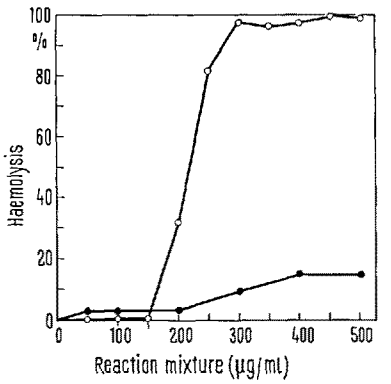
Medicagenic Acid, the Factor Responsible for Hemolytic Activity of Lucerne Saponins

Recent work on lucerne saponins has shown[1] that the various adverse physiological properties of lucerne saponins, such as hemolysis of red blood cells, larval growth depression and inhibition of seed germination, are exhibited to a greater extent by saponin extracts from lucerne roots (S_r) than by those extracted from lucerne tops (S_t). S_t and S_r are very similar in their composition[2] except that the latter is composed of saponins in which the aglycone moiety consists mainly of medicagenic acid and to a lesser extent of soyasapogenols, whereas in S_t the various soyasapogenols are the main aglycones. It was therefore of interest to investigate the relationship between the presence of medicagenic acid in lucerne saponins, and the extent of their biological activities.

A 2% suspension of washed ram red blood cells was prepared in isotonic buffer ($Na_2HPO_4 + KH_2PO_4$), pH ~7.4. The minimal amount of lucerne saponins needed for effecting complete hemolysis was previously determined as follows: varying amounts of S_t or S_r dissolved in 2 ml of the buffer were added to 2 ml of the suspension of the red blood cells and were left at room temperature for 3 h. The solutions were then centrifuged, the supernatant decanted, diluted with an equal volume of buffer and its absorption measured at 540 nm. Complete hemolysis of the standard was effected with water. According to the results in these experiments, appropriate amounts of S_t or S_r were added to 100 ml of the suspension of blood cells and left at room temperature for 3 h. In order to separate the red blood cell membranes, the solutions were then centrifuged at 3000*g*, the supernatant was decanted and the precipitated membranes were washed several times with water. The washings were concentrated under reduced pressure and subjected to thin layer chromatography (TLC) for identification of saponins[2]. Washing of the red blood cell membranes was continued until no lucerne saponins could be detected by TLC in the supernatant. 6–7 washings are usually sufficient. The membranes were then washed with 50 ml boiling absolute ethanol and 50 ml boiling 50% ethanol, in order to liberate any lucerne saponins, which may have adhered to the cell membranes during hemolysis. The combined ethanolic washings, and the combined aqueous washings were evaporated to dryness, the residues were assayed for their hemolytic activity, and subjected to acid hydrolysis. The hydrolysates were subsequently examined by chromatography[1], for sugars and aglycones present.

As shown in the Table, the washings do not differ in regard to sugars identified in them, but they differ completely in the composition of their aglycone moieties. Whereas the aqueous washings contain only those saponins which yield the various soyasapogenols after hydrolysis and are devoid of medicagenic acid, the ethanolic extracts of the red blood cell membranes contain saponins, which have medicagenic acid as their sole aglycone. When these 2 groups of lucerne saponins, both from tops and roots, were assayed for their hemolytic activity, it was found that only those containing medicagenic acid were able to haemolyze red blood cells, whereas the soyasapogenol containing saponins had not hemolytic ability whatever. It seems therefore that, when whole S_t or S_r exhibit hemolytic activity, only the medicagenic acid containing saponins are responsible for this phenomenon.

As lucerne saponins are able to form addition products with cholesterol, which are insoluble in water[3], and as those saponins, which bring about hemolysis could not be removed from the reaction mixture with water, it can be assumed that association of the saponins with certain cell membrane components, possibly cholesterol change the permeability of the membranes, and cause the rupture of red blood cells. Similar conclusions have been



The effect of esterification on the hemolytic activity of medicagenic acid. ○—○, medicagenic acid; ●—●, medicagenic acid dimethylester.

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Chemical composition and haemolytic indexes of lucerne saponins present in aqueous and ethanolic washings of red blood cell membranes

Saponin extract used for haemolysis	Washings	Sugars identified	Aglycones identified	Haemolytic index[a]
S_t	Aqueous	Glucose, galactose, arabinose, xylose, rhamnose, glucuronic acid	Soyasapogenols A, B, C, D, E	None
	Ethanolic	Glucose, galactose, arabinose, xylose, rhamnose, glucuronic acid	Medicagenic acid	2500
S_r	Aqueous	Glucose, galactose, arabinose, xylose, rhamnose, glucuronic acid	Soyasapogenols A, B, C, D, E	None
	Ethanolic	Glucose, galactose, arabinose, xylose, rhamnose, glucuronic acid	Medicagenic acid	3500

[a] For method see SHANY et al.[1].

reached previously[4-6] by showing the ability of other saponins to form complexes with cholesterol in monolayers of cholesterol or in virus and red blood cell membranes.

Having established the relationship between the presence of medicagenic acid in lucerne saponins and their hemolytic activity, it was of interest to examine, whether there are certain structural features in medicagenic acid, which are essential to its ability to haemolyze red blood cells. Comparison of the structures of the various soyasapogenols[7-9], with that of medicagenic acid shows that, the most outstanding difference is the presence of two COOH groups in the latter[10], which are absent from the soyasapogenols. Attempts have therefore been made to examine the hemolytic activity of free medicagenic acid and of its derivative in which the free carboxyl groups are blocked. Medicagenic acid was isolated by semi-preparative thin layer chromatography[2] from an acid hydrolysate of S_r ; a sample of it was converted to its dimethylester with diazomethane as described by DJERASSI et al.[10].

As shown in the Figure, on blocking the free carboxyl groups, medicagenic acid loses practically all of its hemolytic activity. Thus it can be concluded that, in the case of lucerne saponins, the presence of free carboxyl groups of medicagenic acid is essential to their ability to hemolyze red blood cells[11,12].

Zusammenfassung. Die aus Wurzeln von Luzerne isolierten Saponine wirken stärker hämolytisch als diejenigen in den grünen Teilen dieser Pflanze. Die hämolytisch wirksameren Saponine sind reicher an Medicagensäure und es wird angenommen, dass diese für die hämolytische Wirkung der Saponine verantwortlich ist. Die Anwesenheit von 2 Carboxylgruppen in der Medicagensäure scheint für ihre hämolytische Wirkung von ausschlaggebender Bedeutung zu sein, da der Dimethylester dieser Säure nicht mehr hämolytisch wirkt.

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Strain Difference in Sex Ratio Response of Mice to Lactate Dehydrogenase Virus Infection[1]

Initially, one of us[2] reported a significant alteration in the sex ratio of weanlings born to C57BL/Fg mice inoculated, at 10–19 days before conception, with the lactate dehydrogenase (LDH) virus[3]. Then, data were obtained to suggest that the alteration in sex ratio stems from a response of the infected C57BL/Fg sire to the characteristic increase in plasma LDH activity[4]. In this paper, we wish to describe results obtained in related studies with mice of the BALB/cDg strain which have a spontaneously low sex ratio.

Materials and methods. Animals. BALB/cDg male and female mice, 4–6 months old, were bred in the research colonies of The Jackson Laboratory. Strain C57BL/Fg animals were supplied by Dr. F. H. J. FIGGE, University of Maryland School of Medicine.

LDH determinations. Blood was collected by tail bleeding; plasma LDH activities were measured by the method described elsewhere[5].

Sex ratios. Adult BALB/cDg mice were mated (5 females and 2 males per cage) at intervals of 10–14 days after an i.p. injection of mouse plasma (0.1 ml/animal) containing $10^{7.0}$ ID_{50}/ml of the LDH virus. Those females which became pregnant were isolated in separate cages; the number of babies born to each was recorded at birth. Thereafter, mother and progeny were treated as described previously[2] except that all dead offspring were examined for sex as well as gross pathologic lesions. Control animals received an i. p. injection (0.1 ml/mouse) of phosphate-buffered saline (PBS), pH 7.2.

Spermatozoa. Vasa deferentia from normal and 14-day-infected male mice (BALB/cDg and C57BL/Fg) were stripped into 1.0 ml of cold PBS in separate Syracuse watch glasses. After 3 washings, each sperm sample was resuspended in 0.5 ml of PBS, and then frozen and thawed before centrifugation at 2500*g* for 20 min at 0 °C. The resulting supernatant extract was assayed spectrophotometrically for LDH activity.

Results and discussion. As shown in Table I, whereas the sex ratio of offspring born to noninfected BALB/cDg parents was 41:59, that observed among the progeny of matings between LDH virus-infected animals was 49:51 (chi square, 2.45; P 0.12). This finding proved of interest because it suggested a response in sex ratio opposite to the direction reported in previous studies with C57BL/Fg mice[2]. Accordingly, additional experiments were undertaken to investigate certain factors which might be of significance.

The first experiment was designed to see if the plasma LDH levels of noninfected and infected BALB/cDg mice were the same as or distinct from those recorded among comparable groups of C57BL/Fg animals. The results, summarized in Table II, indicate that they are similar. Therefore, the difference in sex ratio response cannot be explained on the basis of a strain difference in: 1. the

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