XII. RESEARCHES ON THE INHIBITION PRODUCED BY CERTAIN SERA ON THE COAGULATING POWER OF RENNET.

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INTRODUCTION.

Among the numerous papers of the last 20 years dealing with the reaction of rennet and its antibodies, those of Morgenroth [1899], Korschun [1902] and Hedin [1906, 1909] have been of particular interest.

In order to compare the effects of enzymes and toxins Morgenroth immunised goats against rennet and was thus able to make a rennet antiserum, which had a strong inhibiting effect upon the activity of rennet. That this inhibiting effect was due to antibodies, formed in the antiserum during the immunisation, and not to some substance in the normal goat serum, is demonstrated by Morgenroth in the following manner. Fresh milk and milk mixed with normal goat serum require exactly the same amount of rennet for their coagulation, whereas milk mixed with the antiserum in the same proportions as milk with normal goat serum only coagulates in presence of a much greater quantity of enzyme. Before Morgenroth's investigations were carried out, Hammarsten and Rödén [1887] had shown that a similar inhibiting effect may be obtained when milk is mixed with normal horse serum. In addition to this, Briot [1900] investigated the blood serum of other animals and showed that the serum of all the species examined had a more or less distinct effect on the coagulating power of rennet. In addition Briot immunised rabbits with rennet and obtained an antiserum similar to that which Morgenroth had made in goats.

In later investigations on the reaction of rennet and its antibodies the preparation of an antiserum has, however, not been attempted, because normal horse serum has a strong inhibiting effect on rennet, which was supposed to be due to a true antibody, and this obviated the necessity of preparing one. Korschun adopts this view in his publication of 1902. His

researches, which are of considerable interest, bring him to the conclusion that normal horse serum contains two substances with inhibiting action on rennet, one having a large range of action, from 20° – 37° , and the other being especially active at 37° . Korschun calls this second substance "pseudo-antilab" (pseudo-antirennet).

A more detailed account of a few of Korschun's experiments will show what led him to accept the above-mentioned theory. In order to determine the coagulating power of milk, Korschun uses the quantitative method, introduced by Morgenroth, which is undoubtedly the most reliable we have. In contradistinction to the others, this method does not indicate the coagulating power of the milk as a function of the time needed for the coagulation of a certain quantity of milk by a constant quantity of rennet, but by the minimal amount of the enzyme, which is sufficient to coagulate this quantity of milk. This method, however, cannot give reliable results, when the coagulation of the milk, that is, the transformation of the colloid caseinogen into precipitated casein is brought about in the usual way, i.e. by heating the milk-rennet complex to about 35° until the transformation has taken place. For by this heating a rather considerable amount of rennet may be destroyed without it having any effect on the caseinogen. Morgenroth's method therefore makes the transformation of the caseinogen take place at lower temperatures (0° – 8°), which do not injure the rennet, so that even very small quantities may remain active for a long time. At the low temperature, however, the casein formed is not precipitated and it is therefore necessary to raise the temperature of the milk-rennet complex to about 35° , when all the caseinogen has been transformed. At 35° the precipitation takes place rapidly, often instantaneously. The transformation of caseinogen into casein proceeds only slowly at 0° – 8° , and to ensure that the reaction should come to an end, the milk-rennet complex has to be kept at low temperature for 12–16 hours.

Morgenroth's quantitative method is very exact, and the same milk tested at various times always indicates the same coagulating power, which is by no means the case, when this power is determined by the time which is taken for the coagulation of a certain quantity of milk with a constant quantity of rennet.

Korschun's method of testing the power of his serum is also much more reliable than the methods previously used. In these earlier methods various quantities of serum and milk were mixed and the time noted which a certain quantity of rennet required for coagulating the mixture of milk and serum at the optimal temperature of the reaction (i.e. 35°). The inhibiting power of

the serum was then expressed by the difference between the time taken for the above-mentioned process and the time required for the coagulation of the same quantity of milk without serum by the same amount of rennet. Such a method, however, only gives quite a rough idea of what really happens, because by mixing serum and milk, both these substances act on the rennet, when this is being added to the mixture, and a part of the enzyme will therefore be adsorbed by the milk, and thus rendered incapable of acting with the serum. Hedin proved, in fact, some years later, that serum has the greatest inhibiting effect on rennet, when the serum and rennet are previously mixed, and milk added to them only when the action of the serum has come to an end. The same way of testing the action of serum on rennet is used in Korschun's experiments. Increasing amounts of rennet solution are added to a constant quantity of serum, and after this mixture has been kept for 15 minutes at 20°, 10 cc. of milk are added. The samples are kept all night (12–16 hours) in the ice room, and then moved to 35° in order to determine in which samples the caseinogen of the milk has been transformed into casein. The tube, which contains the greatest quantity of rennet, and is still liquid, indicates the limit of the inhibiting power of the serum. The degree of this inhibiting power is given by the ratio of rennet used to the smallest amount of enzyme, just sufficient to coagulate 10 cc. of the same milk without serum. For example, if 10 cc. of normal horse serum are able to neutralise the effect of 0.03 cc. of a rennet solution on 10 cc. of milk, whilst 0.0006 cc. rennet is sufficient to coagulate 10 cc. of the same milk without serum, then the inhibiting power of this serum is equal to 50.

Using the above-mentioned method Korschun examines the inhibiting power of fresh normal horse serum, of dialysed and of heated horse serum, and finds that dialysis does not decrease the inhibiting power. By heating the serum, however, its effect is lost. He shows, moreover, that a horse antiserum, made by immunising a goat against horse serum, neutralises the inhibiting effect of the normal horse serum. From these facts he concludes that the inhibiting effect of the normal horse serum is due to a true antibody.

Experimenting with horse serum Korschun observed that this serum neutralises more rennet than might be expected, when the rennet-serum complex is kept at 35° or 40° instead of at 20°, and that the increase of the inhibiting power at 37°–40° is greater, up to a certain point, the longer the rennet-serum mixture is kept at the higher temperature. At 20° on the contrary no such increase in the inhibiting faculty seems to take place. He assumes therefore that in addition to the true antibody, the normal horse

serum contains a second inhibitory substance, the pseudo-antirennet, which is only active at higher temperatures.

As the dialysed serum does not show the greater effect on rennet at higher temperatures, which is shown by normal horse serum, the pseudo-antirennet must according to Korschun be dialysable. On the other hand, a heated horse serum has a strong inhibiting effect at 37° and no effect at 20°; the pseudo-antirennet appears therefore to be highly thermostable.

Hedin's researches point in quite another direction than Korschun's. In a number of publications he discusses the inhibiting influence of different substances on the activity of trypsin and rennet. In regard to rennet he points out that the intensity of the inhibiting effect depends on the order in which rennet, serum and milk are mixed. As already mentioned, he finds that the greatest effect is obtained when rennet and serum are first mixed, and the milk added to this complex, when the action of the serum has been brought to an end. Furthermore, Hedin shows that the longer the complex of rennet and serum is kept, before the milk is added, the greater up to a certain point is the amount of rennet neutralised, and that the maximum amount of the enzyme, which the serum is capable of destroying under the conditions observed, is different for different temperatures, the serum being more active at a higher than at a lower temperature. The concentration of the serum, however, does not seem to have any influence at all on the amount of rennet destroyed, and the inhibiting effect therefore is equally intense, whether the serum used is diluted or not.

Particular interest attaches to Hedin's observation that charcoal affects the rennet in the same way as normal horse serum, thus tending to indicate that the inhibiting effect of this serum is of a less complicated nature than was supposed by Korschun, and that it may possibly be due to a single character of the serum—its adsorbing quality. The further question then arises as to whether the inhibiting effect of the artificially produced rennet antiserum is of a nature similar to that of the normal horse serum, or due to the presence of a true antibody, formed in the serum during the immunisation.

In the experiments discussed below I have examined the inhibiting effect of normal horse serum, in order if possible to obtain agreement between Korschun's and Hedin's theories, which, as already shown, differ in various respects. At the same time the effect of the antirennet of normal horse serum has been compared with the inhibiting power of the antirennet formed in a rabbit serum after an immunisation, with the view of establishing their identity or otherwise.

I. RESEARCHES ON THE INHIBITING SUBSTANCES OF NORMAL HORSE SERUM.

(a) *The true antibody of horse serum and its mode of action.*

The capacity of neutralising a maximum amount of rennet in a very short time (a few minutes) is, according to Korschun, one of the most distinct qualities of the true antirennet, found in normal horse serum. Korschun therefore determines the inhibiting effect of this antibody by mixing horse serum and rennet solution, keeping the complex at 20° for 15 minutes, and measuring the remaining amount of active rennet.

In contradistinction to the action of the true antibody, the inhibiting effect of the so-called pseudo-antirennet only takes place after some time. For the determination of the effect of this substance therefore it is necessary to keep serum and rennet in contact with each other for at least three hours.

From Korschun's work it is evident that the true antibody is fully active at 20°, whereas the pseudo-antirennet does not show any distinct inhibiting effect at this temperature. Furthermore, as the effect of the true antibody does not increase proportionally to the time during which the serum acts on the rennet, it might be expected that the normal horse serum would behave similarly at 20°.

Experiment 1. (Table I.)

In this, as in all other experiments mentioned in this paper, Morgenroth's quantitative method has been used to determine the amount of rennet not neutralised by the serum in question. 10 cc. of milk were added to the rennet-serum complex, after this had been kept for the time required at 20°. The samples were then placed at a low temperature (0°) over night, and the following morning heated in a water-bath to 35°. The caseinogen of the milk was regarded as unaltered in those samples which remained liquid after half an hour at 35°, i.e. in such samples there had not been rennet enough left to transform the caseinogen into casein.

The minimal quantity of rennet solution required for coagulating 10 cc. of the milk used was 0.0015 cc.

The rennet solution in question was a standard solution, made at The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne, according to Morgenroth's instructions. In cases where the amount of rennet used was less than 0.015 cc. the standard solution was diluted (1-10) with saline solution.

The milk used for the experiments was a mixed milk saturated with chloroform, and curdling as mentioned above by the addition of 0.0015 cc. of rennet.

The horse serum in question was a normal horse serum, the durability of which was increased by the addition of some thymol crystals.

All experiments were made in test-tubes, the inner wall of which was covered with a thin film of paraffin in order to prevent alkali from the glass affecting the rennet-serum complex. All liquids used for the experiments were also kept in paraffined tubes, or tubes of Jena-glass.

Table I shows the inhibiting power of normal horse serum at 20°.

TABLE I.

The maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	Time during which the rennet-serum complex is kept at 20° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.04 cc.	0.04 cc.	0.06 cc.	0.07 cc.	0.08 cc.	0.09 cc.	0.09 cc.	0.09 cc.	0.09 cc.

The comparatively large amount of rennet neutralised at the moment of admixture, and which is not increased within the following 15 minutes, represents an actual change within this short period, and is not due to a continuation of the process when the samples were stored at 0° over night. The quantity of rennet neutralised at 0° is actually very small. An experiment, in which liquids, glasses, pipettes, etc., were kept at 0°, showed that the greatest quantity of rennet, neutralised by 0.1 cc. of normal horse serum under these circumstances, amounted to 0.005 cc.

Table I shows that the time during which the serum acts on rennet is not without importance for the inhibiting power of normal horse serum at 20°. This agrees fully with Hedin's observations. According to Korschun's views, however, the objection may be raised, that the effect of the pseudo-anti-rennet sets in at 20°. To examine the inhibiting power of the true antibody in the horse serum, it is therefore necessary to use a method other than that employed in Experiment 1.

Korschun has shown that the two inhibiting substances in the normal horse serum can be separated by dialysing the serum. As distinct from the true antibody, the pseudo-antirennet is dialysable. A dialysed horse serum should therefore give a measure only of the genuine antibody's mode of action.

As seen in Table I normal horse serum instantaneously neutralises a considerable quantity of rennet—0.04 cc. This quantity undoubtedly is

neutralised by the true antibody. A dialysed serum should therefore be capable of destroying a corresponding quantity of rennet in an equally short time, and thereby have reached the maximum of its inhibiting power. As can be seen from Tables II and III, this is by no means the case.

In these tables the results are given of two experiments, made with two samples of horse serum, the one dialysed longer than the other. For the experiment described in Table II, 10 cc. of the horse serum were dialysed for 48 hours in an animal membrane ("Fischblase") against running tap-water. To avoid bacterial action some thymol crystals were added to the serum. During the dialysis the volume of the serum increased to 14 cc. The precipitated euglobulin was separated from the dialysed serum by centrifuging. This euglobulin was also tested for inhibiting power, but proved to be without effect. Korschun has made the same observation. Half of the dialysed serum was used for the experiment, mentioned in Table II, while the remaining part—7 cc.—was brought back into the membrane and dialysed for further 48 hours. The volume of this second part thereby increased to 10.5 cc. This serum was used for the experiment in Table III.

In Fig. 1 the inhibiting powers of the dialysed sera are graphically shown, and compared with the inhibiting power of normal horse serum at 37° (Table IV). Like all the curves in this paper, those in Fig. 1 indicate the effect of the inhibiting substance in question, expressed by the ratio between the maximum amount of rennet, neutralised by the inhibiting substance, and the minimal quantity of rennet (i.e. 0.0015 cc.), required for the coagulation of 10 cc. of the milk used, without any addition of serum.

TABLE II.

The maximum amount of rennet neutralised by 0.14 cc. of a horse serum, dialysed for 48 hours	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.02 cc.	0.08 cc.	0.12 cc.	0.14 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.	0.16 cc.

As seen above, 0.14 cc. horse serum dialysed for 48 hours is equal to 0.1 cc. of normal horse serum.

TABLE III.

The maximum amount of rennet neutralised by 0.21 cc. horse serum, dialysed for 96 hours ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.015 cc.	0.06 cc.	0.11 cc.	0.13 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.	0.15 cc.

0.21 cc. of the horse serum dialysed for 96 hours corresponds to 0.1 cc. normal horse serum.

The curves in Fig. 1 show perhaps more clearly than the corresponding tables that the period for which rennet and dialysed serum are kept before the milk is added to the complex is of decisive importance for the inhibiting power of these sera. The dialysis therefore does not differentiate between the true antibody and the pseudo-antirennet with the loss of the latter, but decreases

Fig. 1. Showing the inhibiting power at 37° of normal and dialysed horse serum.

the total inhibiting power of the serum, a decrease which is evidently greater the longer the dialysis lasts.

Even if the above data do not give any direct information as to the nature of the true antibody of horse serum, two conclusions may be drawn from them. Firstly, that this body cannot be a true antibody in the strict sense of the term, since the power of a true antibody is not decreased by dialysis

of the serum in which it is contained¹. And secondly, they show that there is no such contrast between "true antibody" and "pseudo-antirennet" in their relations to dialysis, as Korschun supposed, which to him was one of the essential arguments for regarding them as two different substances.

(b) *Pseudo-antirennet; its mode of action and its nature.*

When a normal horse serum acts on rennet at 37° its inhibiting effect is much greater than at 20°, as shown in the following table (see Fig. 1).

TABLE IV.

The maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
.....	0.04 cc.	0.12 cc.	0.17 cc.	0.21 cc.	0.29 cc.	0.33 cc.	0.33 cc.	0.33 cc.	0.33 cc.

This observation also agrees with Hedin's statement that the inhibiting power of the serum increases up to a certain equilibrium proportionally, not only to the time, but also to the temperature at which the serum acts on the rennet. Korschun regards this increase in the inhibiting power as being due to the so-called pseudo-antirennet. As this body is supposed to be extremely thermostable, it ought to be separated from the thermolabile "true antibody" by heating the horse serum. An experiment made with a heated serum therefore should show the effect of the pseudo-antirennet better than the normal horse serum was able to do.

Following Korschun's instructions, an experiment like this is best made with a horse serum diluted in the proportions (1 + 5) with distilled water or tap-water, and then boiled for some time. In making the experiment, 10 cc. of the above-mentioned horse serum were therefore mixed with 50 cc. of distilled water, and the mixture kept in a boiling water-bath for one hour. The inhibiting effect of the serum dilution thus treated was determined in the usual way and found to have the following values.

¹ The only apparent cause for a decrease in the power of a true antibody would be that the dialysis had deprived the serum, in which the antibody is contained, of its content of NaCl. But even if NaCl be added to the dialysed horse serum the inhibiting power of the latter is not altered.

TABLE V.

The maximum amount of rennet neutralised by 0.6 cc. of a boiled horse serum dilution (1+5)	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.004 cc.	0.08 cc.	0.20 cc.	0.29 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.	0.31 cc.

0.6 cc. of the boiled serum dilution contains 0.1 cc. horse serum.

The use of so large a quantity of serum dilution made the precipitation of the casein, formed in the samples by the action of the rennet, extremely difficult, as the dilution had lowered the concentration of the calcium salts in the milk too much. Normal conditions were, however, easily brought about by adding some drops of a calcium chloride solution (4 %).

This experiment plainly shows that by heating the horse serum a considerable decrease is effected in the amount of rennet instantaneously neutralised by the true antibody. On the other hand, the heating does not appear to have changed the inhibiting power of the pseudo-antirennet, as the quantity of rennet neutralised at 37° by the heated serum practically agrees with what is destroyed at the same temperature by normal horse serum (Table IV). Notwithstanding this, it would be erroneous to conclude, as Korschun does, that the heating has destroyed the true antibody without hurting the pseudo-antirennet. A closer examination of the conditions, obtaining in the experiment to which Table V refers, shows that a horse serum, diluted (1 + 5) and heated to 70° for a quarter of an hour, has a higher inhibiting power at 37° than the boiled dilution and that an unheated dilution (1 + 5) has a still greater effect at the same temperature. By heating a serum dilution therefore we weaken its total inhibiting power, also that part of it which is due to the pseudo-antirennet. Tables VI and VII and Fig. 2 illustrate this.

TABLE VI.

The maximum amount of rennet neutralised by 0.6 cc. of a horse serum dilution (1+5), heated to 70° for a quarter of an hour ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.006 cc.	0.09 cc.	0.21 cc.	0.29 cc.	0.32 cc.	0.35 cc.	0.38 cc.	0.38 cc.	0.38 cc.

0.6 cc. of the dilution used contained 0.1 cc. horse serum.

TABLE VII.

The maximum amount of rennet neutralised by 0.6 cc. of a horse serum dilution (1+5) ..	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
	0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.37 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.

0.6 cc. of the dilution used contained 0.1 cc. horse serum.

The heating has thus considerably damaged both the true antibody and the pseudo-antirennet, with the difference that the destruction of the first

Fig. 2. Showing the inhibiting power at 37° of heated and unheated horse serum dilutions (1 + 5).

is, so to speak, complete, while the pseudo-antirennet is still active to a high degree and cannot be entirely destroyed. Then it must be assumed that the destruction of the pseudo-antirennet has practically come to an end, since a

serum dilution, boiled for one hour, gives values for its inhibiting power which are but little less than those shown by a dilution heated for a quarter of an hour to 70°. This partial destruction of the pseudo-antirennet and the increase in the inhibiting power by diluting it would hardly be explainable if the pseudo-antirennet, as supposed by Korschun, were a substance present in normal horse serum as an independent body. On consideration the above-mentioned facts rather indicate that the term pseudo-antirennet, as applied to the inhibiting power of heated and unheated serum dilutions, covers at least three properties of normal horse serum, i.e. its faculty of regulating the [H] in its dilutions, its alkalinity and its thermolability.

The importance of the first of these properties is apparent on an examination of the effect which dilution of normal horse serum has on its inhibiting power. Allusion has already been made to Hedin's statement that dilution of the normal serum does not change its inhibiting power; this is not in conformity with my own experiments. In view of the method employed by Hedin in his experiments, it is, however, impossible to attach importance to his deductions. By using Korschun's exact methods it is obvious that the inhibiting power is increased considerably by dilution of the serum. This may be seen from Tables VIII and IX, which concern two experiments made with diluted horse sera.

TABLE VIII.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0.3 cc. of a horse serum diluted (1+2)..	0.04 cc.	0.12 cc.	0.25 cc.	0.28 cc.	0.31 cc.	0.36 cc.	0.36 cc.	0.36 cc.	0.36 cc.
0.3 cc. of the serum dilution used contained 0.1 cc. horse serum.									

TABLE IX.

	Time during which the rennet-serum complex is kept at 37° before milk is added to it								
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours
The maximum amount of rennet neutralised by 0.6 cc. of a horse serum diluted (1+5)..	0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.37 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.
0.6 cc. of the serum dilution used contained 0.1 cc. horse serum.									

This increase in the inhibiting power becomes more apparent on examination of the [H] of the normal horse serum and its dilutions. Tested electrically the [H] of the normal horse serum in question gives the value

$p_{H^+} = 7.99$. Diluting the serum (1 + 5) its alkalinity does not decrease. On the contrary, measured at 37° its $[H^+]$ gives values which vary from $p_{H^+} = 8.20$ to $p_{H^+} = 7.90$, depending on the time at which they are measured, either directly the dilution is made or later. The $[H^+]$ of the dilution (1 + 2) agrees, as does that of the dilution (1 + 5), with the $[H^+]$ of the normal horse serum.

Considering the extreme sensitiveness of rennet towards even very small quantities of alkali, it seems natural to connect the increase of the inhibiting power of the dilutions with the tendency of the serum to maintain normal $[H^+]$ even in its higher dilutions. Indeed we know that the amount of rennet inhibited by an alkali solution is dependent on the $[H^+]$ of this solution only, and not on the exact number of H- or OH-ions present. The same fact must therefore determine the action of the serum dilutions if their $[H^+]$ has any influence on their increased inhibiting power. From this point of view it is, however, also justifiable to suppose that a greater quantity, either of the alkaline solution or of the serum dilutions, will destroy more rennet than a smaller amount is able to do. To explain this we may suppose that 0.1 cc. of alkaline solution of a certain $[H^+]$ neutralises 0.02 cc. rennet solution; 0.6 cc. of the same alkaline solution should then neutralise 6×0.02 cc. = 0.12 cc. rennet. That this occurs when the alkali is kept in six tubes each containing 0.1 cc. is obvious. If 0.6 cc. of the alkali is brought into one single tube, the same amount of rennet should be inhibited, as in this case also we have 0.02 cc. rennet corresponding to each of the alkali's six units of 0.1 cc. I have tried experimentally to ascertain how far this agrees with the actual, and found that 0.6 cc. of a Na_2HPO_4 solution of a certain $[H^+]$ inhibits seven times more rennet than 0.1 cc. of the same phosphate solution. This result is very satisfactory, as experimental errors must always be considerable in work of this kind.

If a greater quantity of alkali is thus able to destroy more rennet than a smaller, the serum and its dilutions must do the same, as their $[H^+]$ is also constant; and 0.6 cc. of a serum dilution (1 + 5) must therefore inhibit more rennet than 0.3 cc. serum dilution (1 + 2), and this 0.3 cc. again more than 0.1 cc. of normal horse serum. The question whether the increase in the inhibiting power of the serum can be due to its property of maintaining normal $[H^+]$ in its dilutions therefore resolves itself into one, as to whether an increase in the inhibiting power, equal to that shown by the serum dilutions, can be caused by an alkaline solution of the same $[H^+]$ as the serum— $p_{H^+} = 7.99$ (at 37°)—and used in the same quantities as this.

The increase in the inhibiting power of the serum dilution (1 + 2) is equal

to 0.03, this value being the difference between the maximum amount of rennet (0.33 cc.) inhibited by 0.1 cc. of normal horse serum at 37° and the greatest quantity (0.36 cc.) destroyed at the same temperature by 0.3 cc. of the serum dilution (1 + 2).

The increase in the inhibiting power of the solution (1 + 5) is 0.06, as 0.3 cc. of the dilution (1 + 2) inhibits 0.36 cc. rennet, and 0.6 cc. of the dilution (1 + 5) inhibits 0.42 cc.

As regards the alkaline solution, it has already been pointed out that the increase in its inhibiting power is proportional to the amount of solution used. 0.3 cc. alkaline solution of a certain $[H]$ therefore inhibits three times as much rennet as 0.1 cc. of the same solution. 0.1 cc. alkali of the concentration required¹ inhibits 0.04 cc. rennet, and 0.3 cc. of this alkali therefore ought to inhibit $3 \times 0.04 = 0.12$ cc. rennet, giving an increase in the inhibiting power equal to $0.12 - 0.04 = 0.08$. On the other hand, 0.6 cc. of the alkali in question² will inhibit $6 \times 0.04 = 0.24$ cc. rennet, which represents an increase in the inhibiting power equal to $0.24 - 0.12 = 0.12$. The experiments upon which these calculations are based are somewhat crude and the values cannot be regarded as absolutely exact. Nevertheless they suffice to show that an increase in the inhibiting power such as is shown by the serum dilutions is to be expected when they are used in increasing quantities.

With respect to the other properties which are necessary for the action of the pseudo-antirennet, the alkalinity and the thermolability of the serum, their importance is best shown by a closer examination of the inhibiting power of a boiled horse serum.

In view of the results of the above-mentioned experiments one might expect the reduction of the inhibiting power of the heated serum dilutions to be due to a decrease of their $[H]$ caused by the treatment. This, however, was not the case, as the $[H]$ of the boiled serum dilution gave roughly the same value as that shown by the unheated dilutions. Nevertheless there is an important relation between the inhibiting power of the boiled serum and its $[H]$. This became evident by a determination of the inhibiting power of 0.6 cc. of the phosphate solution at 37°. The experimental data are given in Table X.

¹ A solution of Na_2HPO_4 and KH_2PO_4 was used, made according to Sørensen's [1909] instructions by mixing 9.75 cc. $n/15 Na_2HPO_4$ and 0.25 cc. $n/15 KH_2PO_4$; tested at 18° this mixture has the $[H]p_H = 8.038$.

² In an experiment already mentioned 0.6 cc. phosphate solution neutralised 0.28 cc. rennet, and not 0.24 cc.

TABLE X.

The maximum amount of rennet neutralised by 0.6 cc. of a phos- phate solution with the [H'] _{P_H} = 8.038 (18°) ..	Time during which the rennet-phosphate complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.003 cc.	0.04 cc.	0.19 cc.	0.23 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.

As can be seen from the curves in Fig. 3 there is a striking agreement between the inhibiting effects of the phosphate solution and the boiled serum

Fig. 3. Showing the inhibiting power at 37° of a boiled horse serum dilution (1+5) and of a phosphate solution of nearly the same [H'].

dilution. There appears no reason to doubt that this agreement is due to the fact that the inhibiting power of the boiled horse serum is an effect of its [H'] only. This will be sufficient to indicate the importance of the alkalinity

of the serum with respect to its inhibiting power. It therefore only remains to be shown that the inhibiting power is dependent also on the thermolability of the serum.

The value of the $[H]$ is clearly as important for the inhibiting power of the serum dilutions as it was found to be for the action of the boiled serum. The inhibiting power of the dilution (1 + 5), for instance, must therefore be regarded as a mixed effect of its $[H]$ and of various other supplementary properties (substances?). In addition to the effect of the $[H]$ the inhibiting power of this dilution therefore also shows the effect of these properties. As can be seen from Table XI they also relate to the pseudo-antirennet, as their inhibiting power increases proportionally to the time the serum acts on the rennet.

TABLE XI.

	Time during which the serum or the phosphate solution acts on the rennet at 37°								
	0	15	1	2	3	4	5	6	7
	min.	min.	hour	hours	hours	hours	hours	hours	hours
The maximum amount of rennet neutralised by 0.6 cc. of a serum dilution (1 + 5)	0.04 cc.	0.12 cc.	0.27 cc.	0.33 cc.	0.40 cc.	0.42 cc.	0.42 cc.	0.42 cc.	0.42 cc.
The maximum amount of rennet neutralised by 0.6 cc. of a phosphate solution of the same $[H]$ as the horse serum	0.003 cc.	0.04 cc.	0.19 cc.	0.23 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.	0.28 cc.
The inhibiting effect of the supplementary properties of the horse serum	0.037 cc.	0.08 cc.	0.08 cc.	0.10 cc.	0.12 cc.	0.14 cc.	0.14 cc.	0.14 cc.	0.14 cc.

Calculations similar to that made in Table XI show that the effect of these supplementary properties of the serum dilution (1 + 5) is greatly reduced by heating it to 70°. In a boiled dilution their effect has completely disappeared. As the thermolability of the serum is thus decisive for the effect of these properties, it must also be of importance for the action of the pseudo-antirennet, since the properties in question, as shown in Table XI, are part of the pseudo-antirennet.

Some interesting light as to the nature of these supplementary properties, or substances, of normal horse serum may be obtained from the experiments already discussed. As they cannot be demonstrated in a boiled horse serum dilution, their effect is obviously due to the proteins of the serum, or perhaps to their colloid qualities. Moreover, the effect of the properties in question increases proportionally to time, and to the temperature at which the serum acts on rennet. This can be seen from Tables I and XII. Table I showed the inhibiting power of 0.1 cc. of normal horse serum at 20°. But the figures

at the same time also practically show the inhibiting effect of the supplementary properties at 20°, as the [H] of 0.1 cc. of normal horse serum is hardly of any importance for the amount of rennet destroyed at this temperature. Experiments showed that 0.1 cc. of the phosphate solution neutralised 0.003 cc. rennet at 20°, an amount which does not increase even if the solution acts on rennet for several hours.

The inhibiting power of the supplementary properties at 37° is shown in Table XII.

TABLE XII.

	Time during which the serum or the phosphate solution acts on the rennet at 37°								
	0	15	1	2	3	4	5	6	7
The maximum amount of rennet neutralised by 0.1 cc. of normal horse serum	min.	min.	hour	hours	hours	hours	hours	hours	hours
	0.04 cc.	0.12 cc.	0.17 cc.	0.21 cc.	0.29 cc.	0.33 cc.	0.33 cc.	0.33 cc.	0.33 cc.
The maximum amount of rennet neutralised by 0.1 cc. phosphate solution of the same [H] as the horse serum	0.003 cc.	0.02 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.	0.04 cc.
The effect of the supplementary properties of the horse serum is therefore equal to	0.037 cc.	0.10 cc.	0.13 cc.	0.17 cc.	0.25 cc.	0.29 cc.	0.29 cc.	0.29 cc.	0.29 cc.

The effect of these properties is thus considerably increased by raising the temperature at which the experiment is made.

Furthermore, some light is thrown on them from Table XII. Compared with the figures in Table XI, which showed their inhibiting power at high dilution, the figures in Table XII indicate that their effect is greater when they act on rennet in concentrated than in diluted form.

The real cause of the appearance of these properties, or substances, cannot be determined from the experiments discussed above. Korschun's "true antibody" is certainly either identical with them or part of them. Nevertheless the use of this expression for the explanation of the action of the normal serum on rennet is not to be recommended. This action cannot be due to a true antibody, but is more probably attributable to physico-chemical effects. On the other hand, Hedin's conclusion that this action is a mere adsorption must undoubtedly only be a partial explanation.

II. THE INHIBITING EFFECT OF NORMAL RABBIT SERUM ON RENNET.

As can be seen from the following table the action of normal rabbit serum on rennet has the same characteristics as that of normal horse serum, with the difference merely that its effect is distinctly weaker.

TABLE XIII.

No.	Material	Temp.	Vol. of serum	Maximum amount (in cc.) of rennet neutralised when the rennet-serum complex is kept for the following times before milk is added to it									
				0	0-25	1	2	3	4	5	6	7	hours
1	Normal rabbit serum	20°	0-1 cc.	0-004	0-008	0-01	0-015	0-03	0-03	0-03	0-03	0-03	0-03
2	Normal rabbit serum	37°	0-1 cc.	0-004	0-025	0-085	0-11	0-14	0-17	0-17	0-17	0-17	0-17
3	Normal rabbit serum dialysed for 48 hours through an animal membrane	37°	0-35 cc. of the dialysed serum (=0-1 cc. undiluted serum)	0-0015	0-02	0-07	0-09	0-15	0-17	0-17	0-17	0-17	0-17
4	Rabbit serum diluted (1+5)	37°	0-6 cc. diluted serum (=0-1 cc. normal serum)	0-004	0-025	0-09	0-13	0-16	0-18	0-21	0-21	0-21	0-21
5	Rabbit serum diluted (1+5) and heated to 70° for 0-25 hour	37°	0-6 cc. diluted serum (=0-1 cc. undiluted serum)	0-0015	0-006	0-12	0-15	0-15	0-15	0-15	0-15	0-15	0-15

The experiments from which these numbers are derived were arrived at in the same way as those dealing with the inhibiting power of normal horse serum. The same rabbit serum was used for all experiments. In addition two other rabbit sera were tested, one of which showed a lower inhibiting power than that used for the experiments quoted. The intensity of the inhibiting power thus varies not only with different species of animals, but also with individuals of the same species, a fact which has already been recognised.

III. THE INHIBITING POWER OF A RENNET ANTISERUM.

The question of the possible identity of artificial antirennet and that found in normal horse serum has already been referred to in the introduction. Previous researches, Korschun's and Morgenroth's [1900] especially, appeared to point in this direction, at any rate as far as those rennet antisera were concerned, which were made by immunising animals, e.g. goats or rabbits, with a true rennet enzyme. The antirennet of the normal serum, or at least a part of it, was regarded as a true antibody, formed in the serum of the animal in question by autoimmunisation with rennet, originating from the mucous membranes of its stomach. As the results of the experiments mentioned in

this paper affect this conception of the inhibiting effect of normal horse serum in various respects, it appeared of interest to ascertain whether the action of the rennet antiserum could be explained in the same manner as that of the normal serum, or whether in this antiserum there occurred a typical antibody with properties similar to those claimed by Korschun for the antibody of normal horse serum.

The rennet antiserum, employed in the experiments discussed below, was obtained in the following manner.

The immunisation was accomplished in The Swiss Institute for Dairy Bacteriology, Liebefeld near Berne. As antigen a pure rennet powder (Merck) with the coagulating power $1-1.5 \times 10^6$ was used. The animals were injected with an extract of this rennet powder in the strength of 1.0 g. of rennet powder to 10 cc. of sterile distilled water. For each injection, given every other day, a fresh quantity of extract was made. At the beginning of the treatment the rabbits used for the immunisation did not show any loss of weight, but later on, after the seventh injection, a rather heavy fall could be noticed. Two of the rabbits died at the end of the treatment with typical anaphylactic symptoms. The subcutaneous injection caused a somewhat considerable local infiltration. Contrary to what was expected, the rennet antiserum was found to be very durable. Even at the end of a year its inhibiting power was entirely unimpaired.

Unfortunately it has been necessary to limit the number of experiments originally planned with this antiserum, as the quantity available was altogether inadequate, especially as each experiment had to be carried out two or three times.

It is interesting to note that the inhibiting power of the rennet antiserum at 20° and 37° agrees well with the properties claimed by Korschun for a serum containing a true antirennet. See Table XIV.

TABLE XIV.

The inhibiting power of rennet antiserum at 20° and 37° .

	Time during which the rennet-serum complex is kept before milk is added to it									
1.	0	15	1	2	3	4	5	6	7	
The maximum amount of rennet neutralised by 0.1 cc. of a rennet antiserum, at 20°	min.	min.	hour	hours	hours	hours	hours	hours	hours	hours
	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.	0.03 cc.
2.										
The maximum amount of rennet neutralised by 0.1 cc. of a rennet antiserum, at 37°	min.	min.	hour	hours	hours	hours	hours	hours	hours	hours
	0.03 cc.	0.045 cc.	0.065 cc.	0.09 cc.	0.115 cc.	0.13 cc.	0.13 cc.	0.13 cc.	0.13 cc.	0.13 cc.

At 20° the antiserum thus neutralises a considerable quantity of rennet, which does not increase even if the rennet-antiserum complex is kept for seven hours at 20°. Furthermore, the effect of the true antirennet, i.e. the capacity of the antiserum for destroying a maximum amount of rennet in a very short time (a few seconds), has considerably increased owing to the immunisation, whereas the effect of the pseudo-antirennet at 37° is still unaltered, as the difference in the inhibiting power between the pseudo-antirennet of the normal rabbit serum and that of the antiserum (Tables XIII, No. 2, and XIV, No. 2) is no doubt due to individual differences in the inhibiting power of sera of the same species, and not to a decrease in the effect of the pseudo-antirennet owing to the dialysis.

At this point, however, the agreement between the effect of the antiserum and the properties claimed by Korschun for the true antirennet ceases. Testing the effect of the antiserum under other conditions, the agreement rather seems to have been accidental. Dialysis, e.g., causes a decrease in the entire inhibiting power of the antiserum (Table XV), just as was the case with the dialysed horse serum.

TABLE XV.

The maximum amount of rennet neutralised by 0.22 cc. of a rennet antiserum dialysed for 48 hours	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.01 cc.	0.03 cc.	0.05 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.	0.08 cc.

3 cc. of antiserum were dialysed for 48 hours, its volume thereby increasing to 6.5 cc. 0.22 cc. of the dialysed antiserum therefore is equal to 0.1 cc. untreated antiserum.

This decrease in the inhibiting power of the antiserum is not consistent with the presence of a true antibody in this serum.

Similarly, it is noteworthy that the effect of the pseudo-antirennet is increased far more by diluting the antiserum (Table XVI) than by diluting normal rabbit serum (Table XIII, No. 4). This would hardly be the case if the pseudo-antirennet, as assumed above, had not been altered by the immunisation.

TABLE XVI.

The maximum amount of rennet neutralised by 0.6 cc. of a rennet antiserum diluted (1+5)	Time during which the rennet-serum complex is kept at 37° before milk is added to it									
	0 min.	15 min.	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours	7 hours	
	0.01 cc.	0.06 cc.	0.20 cc.	0.22 cc.	0.23 cc.	0.24 cc.	0.24 cc.	0.24 cc.	0.24 cc.	0.24 cc.

0.6 cc. of the diluted antiserum contained 0.1 cc. antiserum.

The increase in the effect of the pseudo-antirennet is more explicable if viewed from the same standpoint as the changes in the inhibiting power of normal horse serum caused by dilution. Unfortunately, the experiments made with the antiserum had not left a sufficient quantity for a determination of its $[H']$. Using Sørensen's indicator method it was, however, found that the antiserum was more alkaline than normal rabbit serum, but less alkaline than normal horse serum¹. Dilutions of the antiserum therefore have a smaller $[H']$, i.e. a higher alkalinity, than dilutions of normal rabbit serum, as the sera are able to maintain normal $[H']$ even in high dilutions. As a matter of course the dilutions of the antiserum therefore must destroy more rennet than dilutions of normal rabbit serum.

There is another peculiarity in the inhibiting effect of the antiserum at 20°, which is not so easily explained as the changes in the action of the pseudo-antirennet. As can be seen from Tables XIII, No. 1, and XIV, No. 1, the maximum amount of rennet neutralised by normal rabbit serum at 20° is equal to that destroyed by the antiserum at the same temperature. The velocity of reaction of the destruction is, however, very different for the two sera, the antiserum neutralising the maximum amount in a very short time, normal rabbit serum, on the contrary, in four hours only. As regards the diluted antiserum, the velocity of reaction is less than that of the antiserum itself (Table XVI).

Changes in the velocity of reaction, such as are shown by the antiserum, are well known from the study of the action of adsorbing bodies [Bayliss, 1906]. As was the case for the antibody, these changes are proportional to the concentration of the adsorbing body; adsorption increasing with the concentration. The peculiarity of the inhibiting effect referred to above is therefore possibly due to an increase in the concentration of some substance in the serum during the immunisation.

The researches on the inhibiting power of the antiserum here discussed do not favour the belief that the action of the rennet antiserum is of a special nature. On the contrary, they appear to indicate that this action cannot be due to the presence of a true antibody formed in the serum during immunisation.

¹ This is remarkable, as in his researches on the $[H']$ of the normal serum Michaelis [1914] points out that changes in the normal $[H']$ of the serum are extremely infrequent, at least as far as an increase is concerned.

CONCLUSIONS.

1. The inhibiting effect of normal horse serum on the coagulating power of the rennet enzyme is not, as supposed by Korschun, due to two inhibiting substances, the so-called true antibody and the pseudo-antirennet. Neither can Hedin's explanation of this effect as a mere adsorption be regarded as fully satisfactory.

2. It can be shown furthermore that the $[H]$ of normal horse serum is highly important for its inhibiting power.

3. Normal rabbit serum shows the same characteristics in its effect on rennet as normal horse serum.

4. The inhibiting power of a rennet antiserum made by immunising rabbits with rennet enzyme can hardly be due to the presence of a true antibody, as this serum also tends to show the characteristics of normal horse serum.

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