tive. On the other hand the mucicarmine stain[5] for mucus lends a faint tint to the phagocytosed substance. This, plus its insolubility in water—properties akin to those of the glycoprotein mucin which also stains scarlet to red with periodic acid—indicates that the polysaccharide is bound to a protein, thus constituting a glycoprotein.

The diminished fat, glucose, and possibly protein absorption by the small intestine in Whipple's disease indicates that its etiology resides in a disturbance of function of the intestinal epithelium. It is probably this defect which permits absorption of the unusual glycoprotein which may or may not be related to the mucin discharged into the enteric lumen by the intestine itself. At any rate, the presence of this readily demonstrated glycoprotein in the intestinal mucosa and mesenteric lymph nodes, indicates that Whipple's disease is more than an obscure defect in fat metabolism and is certainly not the result, as is commonly suggested, of a block of the mesenteric lymphatics. Thus the name lipodystrophy intestinalis, first proposed by Whipple and currently in use, would seem to be inappropriate. It would appear desirable to return to the eponym "Whipple's disease" until a name denoting the nature, rather than a complication, of the disease is forthcoming.

[5] Mallory, F. B., Pathological Technic, W. B. Saunders Co., Philadelphia, 1938.

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Inhibitory Effect of Cow's Milk on Influenza Virus Hemagglutination.* (17389)

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The development of theories on the mechanism of infection by influenza viruses has received considerable encouragement in the past few years from studies of the interaction between these viruses and inhibitors of virus hemagglutination found in various mammalian and avian fluids.[1-7] For some lines of inquiry it is desirable to compare the properties of several fluids, as well as those of the inhibitors isolated from them. It is of interest, therefore, that cow's milk, a fluid readily available in considerable quantity, exerts an inhibitory effect on virus hemagglutination. Some aspects of this inhibition phenomenon are described in the present report.

Materials and methods. Fresh raw milk was obtained from individual cows or as a pool from a commercial distributor and was skimmed by centrifugation in the cold room in the laboratory. The cream, which carried an insignificant part of the total inhibitory activity, was lifted off with a spatula and discarded. If necessary, the milk was recentrifuged to remove residual cream or sediment. Raw skim milk (RSM), less than one day old, was used for most of the experiments; if intended for use after one day, the milk was preserved with 1:5,000 Merthiolate (Lilly), which was found not to affect the inhibitory activity.

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[1] Burnet, F. M., *Lancet*, 1948, **254**, 7.

[2] Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 301, 315.

[3] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 442.

[4] Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, **26**, 403.

[5] Svedmyr, A., *Brit. J. Exp. Path.*, 1948, **29**, 295, 309.

[6] Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **88**, 463.

[7] Francis, T., Jr., and Minuse, E., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 291.

TABLE I.
Inhibitory Activity of Raw and Pasteurized Skim Milk Against Hemagglutination by Heated Swine Influenza Virus.

Cow	Type of milk	Titer
1	Raw	4100
2	"	5100
3	"	4100
4	"	6400
5	"	2600
6	"	6400
7	"	6400
8	"	5400
Commercial pool	"	2600
" "	Pasteurized*	460†

* 30 min. at about 71°C by a vat process.
† Conventional endpoint. The slope of the inhibition curve was very shallow. See Table II and its discussion.

Routine inhibition titrations were performed by the constant virus-varying inhibitor technic,[8] employing 3 to 4 hemagglutinating doses (HD) of purified swine influenza virus,[3] which had been heated for 30 minutes at 53°C. In these routine tests virus was first incubated with inhibitor for 30 minutes, then red cells were added, and readings were made after an additional hour at room temperature. The inhibition titer of a fluid is expressed as the reciprocal of the final dilution of the fluid in the reaction mixture (composed of 0.5 ml inhibitor dilution, 0.5 ml virus suspension, and 1.0 ml 2% chicken red blood cells) giving the standard two-plus (++) endpoint of hemagglutination. Buffered saline, consisting of 0.81% sodium chloride and 0.005 M phosphate at pH 7.3, was the diluent throughout. Details of other types of inhibition tests are given with the description of the experiments.

Experimental. Survey. Samples of RSM, prepared from the individual milks of eight cows, were tested for inhibitory activity against heated swine influenza virus, with the results shown in Table I. Samples of a commercial pool of skim milk before and after pasteurization were included.† As seen in

TABLE II. Effect of Commercial Pasteurization and of Heating at 100°C on the Inhibitory Activity of Milk Against Hemagglutination by Heated Swine Influenza Virus.

Milk sample	Reciprocal of final dilution of sample								
	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480
Pool 1, raw whole milk	0	0	0	0	0	1	2	3	3	4
1, after skimming	0	0	0	0	0	1	2	3	3	4
1, skimmed and pasteurized*	1	2—	2—/2	2	2/3—	3—	3	3/4	3/4	4
2, raw whole milk	0	0	0	0	0	1	2	3—	3	4
2, pasteurized†	0	0	0	0	1—	1	2—/2	3	3	4
RSM,‡ 2 min. at 100°C	1	2—	2	2/3—	3—	3—	3—/3	3	3/4	4
" 5 " "	1	2—	2	2/3—	2/3—	3—	3	3	3/4	4
" 10 " "	1	2—/2	2	2/3—	3—	3—	3	3/4	3/4	4
" 15 " "	1	2—/2	2	2/3—	3—	3—	3—/3	3	3/4	4
" 30 " "	2—	2—/2	2	2/3—	3—	3	3	3	3/4	4
" 60 " "	2—/2	2	2	2/3—	3—	3—/3	3	3	3/4	4

* 30 min. at about 71°C (160°F) by a vat process.
† 20 sec. at about 72°C (162°F) by a continuous flow process.
‡ Raw skimmed milk obtained from Pool 1.
The symbols ± to ++++, usually employed to denote degree of hemagglutination, are replaced for convenience by the symbols 1— to 4. The concentrations of red blood cells in the standard suspensions which are assigned these designations have been shown in a previous publication (Fig. 1 of 10).

[8] Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, **179**, 1275.

† The commercial samples studied in the present investigation were obtained from Durham Dairy Products, Inc., through the courtesy of Mr. V. J. Ashbaugh, Mgr.

Table I, the individual titers are remarkably uniform and have values 1 to 2.5 times as great as those of the commercial pool. Titers similar to these were obtained on all other occasions when individual milks or pools were tested. Accordingly, one may conclude that inhibitory activity is a characteristic property of cow's milk.

Heat stability of the inhibitor. Samples of undiluted commercial RSM, prepared by centrifugation, were heated in a boiling-water bath for varying periods, cooled, and titrated for residual inhibitory activity against heated swine influenza virus. Raw and pasteurized samples of whole and skim milk were also tested. The results, presented in Table II, show that the inhibitory activity of RSM is similarly reduced by commercial pasteurization (30 minutes at 71°C) and by heating at 100°C for 2 minutes. Heating at 100°C for periods greater than 2 minutes produces very little further change. Pasteurization of raw *whole milk,* carried out by a different process (20 seconds at 72°C), has little effect on the inhibitor.

From the results with heated RSM, it would appear that the inhibitory activity is associated either with a single substance which has both heat-labile and heat-stable aspects, or with two substances which differ in heat stability. It is interesting that the gradient of inhibition is much more shallow with heated than with unheated skim milk (Table II). On the assumption that the inhibitory activity of milk is associated with a single substance, this difference in gradient is interpreted to mean that the inhibitor becomes weaker, rather than simply more dilute, during heating.[9] Unpublished experiments with the egg-white inhibitor, which is remarkably thermostable, have shown a similar weakening of the inhibitor only after prolonged heating (1 hour or more) at 100°C.

Mechanism of inhibition. To determine whether the inhibitor in milk acted on the virus or on the red cells, an experiment was carried out in which RSM dilutions were incubated for varying periods with heated swine virus or with red cells before the second reagent (red cells or virus) was added. Except for this modification, the general set-up of the tests was like that of the routine inhibition titrations. In all cases readings were made one hour after the second reagent was added. The results, plotted in Fig. 1, show that, when RSM dilutions are first incubated with red cells, the inhibition titer is low and independent of time of incubation. In contrast, when RSM dilutions are first incubated with heated virus, the titer, initially low, rises to a high level as incubation is prolonged. This sort of result has been obtained also in similar experiments with egg-white[3] and saliva.[10] It may be concluded that the inhibitor in milk is effective through its capacity to combine with virus.

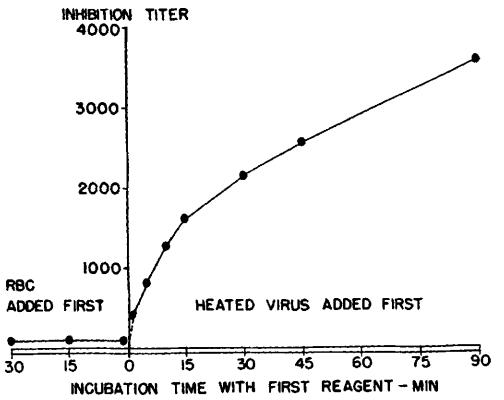

FIG. 1.

Inhibition titer as a function of the order of addition of chicken red blood cells and heated swine influenza virus to dilutions of raw skim milk and the time of incubation at 27°C with the reagent added first.

Comparison of heated and unheated virus. Francis[11] showed that the inhibitory effect of normal serum is greater against suitably heated than against unheated virus. This difference in susceptibility of heated and unheated virus to inhibition, explained in terms of a capacity of unheated virus to destroy inhibitor and the loss of such capacity on heating[1,2] has been observed also with milk. Thus, when unheated swine virus was incubated for varying periods with RSM dilutions before the addition of red cells, the conventional inhibition titer rose to about 64 after 5 minutes

[9] Lanni, F., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 116.

[10] Seltsam, J. H., Lanni, F., and Beard, J. W., *J. Immunol.*, in press.

[11] Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.

TABLE III. Evidence of Inhibitor Destruction by Unheated Influenza Virus A (PR8 Strain) and Lack of Destruction by Heated Virus.

Virus PR8	Incubation with whey,* min.	Total virus nitrogen present, γ								
		4	2	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Unheated	Control†	4	4	4	4	4	3/4—	3—/3	2—	0/1—	0
	1	4	4	3	3—	2—	0/1—	0	0	0	0
	7.5	4	4	3	2/3—	1	0/1—	0	0	0	0
	15	4	4	3/4—	2/3—	1	0/1—	0	0	0	0
	30	4	4	4—	3—	1	0/1—	0	0	0	0
	60	4	4	4	3	2—	0/1—	0	0	0	0
	120	4	4	4	4	3	1—	0	0	0	0
Heated‡	Control†	4	4	4	4	4	3/4—	2/3—	2	0/1—	0
	1	4	3	2/3—	2—	1	0	0	0	0	0
	7.5	4—	3—	2—/2	1	0/1—	0	0	0	0	0
	15	3/4—	2/3—	2—	0/1—	0	0	0	0	0	0
	30	3/4—	2	1	0/1—	0	0	0	0	0	0
	60	3	2	1—	0	0	0	0	0	0	0
	120	3	2—/2	0	0	0	0	0	0	0	0

* Final dilution of whey 1:20 in all tubes except controls.
† Saline substituted for whey.
‡ Heated for 30 min. at 53°C at a concentration of 8 γ N per ml.

of incubation, and then fell progressively, reaching the value 35 after 90 minutes of incubation. After 30 minutes of incubation, the titer was 48; the corresponding titer against heated virus (Fig. 1) was 2150, about 45 times as great. The decrease in titer with unheated virus after 5 minutes of incubation is interpreted as evidence of inhibitor destruction by virus.

Inhibitor destruction by virus. Further evidence of inhibitor destruction by virus is presented in Table III, which shows the results of an experiment carried out with whey and influenza virus A (PR8 strain), purified as previously described.[12] The whey was prepared by bringing RSM to pH 4.6 with N acetic acid, filtering off the precipitated casein, and neutralizing the filtrate to pH 7.0 with NaOH. Progressive dilutions of unheated virus were incubated in constant volume with a constant amount of whey for varying periods at room temperature before red cells were added. The results (Table III) show a slight initial increase in inhibition followed by a progressive decrease, manifested in the partial emergence of the hemagglutinating activity of the virus. Almost identical results were obtained with a partially purified fraction, prepared by half-saturating the whey with $(NH_4)_2SO_4$ (see below); and similar results were obtained with RSM and unheated swine influenza virus. The interesting inhibition optimum seen in Table III has been observed also with saliva[10] and has been explained as a consequence of two oppositely manifested reactions.

Table III shows also the results of a similar experiment carried out with whey and heated PR8 virus. With this virus the inhibition increases progressively as incubation with whey is prolonged, the results emphasizing the difference in behavior of heated and unheated virus noted above.

Partial purification of the inhibitor. Preliminary experiments directed at fractionation have shown that the milk inhibitor occurs in the whey and can be recovered almost quantitatively in the precipitate which forms

[12] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

TABLE IV.
Properties of Fractions Obtained from Raw Skim Milk (RSM).

Fraction (See text)	Inhibition titer*	Nitrogen,* γ per ml	Purification factor†	Activity partition, %
RSM	5,000	5,280	1.00	100
Casein	410	3,830	0.11	8.2
Whey	4,300	1,130	4.0	86
P50	4,200	213	21	84
P60	300	55	5.8	6.0
P100	24	359	0.07	0.5
S100	11	159	0.07	0.2

* Calculated to the original volume of RSM.
† Calculated from the titer-nitrogen ratio with reference to the titer-nitrogen ratio of RSM.

when neutral whey is half-saturated with $(NH_4)_2SO_4$. Less than 1% of the inhibitor is precipitated from neutral whey at 0.33 saturation and less than 10% at 0.4 saturation.‡

An example of the results of fractionation is given in Table IV. A volume of 10 ml N acetic acid was added slowly with vigorous stirring to 100 ml pooled RSM, the pH falling from 6.6 to 4.6. The precipitated casein was obtained by centrifugation, washed with 100 ml 1% NaCl, triturated with 2 ml 10% NaOH, taken up in 100 ml 1% NaCl, and neutralized with N acetic acid. The supernatant whey was neutralized with 2.5 N NaOH and refrigerated for 2 days, during which a slight precipitate formed. This precipitate, which was found to contain about 1% of the initial activity and of the initial nitrogen, was centrifuged off and discarded. The supernatant was brought successively to 0.5, 0.6, and 1.0 saturation with solid $(NH_4)_2SO_4$, added slowly with vigorous stirring. The precipitates, designated P50, P60, and P100, respectively, were taken up in H_2O and dialyzed exhaustively through Visking casing against repeated changes of buffered saline in the cold. The final supernatant at 1.0 saturation, designated S100, was dialyzed against running tap water overnight and then exhaustively against buffered saline in the cold. All of the fractions were analyzed for inhibitor by the routine method and for nitrogen by direct nesslerization.[14] The results show that the inhibitor can be purified about 20 times, with excellent recovery, by this method. These experiments are being continued.

Discussion. The non-dialyzability of the inhibitor and its precipitability by salt suggest that the inhibitor is itself a high molecular weight substance, presumably a protein, or is associated with such a substance.

In its reactions with virus, the milk inhibitor displays the characteristic features of other recently discovered inhibitors, such as those occurring in normal serum, normal allantoic fluid, and egg-white. Thus the inhibitor is more effective against heated than against unheated virus and appears to be susceptible to inactivation by the latter. For this reason, and since all samples of raw milk which have been tested possess roughly the same inhibitory activity, it may be concluded that the inhibitor is a normal component of milk rather than a specific antibody produced in response to casual antigenic stimulation. Experiments now in progress in this laboratory indicate that both the milk and the egg-white inhibitors can be distinguished readily in inhibition experiments from the specific antibody which occurs in convalescent anti-influenza swine serum. For example, antibody is equally effective against heated and unheated viruses, while the other inhibitors are considerably more effective against heated virus. Moreover, the gradient of inhibition in the end-point region is much steeper with antibody than with milk or egg-white.

In view of the reported relation between other naturally occurring inhibitors and mucoproteins,[1,2,4,6,8] it is interesting to note that a mucoprotein, designated *lactomucin*, has

‡ A solution containing 7.857 equivalents of $(NH_4)_2SO_4$ per liter is regarded as a saturated solution.[13]

[14] Lanni, F., Dillon, M. L., and Beard, J. W., submitted for publication.

been isolated from milk[13] and that the reported solubility properties of this substance are roughly those of the inhibitor. The relation between the milk inhibitor and lactomucin is being investigated.

Summary. Milk is capable of inhibiting hemagglutination by influenza viruses, the inhibitory effect being greater against heated than against unheated viruses. The inhibitor, which is non-dialyzable and moderately heat stable, occurs in the whey and can be salted out by half-saturation with ammonium sulfate. Evidence is presented that the inhibitor is a characteristic component of milk rather than a specific antibody.

[13] Sorensen, M., and Sorensen, S. P. L., *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.*, 1938-41, **23**, 55.

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Effect of Dicumarol on Concentration of the Labile Factor.* (17390)

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The observation that the loss of prothrombin activity in stored human plasma could be restored by adding to it rabbit or dog plasma which was markedly depleted of prothrombin by means of dicumarol, led to the discovery of the labile factor and to the assumption that this agent is not affected by dicumarol.[1] The development of a quantitative procedure for estimating the labile factor in blood,[2] now makes it possible to test the validity of this assumption.

Experimental. Rabbits and dogs were given dicumarol orally (5 mg per kilo body weight daily) until the prothrombin time became markedly prolonged. The prothrombin activity was determined by the original one-stage procedure.[3] For the estimation of the concentration of the labile factor, the method of Quick and Stefanini[2] was employed. The method consists essentially in determining the prothrombin time of stored plasma after the addition of a measured amount of the plasma to be tested which has been deprothrombinized by adsorption with tricalcium phosphate. The reduction of the prothrombin time is a measure of the concentration of the labile factor.

Results. From the data in Table I it can be seen that the concentration of the labile factor in normal rabbit plasma is remarkably constant. This is also true of dog plasma, but the concentration is only one-fifth that of rabbit plasma.[2] When dicumarol is administered the prothrombin activity as measured by prothrombin time decreases progressively and after one week may reach a level below one per cent of normal (Table II). In spite of this drastic decrease, the concentration of the labile factor shows no greater fluctuation than may be attributed to the inherent variations of the method. It can be concluded that dicumarol does not influence the labile factor in rabbits and dogs. Preliminary studies indicate that this is also true in humans.

Owren,[4] Fantl and Nance[5] and Seegers and Ware[6] agree at least tentatively that their respective agents, factor V, accelerator factor and Ac-globulin are the same as the labile factor. The finding of Fahey, Olwin and Ware[7] that dicumarol causes at least a moderate decrease in Ac-globulin in dogs is not in agreement with the present results. It is likely that the difference in findings can

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[1] Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

[2] Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1948, **33**, 819.

[3] Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.

[4] Owren, P. A., *Biochem. J.*, 1948, **43**, 136.

[5] Fantl, P., and Nance, M. H., *Med. J. Australia*, 1948, **1**, 98.

[6] Seegers, W. H., and Ware, A. G., *Am. J. Clin. Path.*, 1949, **19**, 441.

[7] Fahey, J. L., Olwin, J. N., and Ware, A. G., PROC. SOC. EXP. BIOL. AND MED., 1948. **69**, 491.