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Inactivation of Pneumococcal Hemolysin by Certain Sterols.*

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It is well known that cholesterol is an effective inhibitor of bacterial hemolysins and saponins; but with the possible exception of the saponins[1] the chemical mechanism of the inhibition is obscure. So far as concerns bacterial hemolysins, the problem is more complex and confused because it is not even certain that the lysins from different bacterial species belong to one chemically defined class of compounds. This situation can be clarified only after systematic studies, of which our examination of pneumococcal hemolysin forms a part.

We have already demonstrated that the lysin can be reversibly oxidized by a variety of agents, and have indicated that its activity is associated with the presence of the thiol grouping in the lysin molecule.[2] In view of the strong inhibitive action of cholesterol, we turned next to an exploration of the effects of this and certain other sterols in an endeavor to learn more concerning the chemistry of the lysin. As will be seen, our present findings indicate, among other things, that the hemolytic activity of this enzyme-like substance is conditioned, in addition, by a second grouping.

Experimental. The hemolysin was an M/15 phosphate (pH 7.6) extract of washed, frozen and thawed pneumococcus (type II) cells; and our hemolytic unit was that amount of extract which would completely hemolyze 2 ml. of a 1 volume percent suspension (pH 7.6) of thrice washed rabbit red cells.

Each sterol suspension was made by dissolving 5 mg. of the compound in 5 ml. absolute methanol, which was then poured slowly into 100 ml. boiling water. This stock 0.00013 M suspension was filtered to remove some small flakes which separated out (these were usually scarcely weighable in amount); it was then cooled and diluted with water for use. In some cases the suspensions were made without boiling. The higher concentrations required for cholesteryl-acetate were obtained by using the methanol solution direct, along with suitable controls to rule out the effect of solvent. The fresh

* Under the term sterol, we have included for convenience both the alcohols and the derivatives.

[1] Windaus, A., *Nachr. Kgl. Gesellsch.* Göttingen, 1916-17, p. 301.

[2] Shwachman, H., Hellerman, L., and Cohen, B., *J. Biol. Chem.*, 1934, **107**, 257.

stock suspensions were of fairly uniform nephelometric density; and occasional colorimetric, gravimetric and nephelometric determinations were made to check and adjust the concentrations of the stock preparations. Nevertheless, the uncertainties involved in the handling of these suspensions were such that it is well to remember that the inhibiting doses reported below represent generally the maximum calculated quantities.

The procedure followed was to allow graded quantities of the sterol (in 0.1 ml. volume) to act on 1 hemolytic unit (in 0.1 ml.) of the lysin for 20 to 30 min., after which red cells were added and the resulting hemolytic activity determined as usual. The minimum quantity of sterol which inactivated one unit was taken as the inhibiting dose.

It may be stated that the minimum inhibiting doses were reproducible and of the same relative magnitudes under any one set of experimental conditions. The variations from time to time were due to uncontrollable differences in the sterol suspensions, the various hemolysin extracts, and the red cells. The ranges given in Table I are the more instructive in that they show the effects obtained under diverse conditions.

The experiments to be reported first were made upon fully active hemolysin. (For present purposes, we consider the lysin to occur in 2 forms: active and inactive. The fully active form is the natural product protected from mild air-oxidation; the fully inactive form is the air-oxidized product, which can be restored to activity by treatment with cysteine or H_2S). The results given in Table I show the high inhibiting potency of cholesterol, and the relative potencies

TABLE I.
Inhibiting Effect of Sterols on Active Pneumococcal Hemolysin.

Common name	Chemical name (a)	Digitonin precipitability	Amount required to inhibit one hemolytic unit (mg.)
Cholesterol (b)		+	0.00001-0.0001
Cholesteryl acetate (c)		—	over 0.2
Allocholesterol	Coprostenol (d) (e)	+	0.00001-0.0001
Coprosterol	Coprostanol (d)	+	0.01-0.05
Cholestenone	Coprostenone (d)	—	0.1-0.3
Pseudocholestene	Coprostene (d)	—	0.1

(a) Nomenclature according to Rosenheim and King, *J. Soc. Chem. Ind.*, 1934, **53**, 91.

(b) Eastman product twice converted to the dibromide and thrice recrystallized from abs. ethanol. Method of Schönheimer, *Z. physiol. Chem.*, 1930, **192**, 86.

(c) Eastman product.

(d) Kindly supplied by Dr. Rudolf Schönheimer.

(e) Prepared by the Windaus method (m.p. 117°), and recently shown by Schönheimer and Evans, *J. Biol. Chem.*, 1936, **114**, 567, to contain admixed cholesterol.

of the other sterols examined. It may be of interest to note that, if cholesterol and lysin react mole for mole, the concentrations of lysin in the test solutions were of the order of 10^{-6} to 10^{-7} molar. This inhibition of the fully active hemolysin is irreversible in the sense that subsequent treatment with cysteine or H_2S does not restore any of the original lytic activity.[3]

Table I shows that the digitonin-precipitable sterols in the series inhibit the lysin in much smaller doses than do the digitonin-negative. (The chemical names of the sterols disclose chemical and structural differences not evident from the common names.) It is seen that the unsaturated alcohols, cholesterol (cholestenol) and coprostenol, differing only in the position of the double bond, are equal in inhibiting potency. (The recently discovered fact that our sample of coprostenol contained admixed cholesterol should not alter this interpretation.) On the other hand, saturation of the double bond as in coprostanol is accompanied by a considerable diminution of inhibiting potency. The digitonin-negative sterol derivatives, all unsaturated, were far less effective as inhibitors. We are inclined to ascribe the fact that some of them did inhibit in much higher concentrations to an effect different from that under discussion.

These findings on pneumolysin are in general harmony with the old observations on tetanolysin[4, 5] and on saponin[6]; and they furnish, in addition, more quantitative comparisons with probably better defined compounds than were then available.

In contrast to the above effects on active lysin, we find that the oxidized, inactive form is influenced by cholesterol very much more slowly, if at all. For example, when a unit of inactive lysin is treated with an inhibiting dose of cholesterol for 30 min. at 37°, the hemolytic activity can be restored almost completely by subsequent treatment of the mixture with cysteine or H_2S. (Here, the quickly regenerated lysin was determined before the cholesterol had a chance to exert its somewhat slower effect.) On the other hand, active lysin similarly treated shows no restoration, except occasionally to a small degree, doubtless due to small amounts of admixed inactive form. A known mixture of the two forms behaves in accordance with this view.

[3] Our suggestion (*J. Bact.*, 1936, **31**, 67) that the inhibitive effect of cholesterol can be reversed was based on results with lysins that turned out to be partly inactive.

[4] Abderhalden, E., and LeCount, E. R., *Z. exp. Path. Therap.*, 1906. **2**. 199.

[5] Walbum, L. E., *Z. Immunitätsf.*, 1910, **7**, 544.

[6] Hausmann, W., *Hofmeisters Beitr.*, 1905, **6**, 567.

It seems reasonable to conclude therefore that the state of oxidation of the lysin conditions its reactivity with cholesterol; and that the presence of the OH group in the sterol molecule is apparently essential for the reaction. It is possible that the spatial configuration of the effective group or groups (as indicated by the parallelism with the digitonin reaction of the compounds under discussion) is also a determining influence; but this will require further study.

Effect of cholesterol on the nitroprusside reaction. The ordinary active hemolysin extract (containing 50 to 150 hemolytic units per ml.) is usually too dilute to give a positive reaction to a nitroprusside test capable of detecting at least 1×10^{-7} mole of cysteine in 0.5 ml. volume. In more concentrated extracts (of about 250 units per ml.) a positive reaction of an intensity corresponding to about 5×10^{-7} mole of cysteine can be obtained. Since a number of quasi-specific reactions[2] of the lysin point to the association of a thiol grouping with its action on the red cell, it becomes of interest to determine if the inhibiting action of cholesterol affects the availability of this grouping. We find that it apparently does not; for a nitroprusside-positive extract treated with 5 to 10 times the inhibiting dose of cholesterol for 15 min. still remained nitroprusside-positive. As would be expected, a parallel experiment with cysteine and cholesterol gave the same result.

It would seem, therefore, that the inactivation caused by cholesterol leaves the SH group of the lysin free; in other words, that the point of attachment (or attack) on the lysin molecule by cholesterol is somewhere else than on the SH group. Consequently, it is reasonable to infer that the lytic activity is associated with at least 2 different groupings: one capable of reversible oxidation and reduction, and the other more or less specific for a certain type of sterol grouping and configuration.

That these functional groupings are not independent has been indicated above and is supported by the following observations. As is well known, the active lysin is taken up quickly and practically entirely by an equivalent amount of red cells. We find, on the other hand, that the inactive form is adsorbed little if at all under similar conditions. For example, 4 ml. red cell suspension were put into each of 2 centrifuge tubes. To tube 1 was added 0.1 ml. inactive extract containing about 2 potential hemolytic units; to tube 2 was added 0.1 ml. phosphate buffer. The mixtures were incubated at 30° for 25 min. and then spun out. The supernatant fluid from tube 1 was then added to the sedimented cells of tube 2, and *vice versa*. The cells were resuspended, and 0.1 ml. of 0.06 M cysteine was added to each suspension, after which the tubes were incubated.

The final result was as follows: tube 1 showed no sign of hemolysis; tube 2 showed 85% hemolysis, equal to that in a third, control tube containing lysin, red cells and cysteine in the same proportions.

These results, taken together with the fact that much free cholesterol occurs in the red cell, may mean that the red cell sterol attaches active pneumococcal lysin as an antecedent to hemolysis. But we are not yet prepared to accept this view without more conclusive data. It is, however, of interest in this connection that cholesterol-treated red cells remain vulnerable to the hemolysin, although the rate of hemolysis is retarded more or less.

The effect of contaminants (peroxides). The picture presented above seems fairly self-consistent; nevertheless, owing to the nature of our materials, it is necessary to consider the possibility of another factor, *viz.*, peroxide formation, as a source of significant error in interpretation. This arises from 2 related facts. In the first place, hemolysin exposed to H_2O_2 is affected rather rapidly. Secondly, cholesterol is known to undergo spontaneous oxidation in air,[7] a process which apparently involves a peroxide stage, for we find a week-old stock 0.00013 M suspension of the purified sterol to give a pronounced test for peroxide with ferrous thiocyanate reagent[8] and a weaker test with acidified KI. The freshly made suspension is negative, and in addition shows antioxidant action by greatly retarding the spontaneous oxidation of the thiocyanate reagent.

Granting these facts, does peroxide formation account for the inhibiting action of cholesterol? Our observation that cholesterolized lysin retains its free SH grouping is one point against such a view. We find, however, that the sterol, pretreated with a crude catalase preparation, retains some 80 to 90% of its inhibiting potency. This, taken along with the fact that traces of peroxide can be detected in saturated solutions of our cholesterol in methanol (although not detectable in the highly diluted suspensions actually employed) would indicate that only a small part of the inhibition can be assigned to peroxides.

It is hoped that concentrations of the lysin now in progress will provide material better suited for analysis of the various aspects of the problems here touched upon.

Summary. I. It is shown that the inhibiting effect on pneumolysin by cholesterol and certain related sterols is apparently determined primarily by the presence of the OH group in the sterol struc-

7 Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1904-5, **43**, 316.

8 Young, C. A., Vogt, R. R., and Nieuwland, J. A., *Ind. Eng. Chem., Anal. Ed.*, 1936, **8**, 198.

ture and secondarily by the double bond. Possible peroxide formation would seem to account for only a small portion of the cholesterol effect. 2. Active pneumolysin is inhibited promptly, while inactive (air-oxidized) lysin is affected slowly if at all by cholesterol. Likewise, active lysin is adsorbed by red cells, while the inactive form is not. That is, the state of oxidation of the lysin conditions its reactivity with the sterol and with its adsorption on red cells. 3. The free thiol grouping on the active lysin molecule remains free after treatment with excess cholesterol. The lytic activity, therefore, is associated with at least 2 functional groupings, one reversibly oxidizable, and the other more or less specific for a certain sterol grouping and configuration.

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Synthesis of Protein and Amino Acids in Mice with the Aid of Deuterium.

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Schoenheimer and Rittenberg[1] have proposed that if non-labile deuterium is found in the molecule of a fatty acid isolated from tissues of mice which were receiving heavy water, this finding is then indicative of the synthesis of the fatty acid in the animal body. In connection with the study of the synthesis of protein and amino acids in animals, we were interested to ascertain whether or not a similar criterion, as used by Schoenheimer and Rittenberg, is applicable to protein and amino acid synthesis. Barbour, *et al.*,[2] on the assumption that one hour after a single injection of D_2O into mice a maximum of exchangeable deuterium will be fixed in the tissue, conclude that inasmuch as "mice drinking 15% D_2O for 2 months have 3 times the concentration of deuterium (relative to body water deuterium) in the tissue as mice receiving a single injection of D_2O," the difference in the deuterium content of the tissue indicates the fixation of deuterium in tissue in stable form. It occurred to us that the isolation of tissue protein and of amino acids derived therefrom and the analysis of the isolated products from which all deuterium in labile position has been removed would constitute more direct evidence for the fixation of deuterium in the

1 Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, 1935, **111**, 163.

2 Smith, P. K., Trace, J., and Barbour, H. G., *J. Biol. Chem.*, 1936, **116**, 371.