VOLUME XV JULY, 1915 No. 1

ON A COMPLEMENT-STIMULATING SUBSTANCE IN COW'S MILK.

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IN a former communication (1) we showed that a substance acting as complement is present in milk under both normal and abnormal conditions, and that this substance displays characteristics which lend a certain amount of doubt as to its true complementary nature. Expression was also given to the idea, that the haemolytic system—heated ox-serum + guinea-pig corpuscles—is of a peculiarly sensitive character in regard to ox-complement in the presence of milk.

We have made a considerable number of experiments directed towards the elucidation of these points, but whilst an answer to the former has not been satisfactorily obtained the latter has been well substantiated.

THE NATURE OF THE COMPLEMENTARY SUBSTANCE IN MILK.

(*a*) *The effect of heating to* 55° *C. for* 30 *minutes.*

In all cases this was effective in destroying the complement activity of milk.

This however is also stated to be true of certain pseudo-complementary substances such as oleates, in presence of protein.

(*b*) *The effect of filtration through porous porcelain, under pressure.*

The complementary substance of milk does not appear to pass such filters, the whey obtained being quite inactive. A similar result is obtained when ox-complement is added to milk before filtration.

(*c*) *The effect of the removal of calcium salts on the complement activity of milk.*

It is usually held that the presence of calcium salts has an inhibitory effect on complement activity. As milk contains a considerable percentage of calcium salts, experiments were made with a milk, which ordinarily showed strong haemolytic activity, both before and after removal of calcium by the addition of sodium oxalate.

If 0·4 per cent. of solid sodium oxalate be added to milk and the mixture allowed to stand for a short time and then centrifuged, practically the whole of the calcium in the milk is replaced by sodium, and calcium oxalate is precipitated. At the same time the milk undergoes a decided change in reaction; for instance a milk showing 15 degrees of acidity (*i.e.* 100 c.c. of milk require 15 c.c. of N/10 NaOH to produce a pink colour with phenol-phthalein) after treatment with sodium oxalate only shows an acidity of about 4–5 degrees. Milk so treated with sodium oxalate shows an identical haemolytic action before and after treatment even when no attempt was made to restore the original hydrogen-ion concentration. When this was done, the haemolytic activity of the treated milk appeared to be somewhat less than that of the untreated milk.

(*d*) *The effect of shaking.*

Many investigators hold that complement is destroyed fairly readily by agitation of a more or less severe character. A great number of experiments were made with (1) milk which possessed naturally a strong haemolytic activity, (2) milk, inactivated at 55°, to which an addition of ox-complement had been made, (3) saline with a similar addition of ox-complement. These mixtures were placed, in quantities of 20 c.c., in strong glass test tubes (12·5 cm. × 2·5 cm.) and shaken in a Hearson shaker, both with and without the addition of glass beads. The shaking was very vigorous (about 250 vibrations per minute) and

tests of the haemolytic activity were made after intervals of 15, 30, 45 and 60 minutes. The temperature of shaking was that of the room (*circa* 65°–70° F.). These experiments led to very little information of a direct character, as the greatest difficulty was experienced in bringing about any appreciable destruction of complement. There was, perhaps, very slight evidence of such destruction but not of sufficient magnitude to throw any light on the character of the substance which naturally occurs in milk.

During the course of these experiments, however, it was noticed that, whereas the concentration of ox-complement in the saline and in the inactivated milk was the same in any individual experiment, yet, on testing the two series for haemolytic activity, the haemolysis in the milk series was relatively very much more powerful than in the corresponding saline series, and in fact the difference was of a very marked character. This observation led to a careful investigation into a possible stimulating effect of milk on complement.

THE PRESENCE OF A SPECIFIC COMPLEMENT-STIMULATING SUBSTANCE IN MILK.

Determinations of the limits of haemolytic activity were made with the system—heated ox-serum 0·3 c.c. + guinea-pig corpuscles (1 : 20) 0·5 c.c. + diluted complement 1 c.c.—using various substances as the complement diluent and also complement from various sources.

The following signs are used to signify the amount of haemolysis obtained:

+ + + +	=complete haemolysis.
+ + +	=nearly complete, but slight deposit of unlaked cells.
+ +	=partial haemolysis, with increasing deposit of cells.
+	=partial haemolysis, with increasing deposit of cells.
sl. +	=partial haemolysis, with increasing deposit of cells.
—	=no haemolysis.

It will only be necessary to state once and for all that all the required controls were made in conjunction with the following experiments. No experiment is recorded in which there was any failure in this respect.

The dilutions employed were made with the utmost care, as far as possible the same pipettes being employed in each series.

Exp. 1. *Diluents.* Saline and inactivated cow's milk.
Complement. Ox.

Dilutions	Haemolysis in Saline	Haemolysis in Milk
1 : 20	+ + + +	+ + + +
1 : 50	+ + +	+ + + +
1 : 200	—	+ + +

Exp. 2. *Diluents.* Saline and two different samples of inactivated cow's milk, both of which gave reactions for the presence of complementary substance before inactivation.
Complement. Ox.

Dilutions	Haemolysis in Saline	Haemolysis in Milk 1	Haemolysis in Milk 2
1 : 25	+ + + +	+ + + +	+ + + +
1 : 50	+ +	+ + + +	+ + + +
1 : 100	sl. +	+ + + +	+ + +
1 : 150	—	+ + +	+ +
1 : 200	—	+ +	+
1 : 250	—	+ +	+
1 : 300	—	+	sl. +
1 : 400	—	sl. +	v.sl. +
1 : 500	—	—	—

The haemolysis obtained in 1 c.c. of each of these milks before inactivation was (1) + +; (2) + + + +. It is to be noted that the latter had a somewhat slighter stimulating action.

Exp. 3. As Exp. 2 but using as diluents saline and a farmer's mixed milk, inactivated and uninactivated.

Dilutions	Haemolysis in Saline	Haemolysis in Inactivated Milk	Haemolysis in Uninactivated Milk
1 : 25	+ + + +	+ + + +	
1 : 50	+ + + +	+ + + +	
1 : 100	? v.sl. +	+ + + +	identical
1 : 150	—	+ + +	with the
1 : 200	—	+ + +	inactivated
1 : 250	—	+ +	milk
1 : 300	—	+ +	series
1 : 400	—	+	
1 : 500	—	+	

From the above three experiments, the stimulating action of milk on ox-complement is easily seen, and the inactivation of the milk by heat at 55° C. neither contributes nor detracts from the effect. This

https://doi.org/10.1017/S0022172400006070 Published online by Cambridge University Press

stimulating effect was always obtained though in varying degrees of intensity. The thermostability of the stimulating substance was next ascertained.

EXP. 4. *Diluents.* Inactivated cow's milk and the same milk heated in boiling water for 10 minutes.

Complement. Ox.

Dilutions	Haemolysis in	
	Boiled Milk	Inactivated Milk
1: 25	+ + +	+ + + +
1: 50	+	+ + + +
1: 100	—	+ + +
1: 150	—	+ +
1: 200	—	+
1: 250	—	+
1: 300	—	sl. +
1: 400	—	v.sl. +
1: 500	—	—

The heating of milk to the temperature of boiling water destroys or changes the substance which produces the stimulating effect. The same experiment was carried out in order to determine as nearly as possible at what temperature the destruction of this substance occurs. For this purpose milk was heated (1) at 55° C. for 30 minutes, (2) at 67–68° C. for 10 minutes, (3) at 72–73° C. for 10 minutes, and (4) in boiling water for seven minutes.

The results were as follows:

Dilutions	Haemolysis in				
	Saline	Milk 55°	Milk 67°	Milk 72°	Milk 100°
1: 10	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
1: 25	+ + +	+ + +	+ + +	+ + +	+ +
1: 50	+	+ +	+	+	sl. +
1: 75	sl. +	+	sl. +	v.sl. +	v.sl. +
1: 100	—	sl. +	—	—	—
1: 150	—	v.sl. +	—	—	—

This series did not show the marked differences which were sometimes obtained, but it is not easy to express on paper differences which are easily detected by the eye. There was however distinct inhibition by the boiled milk, distinct stimulation by the milk heated at 55°, slight inhibition by the milk heated at 72°, and practically no effect in either direction by the milk heated at 67°. It would appear that the destruction of this stimulating substance begins in the neighbourhood of that temperature at which albumin coagulation occurs, but as will be seen (Exp. 5) the lact-albumin does not appear to be concerned in the stimulating effect.

Exp. 5. The filtration of cow's milk through Doulton filters in order to see whether the stimulating substance would pass with the whey.

Diluents. In all cases: saline, inactivated cow's milk, and inactivated filtered whey of same milk.

Complement. In all cases: ox.

(1)	Dilutions	Haemolysis in Saline	Milk	Whey
	1: 50	+ + +	+ + + +	+ + + +
	1: 100	—	+ + + +	+ + +
	1: 200	—	? v.sl. +	—
	1: 300	—	—	—
	1: 400	—	—	—

In this case the stimulating substance appeared to pass the filter.

(2)	Dilutions	Haemolysis in Saline	Milk	Whey
	1: 25	+	+ + +	+ +
	1: 50	?	+ +	?
	1: 100	—	sl. +	—
	1: 200	—	—	—

The whey had a slight stimulating influence, but was not so energetic as the milk itself.

(3)	Dilutions	Haemolysis in Saline	Milk	Whey	Whey heated to 75° C. for 5 minutes and filtered
	1: 10	+ + + +	+ + + +	+ + + +	+ + + +
	1: 25	+ + +	+ + +	+ + +	+ + +
	1: 50	? v.sl. +	+	? v.sl. +	sl. +
	1: 75	—	sl. +	—	—
	1: 100	—	? v.sl +	—	—
	1: 150	—	—	—	—
	1: 200	—	—	—	—

The whey showed a slight stimulating power over saline and the heated whey had a slightly more powerful action, but neither had the same effect as the milk itself.

(4)	Dilutions	Haemolysis in Saline	Milk	Whey
	1: 25	+ +	+ + + +	+ + +
	1: 50	v.sl. +	+ +	sl. +
	1: 100	—	sl. +	—
	1: 150	—	—	—
	1: 200	—	—	—

As in the other experiments the whey appears to have a slight stimulating effect as compared with the saline, but is not so active as the milk itself.

As a result of these experiments, it is evident that the greater part of the stimulating substance remains with the casein on the filter, or else is destroyed or altered during its passage.

EXP. 6. Similar to Exp. 4 but using guinea-pig complement in the place of ox-complement.

Diluents. Saline and
Cow's milk (*a*) Heated in boiling water 5 minutes.
„ (*b*) „ at 72° C., 10 minutes.
„ (*c*) „ at 55° C., 30 minutes.
Complement. Guinea-pig.

Dilutions	Haemolysis in Saline	(*a*)	(*b*)	(*c*)
1: 10	+ + + +	v.sl. +	+ + + +	+ + + +
1: 25	+ + + +	—	+ + +	+ + +
1: 50	+ + +	—	+ +	+ +
1: 75	+ +	—	+	+
1: 100	+ +	—	v.sl. +	sl. +
1: 150	+	—	—	v.sl. +
1: 200	v.sl. +	—	—	—
1: 250	—	—	—	—

These results are remarkable. Cow's milk heated at 55° C. for 30 minutes exerts *no* stimulating effect on guinea-pig complement, in fact there is inhibition (probably due to the calcium salts present). Heating at 72° C. renders cow's milk a little more inhibitory and heating in boiling water renders the milk almost completely inhibitory to guinea-pig complement.

The distinction between these two forms of complement is shown again in the following experiment.

The guinea-pig complement was diluted 1: 1 with saline before making the further dilutions with saline and milk.

Dilutions	Ox-complement Haemolysis: Saline	Ox-complement Haemolysis: Milk	G.P. complement Haemolysis: Saline	G.P. complement Haemolysis: Milk
1: 10	+ + + +	+ + + +	+ +	sl. +
1: 25	+ +	+ + + +	sl. +	—
1: 50	? v.sl. +	+ + + +	—	—
1: 75	—	+ + +	—	—
1: 100	—	+ +	—	—
1: 150	—	sl. +	—	—
1: 200	—	—	—	—

The stimulating action of cow's milk on ox-complement and its inhibitory action on guinea-pig complement is easily seen.

The next step was to ascertain whether the same stimulating effect was produced on ox-complement, by milk from heterogeneous sources.

Exp. 7. *Diluents.* Saline, inactivated cow's milk and inactivated asses' milk.

Complement. Ox.

Dilutions	Haemolysis in Saline	Cow's Milk	Asses' Milk
1: 10	+ + + +	+ + + +	+ + + +
1: 25	+ +	+ + + +	+ + +
1: 50	v.sl. +	+ + +	v.sl. +
1: 75	—	+ + +	?
1: 100	—	+ +	—
1: 150	—	sl. +	—
1: 200	—	—	—

Asses' milk has apparently neither stimulating nor inhibitory action on ox-complement, the saline and asses' milk series being practically identical.

The experiment was then varied by using other sources of complement both with cow's milk and also with the homologous milk.

Exp. 8. *Diluents.* Saline, inactivated cow's milk and inactivated asses' milk.

Complement. Ass.

Dilutions	Haemolysis in Saline	Cow's Milk	Asses' Milk
1: 10	+ + +	+ + +	+ + +
1: 20	+	+	+
1: 25	v.sl. +	v.sl. +	v.sl. +
1: 33	—	—	—
1: 50	—	—	—
1: 65	—	—	—
1: 100	—	—	—

It is evident that asses' milk does not stimulate asses' complement in the same way that cow's milk stimulates ox-complement.

Exp. 9. *Diluents.* Saline, inactivated cow's milk and inactivated human milk.

Complement. Human.

Dilutions	Haemolysis in Saline	Cow's Milk	Human Milk
1: 10	+ + + +	+ + +	+ +
1: 25	+ + +	+	—
1: 50	+ +	—	—
1: 75	+	—	—
1: 100	sl. +	—	—
1: 150	—	—	—

In this case both the milks have a strong inhibitory action on human complement, the action being greater in the case of the human milk. This can scarcely be due to calcium salts as probably there is a smaller percentage of these in the human milk than in the cow's milk.

As the result of these experiments, it was evident that the stimulating effect is only exerted in an entirely homologous system. An experiment was therefore made, using a parallel system, in order to see if the same result would be obtained, as had been, in the case of cow's milk.

The parallel system was: sheep's corpuscles (1 : 20) 0·5 c.c. + inactivated human serum 0·5 c.c. + human-complement + human milk. This is strictly analogous to the original system: guinea-pig corpuscles + heated ox-serum + ox-complement + cow's milk. Three parallel tests were made using as diluents (1) human milk, (2) saline, (3) cow's milk.

The results were as follows:

	Haemolysis in		
Dilutions	Saline	Cow's Milk	Human Milk
1 : 10	+ +	v.sl. +	—
1 : 25	v.sl. +	—	—
1 : 50	—	—	—
1 : 75	—	—	—
1 : 100	—	—	—
1 : 125	—	—	—

The result is quite the reverse of the original system, and it is necessary to suppose that the stimulating action is specific for the original system only.

It was necessary however to ascertain whether the stimulating effect was directed towards the complement, or whether it was the amboceptor that was affected. In order to ascertain this, sheep's corpuscles and horse-serum rendered haemolytic to these corpuscles were used. The system was: 0·4 c.c. horse-serum (100 times the minimum haemolytic dose) + 0·5 c.c. sheep's corpuscles (1 : 20) + 1 c.c. of the dilution of ox-complement. The diluents were saline and inactivated cow's milk. The results were as follows:

	Haemolysis in	
Dilutions	Saline	Cow's Milk
1 : 5	+	+
1 : 10	sl. +	sl. +
1 : 20	v.sl. +	v.sl. +
1 : 50	—	—
1 : 75	—	—
1 : 100	—	—

https://doi.org/10.1017/S0022172400006070 Published online by Cambridge University Press

The haemolysis was identical in each case whilst a parallel experiment using the original heated ox-serum and guinea-pig corpuscles system showed the usual stimulating effect. The use of the above system in which sheep's corpuscles play a part is rendered somewhat difficult on account of the agglutination of the corpuscles which occurs. Further, it has been noticed constantly that towards the delicate system—sheep's corpuscles + the corresponding horse-serum amboceptor—ox-serum appears to contain but little complement. For instance, whereas with this system complete haemolysis occurred in a few minutes with 1 : 10 guinea-pig complement, haemolysis was not complete in two hours, even when undiluted ox-complement was employed, though possibly the agglutination of the sheep's corpuscles played some part in this inhibition.

The exhibition of this stimulating effect of milk on haemolytic action is to a certain extent dependent on the quantity of inactivated ox-serum used as amboceptor. The divergence between the results in saline and milk is most marked when the least possible quantity of amboceptor is used. If excessive amounts of ox-serum amboceptor be used, the result may be reversed.

The results recorded above appear to admit of little explanation, though they explain to a certain extent some of the divergent views which have been published as to the presence of complement in milk.

The following view has been expressed by Liebermann and V. Fenyvissy.

"One can attain, by the use of mixtures of sodium soaps with various substances present in normal sera which contain natural complement, much stronger haemolysis of sensitised corpuscles than normal. As particularly useful in this connection may be noted, combinations which besides soap, contain serum globulin and calcium chloride and an alkalinity suitable to the combination." How very closely milk corresponds to this necessary combination is obvious. At the same time, there seems no reason why the exhibition of this stimulating power should be confined to cow's milk and to one particular haemolytic system.

REFERENCE.

(1) Hewlett, R. Tanner and Revis, C. (1914). On the presence of so-called "Complement" in Milk. *Journal of Hygiene*, xiv. 481–497.