experiment**—The time-dilution curve, i. e. the relation between the dilution of saponin and the time taken for producing 100% hemolysis was obtained by the method described in Ponder's book.<sup>9)</sup> To investigate the pH influence on hemolytic activity the phosphate-bicarbonate buffer of different composition isotonic with red cells were used to prepare human red cell suspensions and saponin solutions; the pH was 6.06, 7.10, and 7.59. In each pH solution the hemolysis experiment was carried out at ca. 25°.

**Film Experiment**—Cholesterol was purified by the dibromide method, m.p. 148.5°. The total lipid fraction of bovine red cell was extracted with EtOH-ether mixture as reported by Parpart and Ballentine.<sup>10)</sup> The apparatus used in this film experiment was the same as that used in the previous work<sup>11)</sup> of one of the authors. The tray was 55.0×14.3×0.9 cm. (at the deepest). The surface tension of saponin solution and surface pressure of the film were measured by the hanging plate method of Wilhelmy type. 5% of NaCl solution was used as a substrate in every film experiment, and the acidic solution of pH 3 was prepared by adding HCl. The expansion curve, i. e. the change with time in the occupied area of film at the constant film pressure was measured as follows: The film spread on the substrate solution was compressed to 8 dynes/cm. and the saponin stock solution was injected into the substrate to reach every part of the tray. Keeping the film pressure constant, which was the sum of film pressure without saponin and surface pressure of the substrate solution involving saponin, one of the glass barriers was gradually moved and the change in occupied area of the film between two barriers was read.

## Results and Discussion

The time-dilution curves of human red cell suspension for various saponins at ca. 25° are plotted in Fig. 1. As shown, the strength of hemolytic activity of these saponins was in the order of dioscin, digitonin, camellia-saponin, impure thea-saponin, and pure thea-saponin. With 20,000 dilution of panax-saponin and with 2,000 dilution of potassium glycyrrhate the hemolysis was incomplete even at log 4 sec., showing that these saponins had only a weak hemolytic activity. In the case of dioscin and digitonin, being a steroidal saponin, the pH influence on hemolytic activity was not recognized, but in the case of camellia- and thea-saponins, being an acid saponin, the hemolytic activity was stronger in the acid solution. This result was in accordance with that found by Ruysen and Loos<sup>2)</sup>. Also, K-salts of acid saponins showed nearly the same activity as the free saponins. The expansion curves of cholesterol monolayer and film of total lipid fraction of bovine red cell at 8 dynes/cm. due to the injection of saponins are shown in Figs. 2 and 3, respectively.

Under the figure in Fig. 2, the values of surface tension lowering of saponins (the dilution of these saponins in substrate solution were all 1/200,000), which were the values taken after a lapse of the minutes since saponin had been injected, are noted. The order of the strength of expanding power of saponins for these films was dioscin, digitonin, camellia-saponin, impure thea-saponin, pure thea-saponin, panax-saponin, and potassium glycyrrhate. This order was the same both in the case of cholesterol mono-

\* The solubilities of these acid saponins were examined as follows: A definite amount of saponin was added to 100 cc. of distilled water, heated at 100° for 20 mins., left in 37~38° thermostat, and then filtered. 20 cc. of this filtrate was titrated with 0.02 N NaOH with phenolphthalein as the indicator. It was found that the solubilities of camellia- and thea-saponin were 0.0625 g./100 cc. and 0.0463 g./100 cc., respectively. That of dioscin was measured gravimetrically to be lower than 5 mg./L.<sup>4)</sup>

9) E. Ponder: "Hemolysis and Related Phenomena," New York, (1948).

10) A. K. Parpart, R. Ballentine: "Modern Trends in Physiology and Biochemistry," Academic Press, 135(1952).

11) E. Kimoto: Kyushu Med. Sci., 4, 35, 165(1953).

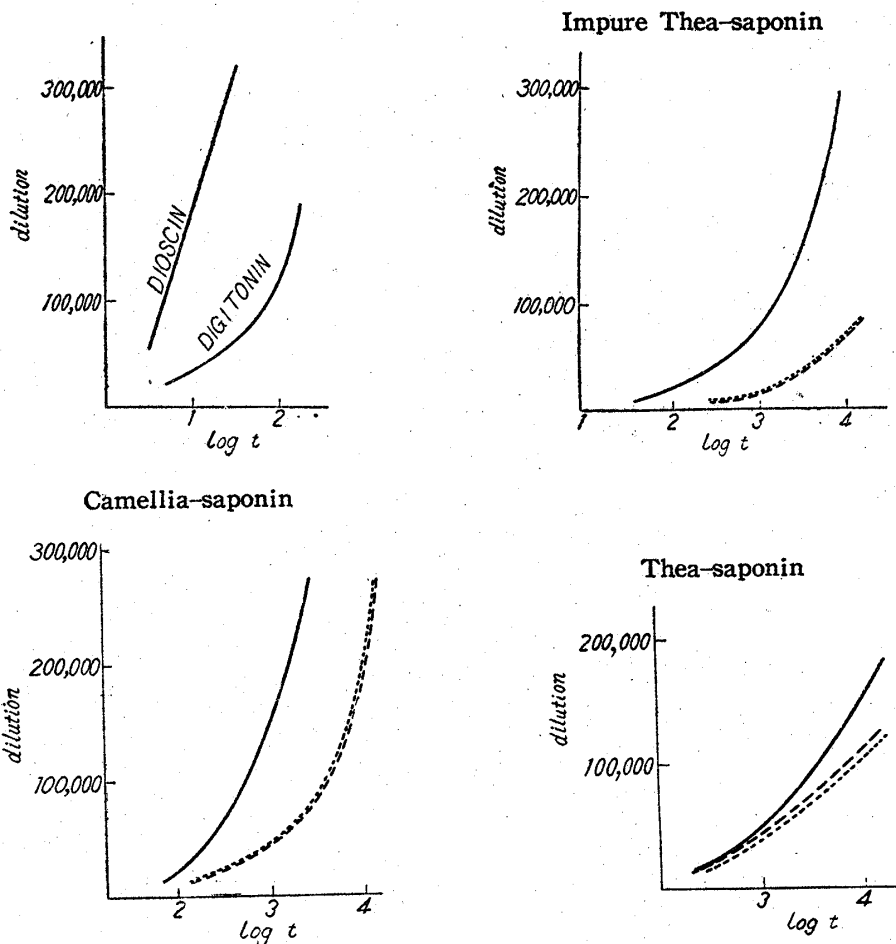


Fig. 1. Time-Dilution Curves for Various Saponins  
Ordinate, dilution of saponin; abscissa, log of time in seconds.  
solid line, pH 6.06; dotted line, pH 7.10; broken line, pH 7.59.

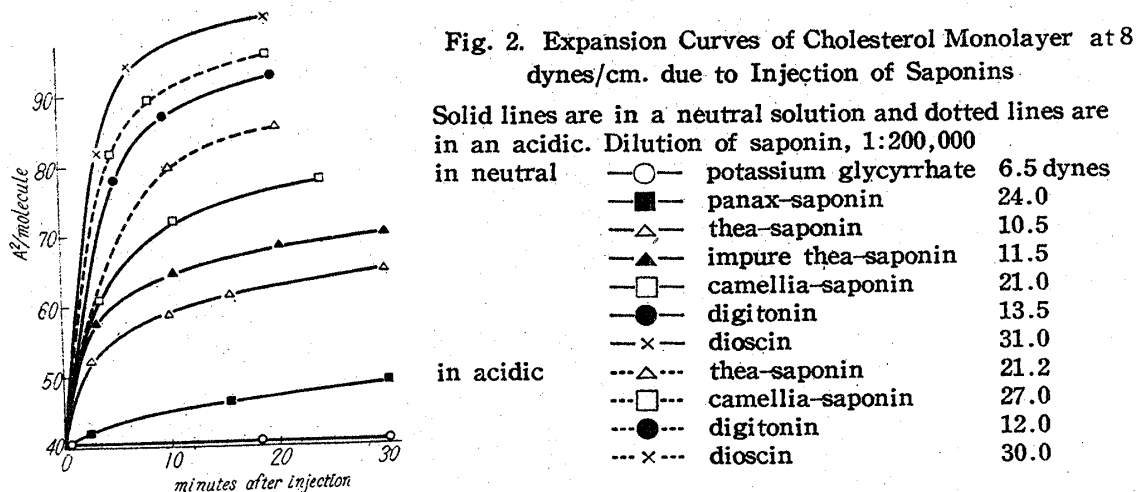


Fig. 2. Expansion Curves of Cholesterol Monolayer at 8 dynes/cm. due to Injection of Saponins

Solid lines are in a neutral solution and dotted lines are in an acidic. Dilution of saponin, 1:200,000 in neutral

in acidic

layer and in that of film of total lipid fraction of red cell membrane, and moreover the extent of expansion for the former was not much different from that for the latter. In the acid saponins, such as camellia-saponin and thea-saponin, they expanded the cholesterol film far more strongly in acid solution than in a neutral solution, similar to the pH dependence in the hemolytic activity, but in the steroidal saponins, such as dioscin and

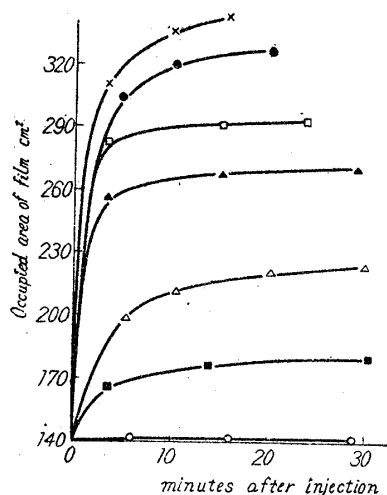


Fig. 3. Expansion Curves of Film of Total Lipid Fraction of Bovine Red Cells at 8 dynes/cm. due to Injection of Saponins

digitonin, the strength of expansion was nearly the same in both solutions. Windaus<sup>12)</sup> reported that digitonin formed a 1:1 complex with cholesterol and precipitated it quantitatively. Tsukamoto and Kawasaki<sup>4)</sup> reported that dioscin also precipitated cholesterol, but not quantitatively. The authors observed the precipitation reaction between cholesterol and other saponins by the Windaus method. Both camellia- and thea-saponin formed a precipitate with cholesterol with a yield of ca 62%. The yield in panax-saponin was far lower than in the above-mentioned saponins and its precipitate was easily solubilized on adding a small amount of ethanol. Potassium glycyrrhate caused no precipitate with cholesterol.

From the above experimental results, it was found that in the saponins studied the hemolytic activity, the capacity to form a precipitate with cholesterol, and the power of expansion for cholesterol film, ran parallel to each other, and that the surface lowering (surface activity) of these saponins did not necessarily run parallel to the hemolytic activity but, in the case of saponins having the identical capacity to precipitate cholesterol, such as camellia- and thea-saponins, the stronger the surface activity, the stronger were the hemolytic activity and film expansion.

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### Summary

With six kinds of pure saponins, including dioscin, digitonin, camellia-saponin, thea-saponin, panax-saponin, and potassium glycyrrhate, the hemolytic activity (the time-dilution curve for the hemolysis of human red cells) and the power of expansion (expansion curve) for cholesterol monolayer and for the film of total lipid fraction of bovine red cells were measured. It was found that in these saponins the hemolytic activity, the capacity to form a precipitate with cholesterol, and the power of expansion for these films ran parallel and that the acid saponins such as camellia- and thea-saponins were stronger in the hemolytic activity and in the power of expansion for cholesterol monolayer in acid solution than in a neutral one. On the other hand, in the steroidal saponins, such as digitonin and dioscin, their strength was nearly the same in both solutions. Moreover, it was found that the impure thea-saponin had a stronger hemolytic activity and film-expanding power than the pure one.

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12) A. Windaus: Ber., 42, 238 (1909).