A STUDY OF THE INTERACTION BETWEEN SARCOLYSIN AND SERUM PROTEINS USING A BACTERIOPHAGE MODEL

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The reaction between alkylating compounds (mustard gas, nitrogen mustards, etc.) and certain proteins has been studied extensively (see surveys in [1, 7, 9-11]). It has been found that alkylating agents interact with the carboxyl, hydroxyl, amino, and SH groups of proteins, and can also cause hydrolysis of proteins. The investigations of Soviet authors have demonstrated the rapid fixation of sarcolysin (3-[p-(bis-2-chloroethyl amino)phenyl]alanine) by the proteins of whole human blood serum [4, 6].

To discover on account of which individual protein fractions fixation of the haloidalkylamine takes place, and to determine the kinetics of this process, it is important to investigate the interaction between sarcolysin and whole blood serum and its individual protein fractions.

The biological activity of alkylating agents may also be estimated from their antiphage action [2, 3]. On the basis of general considerations and some experimental findings [2] we postulated that, as a result of the fixation of the alkyl groups of the haloidalkylamine with the reactive centers of the protein, its antiphage activity must fall. Hence, the use of bacteriophage as a specific biological indicator may be a means of determining the fixation of the test substance in relation to the lowering of its antiphage activity, which is capable of being established accurately.

EXPERIMENTAL METHOD

Fresh serum was prepared from donors' blood and as a first step the serum albumin and γ-globulin were separated from it by Cohn's method. Electrophoresis with a moving boundary showed that other fractions were present in these proteins to the extent of 4-6%. A complex protein preparation—serum polyglobulin (SPG), containing 80% of γ-globulins and 20% of β-globulins, was also used in the experiments. The SPG contained immune β_2-globulins and the metal-binding protein, transferrin. The protein concentration was determined by the biuret method and adjusted to 1% with physiological saline.

To 0.9 ml of each of the protein solutions 2 mg sarcolysin in 0.85% NaCl solution (0.1 ml in volume) was added. The reaction took place at 37° for a predetermined period. Binding of sarcolysin by the serum proteins was determined from the decrease in the antiphage action of this substance from the control level. For this purpose one of the phages most sensitive to chloroethylamines was used, namely, coli-phage O26, which was added in a dose of 10^7 infection phage particles in a volume of 0.1 ml to the tube containing a mixture of protein and sarcolysin solutions. After exposure for 5 min, a series of dilutions was prepared, in which the number of phage particles remaining active was determined by the agar layer method [5]. Each experiment was accompanied by 2 control tests, the first to verify the antiphages action of the sarcolysin. For this purpose, to 0.9 ml of physiological saline was added 0.1 ml of sarcolysin solution (2 mg), and then 0.1 ml phage. The second control was for the accuracy of determination of the number of phage particles in the whole serum and its fractions. To 0.9 ml of each protein solution, 0.1 ml of physiological saline (instead of an equal volume of sarcolysin) and 0.1 ml of phage were added. A series of dilution was then prepared from both controls and the experimental tube, and the number of active phage particles in the dilutions was counted. The residual phage activity in the experimental samples was expressed as a percentage

TABLE 1. Lowering of Antiphage Activity of Sarcolysin after Interaction with Whole Blood Serum

Duration of incubation of mixture of sarcolysin (2 mg) with serum	Number of phage particles remaining active (as % of control)
30 sec	17 ±4
60 min	27 ±6
3 h	30 ±4
6 h	50 ±12
12 h	75 ±14
24 h	100

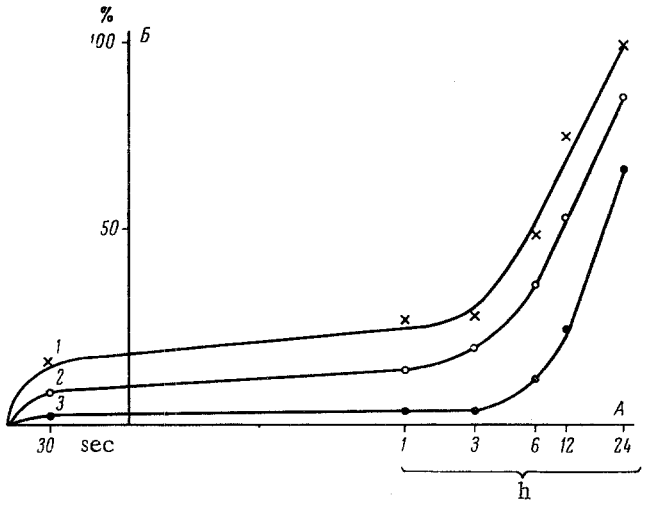

Fig. 1. Changes in the antiphage activity of sarcolysin after its interaction with whole serum and its fractions. A) Log of time of interaction of sarcolysin with solutions of serum proteins; B) Number of phage particles (in %) retaining their activity after treatment with a mixture of sarcolysin and serum proteins; 1) Serum; 2) SPG; 3) Albumin.

of the initial number of particles, determined from the results of the second control and taken as 100%. The experiments were taken into consideration only if no infection phage particles were present in the first control. The intensity of fixation of sarcolysin by the serum proteins could be estimated indirectly from the number of phage colonies obtained from the experimental samples.

EXPERIMENTAL RESULTS

The survival rate of the phage particles in whole human blood serum was determined in preliminary experiments. Certain batches of serum had an inhibitory action on phage after exposures of as little as 5 min, while others had no such effect after 30-60 min. The results of our experiments are in agreement with those in the literature [8]. It should be noted that individual fractions of serum proteins did not possess antiphage properties.

In subsequent experiments attempts were made to determine the extent to which whole blood serum, not possessing antiphage activity, could bind sarcolysin, to study the kinetics of this process, and to establish the variability of the results obtained by means of this method. The results of these experiments, after statistical analysis, are given in Table 1.

The ability of sarcolysin to undergo fixation by different samples of serum varied within wide limits. To discover the causes of these variations and the degree of fixation of sarcolysin with individual fractions of serum proteins, seecial series of experiments were carried out with solutions of human serum albumin, γ-globulin, and SPG. During the analysis of the results of these experiments, attention was drawn to the fact that γ-globulin hardly interacted with sarcolysin, which almost completely preserved its antiphage activity. The number of infection phage particles was approximately the same as in the control sample, containing sarcolysin solution alone, regardless of the duration of incubation with γ-globulin. The other protein fractions and whole serum possessed a marked ability to depress the antiphage action of sarcolysin.

It is clear from Fig. 1 that, with an increase in the time of interaction between sarcolysin and protein, the antiphage activity of the former fell progressively and the number of infection phage particles remaining in the sample rose correspondingly. Analysis of the kinetics of the reaction demonstrated that the process took place in three phases. During the first few seconds an appreciable fixation of sarcolysin by the blood serum proteins was observed. These findings are in agreement with the results of investigations of the binding power of whole serum using other methods [4, 6]. During the next 3-5 h, fixation of the preparation took place comparatively slowly, and the rate increased appreciably towards the end of the first day.

Since, in all the experiments shown in Fig. 1, equal concentrations of protein solutions were used, the differences in the degree of depression of the antiphage action of sarcolysin indicated differences in the degree of interaction between the individual protein fractions and the alkylating agent. Whole serum reacted most intensively with sarcolysin; next in order to depression of the binding power was the complex of β - and γ-globulins (SPG), followed by albumin, and, finally, by γ-globulin which practically did not interact with the preparation. However, this order was not constant, and changed with an increase in the time of incubation of the protein with the solution of haloidalkylamine.

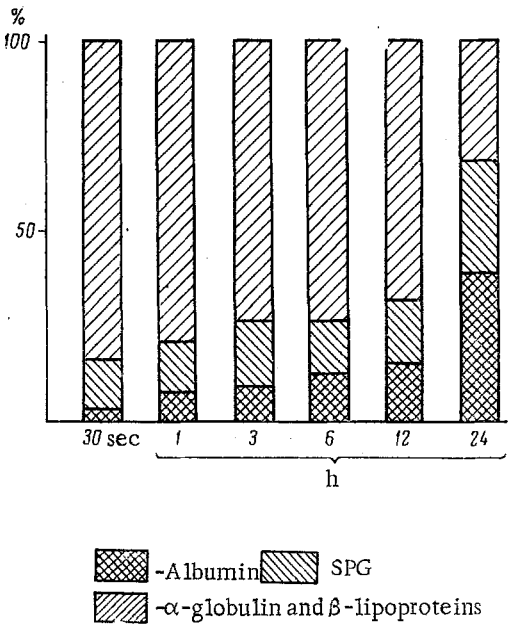

Fig. 2. Relative role of individual protein fractions in inactivation of sarcolysin.

To compare and estimate the relative role of the individual fractions in the depression of the antiphage activity of sarcolysin, the observed effect had to be compared with the relative content of these fractions in the serum. This rested on the assumption that the degree of inactivation of the alkylating substance is proportional to the concentration of protein in the solution.

The results given in Fig. 2 show that the relative role of albumin in the binding of sarcolysin gradually increased in time, and reached 40% after 24 h. Since the γ-globulin practically did not interact with the haloidalkylamine, the binding power of the SPG was evidently due to the other fractions composing this complex and, in particular, to the components of the β-globulin fraction. The degree of antiphage activity of the sarcolysin after its interaction with SPG remained approximately constant for 12 h, and thereafter rose slightly for 24 h (from 14-16% to 23-30%). It may be concluded from these results that during the first phase most of the sarcolysin was bound, not with albumin, or β- and γ-globulins, accounting for 85-90% of the total content of the serum proteins, but with other components of the blood serum. This suggests that the lowering of the antiphage action of sarcolysin took place on account of the α-globulins or β-lipoproteins, components not present in the fractions which we investigated. The α-globulin fraction was a complex system of individual proteins, differing in their antigenic structure and performing different physiological functions. The relative proportion of these components in this particular fraction varied within comparatively wide limits. This feature probably accounted for the variability observed in the antiphage activity of individual samples of donors' sera.

To examine the mechanism of inactivation of the alkylating agents in the presence of proteins an important step was to determine the absolute amount of sarcolysin bound by the individual proteins and by whole serum. The preliminarily established relationship between the degree of inactivation of phage and the amount of sarcolysin in the reaction mixture (Table 2) was used to plot a calibration curve by means of which the amount of sarcolysin bound with proteins after definite time intervals could be computed approximately.

This method enables the degree of fixation to be determined with an accuracy not exceeding 10-15%, although for the preliminary calculations this was good enough. Curves showing the amount of sarcolysin fixed by serum and individual proteins at different time intervals are shown in Fig. 3. As in the case of Fig. 1, they demonstrate the existence of three phases of the process of interaction between the alkylating substance and proteins: all the curves have two inflections—one in the time interval to 1 min, and the other between 3 and 6 h. The question accordingly arises: to what can these three phases of the process of association of protein with sarcolysin be due?

It may be postulated on the basis of our findings that the rapid fixation of sarcolysin in the initial period is due to the formation of a complex with one or several components of the α-globulin fraction. The reactivity of the β_1-metal-binding globulin and of the β_2-globulins contained in SPG remained almost constant and rose only at the end of the first day, whereas the amount of sarcolysin bound by albumin rose progressively. The investigated process took place at a pH exceeding the isoelectric point of albumin, so that the chance of interaction with a carboxyl group was slight, and the haloidalkylamines hardly react at all with the amino group [11]. However, during incu-

TABLE 2. Relationship Between Antiphage Activity of Sarcolysin and it Concentration

Number of phage particles remaining active (in %)	0	1,4	6	29	51	80	100
Concentration of sarcolysin (in μg/ml)	2 000	1 500	750	500	375	125	62,5

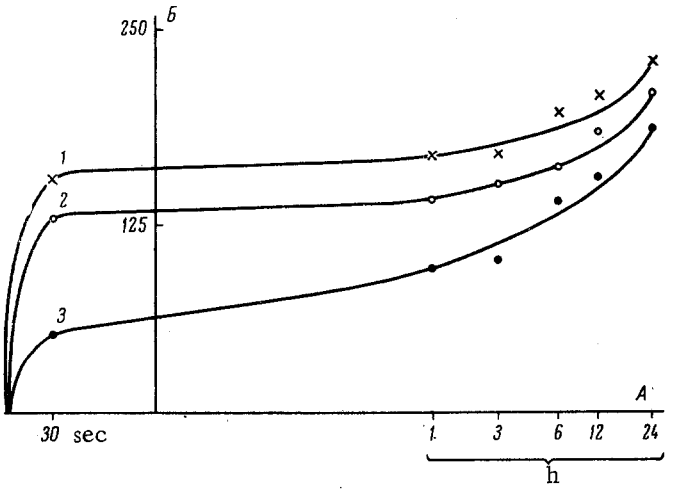

Fig. 3. Kinetics of fixation of sarcolysin by serum proteins. A) Log of time of incubation of sarcolysin with solutions of serum proteins; B) Amount of fixed sarcolysin (in μg/mg protein); 1) Serum; 2) SPG; 3) Albumin.

bation of protein in an excess of sarcolysin, partial denaturation of the protein evidently takes place, leading to an increase in the fixation of sarcolysin, which brings about a lowering of its antiphage action at the end of the first day.

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