THE REACTION OF COW'S MILK TO BLOOD SERUM PRECIPITIN.

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(Received for publication, January 20, 1926.)

Osborne and Wakeman[1] isolated in a high degree of purity four proteins from cow's milk: casein, lactalbumin, lactoglobulin, and an alcohol-soluble protein. By chemical methods Crowther and Raistrick[2] had previously been unable to show that the lactoglobulin differed from serum globulin. Schlossmann and Moro[3] had called attention to the fact that a precipitin prepared by injecting animals with milk whey reacts with blood serum. Wells and Osborne[4] using the anaphylactic phenomenon showed that the four proteins obtained by Osborne and Wakeman were antigenically distinct. They further called attention to the fact that lactoglobulin sensitized guinea pigs to beef serum, and that test animals sensitized to beef serum reacted when subsequently injected with lactoglobulin. The inference then is that serum globulin and lactoglobulin are the same, since they cannot be differentiated by chemical or serologic methods. Since it has been well established that globulin is a normal constituent of milk, it should be possible to recognize its presence in milk when tested with a suitable serum precipitin. Furthermore, if the reaction of normal milk to a specific serum precipitin is once established, departures from the normal can readily be detected.

EXPERIMENTAL.

Rabbits were immunized with cow serum until a high titered precipitin had been obtained. The sera were mixed and stored in the refrigerator. Throughout,

[1] Osborne, T. B., and Wakeman, A. J., *J. Biol. Chem.*, 1918, xxxiii, 7.

[2] Crowther, C., and Raistrick, H., *Biochem. J.*, 1916, x, 434.

[3] Schlossmann, A., and Moro, E., *Münch. med. Woch.*, 1903, l, 597.

[4] Wells, H. G., and Osborne, T. B., *J. Infect. Dis.*, 1921, xxix, 200.

TABLE I.

The Reaction of Cow's Milk to Serum Precipitin.

Cow No.	Dilutions of milk tested with 0.1 cc. of serum precipitin.						Number and kind of bacteria per 1 cc. of milk.
	1:20	1:40	1:80	1:160	1:320	1:640	
1	+++*	++	++	+	−	−	540. No streptococci.
2	+++	++	++	+	+	−	7,500. 90 per cent micrococci and 5 per cent hemolytic streptococci.
3	+++	++	++	+	−	−	70. No streptococci.
4	+++	+++	++	+	−	−	600. 40 per cent non-hemolytic streptococci.
5	++	++	+	−	−	−	1,100. No streptococci.
6	+++	++	+	+	−	−	900. 15 per cent hemolytic streptococci.
7	+++	+++	++	++	+	−	19,200. 85 per cent hemolytic streptococci.
8	+++	++	+	±	−	−	800. No streptococci.
9	+++	+++	++	+	−	−	2,800. 20 per cent hemolytic streptococci.
10	+++	+	+	±	−	−	75. No streptococci.
11	+++	++	+	+	−	−	680. Micrococci.
12	+++	+++	++	+	+	±	2,000. 85 per cent hemolytic streptococci.
13	+++	++	+	±	−	−	810. No streptococci.
14	+++	++	+	+	±	−	5,600. 70 per cent hemolytic streptococci.
15	+++	+++	++	++	+	±	1,060. 10 per cent hemolytic streptococci.
16	+++	++	+	+	−	−	2,800. 80 per cent non-hemolytic streptococci.
17	+++	+++	++	+	+	−	1,600. No streptococci.
18	+++	++	++	+	±	−	940. 60 per cent non-hemolytic, and 5 per cent hemolytic streptococci.
19	+++	++	++	+	±	−	1,450. A few non-hemolytic streptococci.
20	+++	++	+	±	−	−	100. No streptococci.

* The amount of precipitation is recorded in the following manner: +++, the maximum, a heavy precipitate; ++, a more moderate accumulation at the bottom of the tube; +, a slight but well defined precipitate; ±, a trace of sediment which fails to appreciably cloud the tube on agitation.

a precipitin was used which in amounts of 0.1 cc. would detect $\frac{1}{40,960}$ cc. of cow serum.

The milk was largely freed of fat by permitting it to stand in the refrigerator overnight. The tests were made by diluting the milk with NaCl solution, and adding to each tube containing 1 cc. of the diluted milk 0.1 cc. of the specific serum. The tubes were incubated 2 hours and refrigerated overnight.

Experiment 1.—At various times milk from 20 cows was obtained directly from the milk pails. In addition to the tests for serum proteins, samples were plated in blood agar and films were examined for the presence of excessive numbers of leucocytes and bacteria. The results of the precipitin tests and plate cultures are recorded in Table I.

It can safely be said that normal milk when tested with amounts of specific blood serum precipitin which will detect $\frac{1}{40,960}$ cc. of cow serum, will react in dilutions of 1:80 and 1:160. Six of the samples

TABLE II.

The Reaction of Fore Milk and of Strippings When Tested with Serum Precipitin.

Cow No.		Dilutions of milk.				
		1:40	1:80	1:160	1:320	1:640
21	Fore milk.	++	+	+	−	−
21	Strippings.	++	+	+	−	−
22	Fore milk.	+++	++	+	+	−
22	Strippings.	+++	++	+	±	−

reacted in a dilution of 1:320, and two but slightly at 1:640. In the main, then, it appears that there is a definite level at which milk will react. Several of the cows harbored a considerable number of organisms in the milk. The reactions usually occurred at a higher dilution in such instances, although this was not always the case.

It seemed probable that the milk withdrawn from the udder at the end of milking might be richer in blood protein than that obtained at first. That such is not the case is indicated in Table II.

It is true that milk drawn from normal udders will react in rather definite dilutions to a definite amount of serum precipitin. The end-point varies within relatively narrow limits, but is not greatly influenced by the presence or absence of moderate numbers of streptococci or the other usual udder bacteria.

It is well known that during pregnancy when the udder is no longer secreting there accumulates a distinctly serous liquid. This rapidly increases in amount toward the end of pregnancy. With birth and the consequent frequent emptying of the udder the appearance of the secretion changes. The reaction of the colostrum and of the milk shortly after parturition is given in Table III.

TABLE III.

The Reaction of the Colostrum and of the Milk Shortly after Parturition to Serum Precipitin.

Cow No.		Dilutions of udder fluid or milk.								
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480
1	Before calving.			+++	+++	+++	+++	+++	++	+
	Day of calving.			+++	+++	+++	++	+	+	±
	Days after calving.									
	1		+++	+++	++	+	+	±	−	−
	2		+++	++	++	+	±	−	−	−
	3		+++	++	+	±	−	−	−	−
	4	+++	+++	++	+	±	−	−	−	−
	6	+++	+++	++	+	±	−	−	−	−
	7	+++	++	+	+	±	−	−	−	−
2	Day of calving.		+++	+++	++	+	+	±	−	−
	Days after calving.									
	1		+++	+++	+++	++	+	±	−	−
	2		+++	+++	++	+	+	±	−	−
	3		+++	++	+	+	+	−	−	−
	4		+++	++	+	+	±	−	−	−
	5		++	+	+	+	−	−	−	−
	7		++	+	+	−	−	−	−	−
	9		+	+	+	−	−	−	−	−
	14	++	+	+	−	−	−	−	−	−

The results of the precipitin tests show that the udder fluid just before parturition is rich in serum proteins. Their concentration within the udder is temporary; at first they diminish rapidly as soon as the udder is regularly emptied, and then fall gradually to the normal level.

The presence of serum in the milk from inflamed udders has led several to suggest that its presence in excessive amounts may be used

for diagnostic purposes. Schern[5] has shown that rennet fails to coagulate milk from inflamed udders. This he attributes to the transudation of blood serum which is said to contain anti-rennet. Baker and Van Slyke[6] showed that the milk from diseased udders was on the whole more alkaline when tested with brom cresol purple than normal milk.

It seemed desirable to test the blood protein of several samples of milk drawn from inflamed udders. In Table IV the results of these tests and a general description of the character of the secretion are recorded.

From the observations recorded in Table IV it becomes apparent that even in milder cases of mastitis, such as that of Cow 1049, there is a considerable increase in the serum content of the milk from the affected quarter. As the process becomes more severe serum passes into the udder to a greater degree. The increase of serum is, however, confined only to the involved quarter, since the results of a number of tests of the milk from the uninvolved quarters have given reactions at only the dilutions which may be considered the normal level.

The ability of the precipitin test to readily detect abnormalities in the secretion has been shown. It must be borne in mind, however, that the cases of mastitis could also be detected by careful clinical examination. However, under usual herd conditions it is possible that milk from definitely involved quarters or from fresh cows may enter the herd supply. That such a test would be of considerable importance as an aid in detecting such undesirable additions is brought out in Table V. In this experiment samples of bottled milk were mixed with various amounts of exudate from a severely involved quarter. The various dilutions of the mastitis exudate in market milk were then tested with the serum precipitin.

It has been shown that the addition of even as little as one-eightieth part of mastitis milk to the bottled milk will increase the serum titer four- to eightfold. It might be said that the addition of the mastitis milk would so change the character of the herd milk that it would become readily apparent by methods in use; such was not the case.

[5] Schern, cited by Klein, L. A., Principles and practice of milk hygiene, Philadelphia and London, 1917.

[6] Baker, J. C., and Van Slyke, L. L., *New York State Agric. Exp. Station, Techn. Bull. 71,* 1919.

TABLE IV.

The Reaction of Milk Obtained from Cases of Mastitis to Serum Precipitin.

	Dilutions of milk from quarters of udder affected with mastitis.						
	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Cow 1049.—Mastitis of 2 days standing. Milk normal in color but contains tiny flocculi. Fails to coagulate on boiling. 5,500 non-hemolytic streptococci per cc.	+++	++	+	+	±	−	−
Cow G.A. 8.—Mastitis of 3 wks. standing. Atrophy of affected quarter. Milk normal except for tiny flocculi. 8,500 non-hemolytic streptococci per cc.	+++	++	+	+	−	−	−
Cow 4836.—Mastitis of 2 days standing. Milk yellow in color, thick in consistency, and contains yellow flocculi. 100 non-hemolytic streptococci per cc.	+++	++	+	+	+	+	±
Cow 1375.—Mastitis of 3 days standing. Milk tinged with pink and contains small flocculi. Fails to coagulate on boiling. 12,000 non-hemolytic streptococci per cc.	+++	+++	++	+	+	±	−
Cow 3321.—Within 24 hrs. of onset. Milk slightly watery, otherwise normal. Fails to coagulate on boiling. 200 non-hemolytic streptococci per cc.	+++	+++	++	+	+	±	±
Cow 4910.—Mastitis of 5 days standing. Recovering. Milk yellow and contains flocculi. 20 non-hemolytic streptococci per cc.	+++	+++	+++	+++	++	+	±

The reaction of the mastitis milk was definitely alkaline, since it became a deep purple on the addition of brom cresol purple. The bottled milk was distinctly acid when tested with the same indicator. The

addition of the mastitis exudate in the dilutions specified in the table failed to appreciably change the reaction of the milk.

It seemed probable that pasteurization of the milk might influence the precipitin test, provided the milk was heated sufficiently to coagulate the protein. To ascertain at what temperature the blood protein

TABLE V.

The Effect of Adding Mastitis Exudate on the Serum Content of Milk.

	Dilutions of milk.								
	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	1:40,960
Market milk	++	+	−	−	−	−	−	−	−
Mastitis exudate				+++	++	++	++	++	±
1 part of mastitis exudate diluted with 10 parts of market milk	+++	+++	+++	+	+	±	−	−	−
Diluted with 20 parts	+++	+++	++	+	±	±	−	−	−
" " 40 "	+++	++	++	+	±	−	−	−	−
" " 80 "	+++	++	+	+	±	−	−	−	−

TABLE VI.

The Effect of Heating Milk on the Precipitin Test.

	Dilutions of milk.				
	1:40	1:80	1:160	1:320	1:640
Raw milk	+++	++	+	±	−
Heated 20 min. at					
55–56°C.	+++	++	+	±	−
62–62.5°C.	+++	++	+	±	−
65–66°C.	+++	++	+	±	−
68–69°C.	−	−	−	−	−

in milk was no longer capable of reacting with the serum precipitin the following experiment was performed.

Mixed milk partially freed of fat was divided into lots and heated for 20 minutes in the water bath at various temperatures. Each lot was then tested with serum precipitin. The results are shown in Table VI.

It is clear that the reacting protein in milk is relatively resistant to heat, since milk heated at a temperature between 65° and 66°C. for 20 minutes still reacted as well as the raw sample. If, however, the temperature is increased to 68–69°C. for the same period, the milk ceases to react.

In addition to the observations cited a number of samples of milk obtained from cows harboring a considerable number of streptococci in the udder have been tested. The milk showed no gross abnormalities. Since the series is small only two cases will be cited. Cow 3954 had had several attacks of mastitis in the previous lactation period. 18 days after parturition plate cultures showed 20,000 non-hemolytic streptococci per cc. The milk reacted in a dilution of 1:1,280. In Cow 4784, blood had been observed on two occasions in the milk. 1 month after the last attack the milk contained 50,000 streptococci per cc. and gave a strong precipitin reaction in a dilution of 1:1,280. Similar results were obtained in two other instances.

DISCUSSION AND SUMMARY.

It seems well established that during normal lactation there passes a slight but well defined quantity of blood protein into the udder. When a specific serum precipitin is applied to diluted milk the reaction occurs at a relatively uniform level of dilution. This line may well be considered the normal level of blood protein elimination. In the main the serum protein level is the same when mastitis streptococci are present as when they are absent, provided they are not too numerous. On the other hand it has been shown that when streptococci are present in large numbers the milk while apparently normal reacts at dilutions considerably higher than the normal.

In the non-lactating udder, particularly during the latter end of gestation, serum proteins accumulate. Little and Orcutt[7] have shown that certain antibodies tend to become greatly concentrated within the udder. Howe[8] and others have shown that the colostrum of cows is rich in blood proteins. It is to be noted, however, that colostrum while rich in serum proteins usually reacts with serum precipitin to

[7] Little, R. B., and Orcutt, M. L., *J. Exp. Med.*, 1922, xxxv, 161.
[8] Howe, P. E., *J. Biol. Chem.*, 1922, lii, 51.

about one-half the titer of the blood serum itself. It may well be that certain proteins of the blood passing into the acini are reabsorbed, leaving a portion of the antigen within the udder. Thus a gradual accumulation of antigen may occur. Shortly after parturition the udder may be drained with a consequent marked fall in serum content. There, however, apparently exists for the next few days considerable permeability of the capillaries for serum proteins. This is borne out by the reaction of the milk to the serum precipitin, since it may react above the normal level for as long as 10 days or 2 weeks after parturition.

During the course of inflammation there may occur a considerable outpouring of blood protein. In the severe cases the exudate may give a titer at as great a dilution as the blood serum. In other milder cases where the inflammation may be confined only to the mucosa of the milk cystern, there is usually little that is abnormal in the appearance of the milk. The precipitin test, however, indicates that serum proteins are present in definitely greater concentration than in normal milk.

Of practical interest is the effect of heating the milk on the reaction of serum precipitin. Milk heated to a temperature as high as 66°C. for 20 minutes will react at the same dilutions as the raw milk. Milk pasteurized at 68–69°C. for 20 minutes will no longer react. The precipitin test, then, affords a ready means of detecting milk heated above 68–69°C.

It is assumed that the precipitin test may have some further practical application. The evidence presented indicates a well defined serum proportion in the milk which may be increased during inflammation of the udder. The experiments in which mastitis exudate was mixed with varying quantities of market milk indicate that such mixtures can readily be detected with serum precipitin. It seems not improbable that some such procedure might be applied as a presumptive test to the product of small herds or to the mixed milk of a number of cows.