LXV. THE RATE OF INACTIVATION BY HEAT OF PEROXIDASE IN MILK. I.

BY SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute.

(Received Nov. 16th, 1914.)

The presence of peroxidase in fresh cow's milk can be detected by the usual reagents for that enzyme. Hydrogen peroxide and *p*-phenylenediamine are considered the most sensitive reagents for the purpose and are mostly employed in testing milk for peroxidase (Storch's reaction). A dark blue-grey colour is imparted almost instantaneously to the milk if the reaction is positive.

That peroxidase is an actual constituent of the milk, and not due to bacterial contamination, is evident from the fact that initially sterile cow's milk, or milk rendered sterile directly after milking by antiseptics like chloroform or toluene, gives a positive reaction. On the other hand, bacterial cultures from the most common organisms in milk fail to react for peroxidase [Lane-Claypon, 1913, p. 11].

Everything seems to point to the fact that the enzyme is associated with the albumin fraction of the milk. Julius Meyer [1910] showed that when caseinogen is brought down by means of trichloroacetic acid, acetic acid, natural acidity or calcium chloride, and properly washed, it does not react for peroxidase, while casein brought down by means of rennet gives but a faint reaction. Again, when milk is centrifuged the enzyme remains in the fluid and does not go up with the cream [Lane-Claypon, 1913, p. 12]. Yet when the precipitate brought down from whey by saturating it with ammonium sulphate is redissolved, the solution reacts very intensely for peroxidase [Lane-Claypon, 1913, p. 10].

Like most enzymes, peroxidase can be inactivated by heat, or at least, when the milk is heated beyond certain temperatures for some time no perceptible reaction is manifested when the usual reagents are employed.

This fact Storch found could be utilised as a reliable test for detecting whether the milk had been heated to 80° C.

Van Eck [1911], investigating the process of inactivation of peroxidase in milk by heat, found that it proceeds as a reaction of the first order and, making use of the formula for a unimolecular reaction, was able to obtain a constant. This was confirmed by the author, who further investigated the matter with the view of ascertaining whether the peroxidase reaction in milk could be employed with any certainty as a test for pasteurisation. The temperature coefficient for the inactivation of peroxidase in milk and the influence of acidity and alkalinity and neutral salts on the rate of inactivation were studied.

EXPERIMENTAL METHOD.

The technique employed was very similar to that used by van Eck. There is no method for the determination of the absolute quantity of peroxidase, and therefore the amount of the enzyme present in the samples was determined relative to that of the raw milk used in the experiment, and expressed as a percentage of the same. It was carried out colorimetrically. For the standard scale 38 test tubes were used, each containing from 0.1 cc. to 5 cc. of raw milk and made up to 10 cc. with boiled milk. The experimental tubes contained 5 cc. of the sample under investigation, and 5 cc. of boiled milk. To each of the tubes, both experimental and scale, 0.3 cc. of 1 % hydrogen peroxide and 0.25 cc. of 2 % *p*-phenylenediamine were added. Half an hour was allowed to elapse between the addition of the reagent and the comparison of the samples with the scale. After that period no perceptible change could be discerned to take place in the shades of the tubes during the time of the estimation. A fresh scale was made up for each experiment and the raw milk employed invariably was of the same sample as the milk used for investigation. New solutions of hydrogen peroxide and *p*-phenylenediamine were also made up every time, for the former is very unstable and the latter, like guaiacum tincture, after remaining for some time in solution, will react with milk without the addition of hydrogen peroxide. The experimental error of this method varies from 5 % when the peroxidase content is high to 2 % when the amount of peroxidase to be estimated is low.

The milk for investigation was heated in a beaker covered with a cork lid, through which passed a thermometer graduated in tenths of a degree and a stirrer, and which was provided with an opening for the removal of

the samples. The beaker was placed in an asbestos-jacketed water-bath, having a layer of liquid paraffin on the surface to diminish the evaporation, and regulated by a toluene regulator. Like the beaker, the bath contained a stirrer driven by a water-motor. In starting the experiment the milk in the beaker was quickly brought up to the desired temperature in a boiling water-bath, and then without loss of time placed in the regulated bath and the stirrers set going. As soon as the temperature in the beaker became constant the first sample was taken. The taking of the samples was then repeated after the desired intervals. In removing the samples warmed pipettes were used in order not to cool down the milk in the beaker. The test tubes containing the freshly withdrawn samples were immediately placed in cooled water so that the temperature of the milk was rapidly brought down. The entire procedure of removing the samples was carried out regularly in ten seconds.

DETERMINATION OF THE TEMPERATURE COEFFICIENT.

In order to determine the temperature coefficient of the rate of inactivation by heat of the peroxidase in milk, it was necessary to ascertain the constants for several temperatures from which the coefficients could be calculated. Preliminary experiments showed that heating of milk at 65° C. for four hours made hardly any perceptible impression on the peroxidase content. On the other hand, at 72° C. the inactivation of the enzyme proceeded at a rate much too fast for any reliable results to be recorded. Temperatures of 71°, 70° and 69° were found to be very convenient and were accordingly chosen for the determination of the constants. The following tables give the results obtained for these three temperatures.

TABLE I.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.005066$
0	69.05° C.	63	77
20	68.95	61	61
40	68.9	49	48.3
60	68.9	37	38.2
80	68.9	31	30.3
101	68.9	25	24
120	68.85	19	19
140	68.85	17	15

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.005066 \text{ where } t = 100 \text{ mins., } C_1 = 61, C_2 = 19$$

TABLE II.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of per-oxidase calculated from $K=0.01333$
0	70.25° C.	61	55.7
10	70.1	41	41
20	70.1	30	30.1
50	70.07	12.5	12
60	70.05	9.5	8.8
70	70.05	6.5	6.5
80	70.05	5.5	4.8

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.01333 \text{ where } t=60 \text{ mins., } C_1=41, C_2=6.5.$$

TABLE III.

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of per-oxidase calculated from $K=0.02698$
0	71.15° C.	37	35.5
5	71.08	26	26
10	71.05	18	19.1
15	71	13	13.9
20	70.98	9.5	10.2
25	70.98	8	7.5
30	70.9	5.5	5.5
35	70.9	4.5	4

$$K = \frac{1}{t} \log \frac{C_1}{C_2} = 0.02698 \text{ where } t=25 \text{ mins., } C_1=26, C_2=5.5.$$

The last column in these tables gives the percentage of peroxidase calculated from the constant (K) obtained from the equation of a unimolecular reaction. The first and last readings were not utilised for calculating the constant, as the possibility of error was more likely in these cases than in any other. In the first sample the temperature is usually not quite constant and the high content of the enzyme makes accurate estimation harder than in the other samples, while in the last sample the experimental error of the colorimetric method is too significant in comparison with the low peroxidase content to be determined. The second reading and the last but one were therefore employed. Considering that the method which was employed for the estimation of the enzyme cannot claim a very high degree of accuracy, the figures calculated from the constants agree well with the figures actually obtained by experiment and this shows clearly that the inactivation of the enzyme proceeds as a reaction of the first order. It is also to be observed that constants obtained by calculating from different data in the tables gave results which approximated to those given above. The error in

the constant is probably between 5–10 %. Fig. 1 gives a graphic representation of the rates of inactivation at the three temperatures.

Fig. 1.

Calculating the temperature coefficient from the three constants, we obtain the following figures:

TABLE IV.

Temperature	Constant	Difference in temperature in degrees	Temperature coefficient for one degree
68.9° C.	0.005066	—	—
70.075	0.01333	1.175	2.278
70.95	0.02698	0.875	2.18
70.95	0.02698	2.05	2.236
Mean temperature coefficient	..	..	2.231

The mean temperature coefficient is therefore 2.23 per degree. This high value is of the same order as that found by Famulener and Madsen [1908] for the inactivation of tetanolysin and vibriolysin nasik by hot water. For the former the authors obtained the value of 2.4 and for the latter a value

a little under 2 for one degree. Chick and Martin [1910] also found the temperature coefficient for the coagulation of egg albumin by heat to be equal to 1.9 per degree.

The results of Tables I, II and III moreover show the impossibility of applying the peroxidase reaction as a direct test for pasteurisation. The term "pasteurisation" has a very broad application as to the temperature and time of heating the milk. It is not uncommon in some dairies in "pasteurising" to heat the milk up to 80° C. (176° F.), but according to the definition laid down by the Department of Health of the City of New York, pasteurisation consists in heating the milk at not less than

158° F. (70° C.)	for at least	3 mins.
155° F. (68.3° C.)	"	5 "
152° F. (66.7° C.)	"	10 "
148° F. (64.4° C.)	"	15 "
145° F. (62.8° C.)	"	18 "
140° F. (60° C.)	"	20 "

From the constant obtained above for 70° C. it can be calculated that 91.2 % of the original peroxidase would remain after heating the milk for 3 minutes at 158° F. (70° C.). Such an amount of peroxidase could be differentiated with difficulty by the colorimetric method from the full amount of 100 %, even if the original sample of raw milk could be used for the scale. In testing milk, however, for pasteurisation the original raw milk is never available and although different samples of raw milk differ apparently but little in intensity in the peroxidase reaction, the possibility of small variations is by no means excluded. This would make the differentiation still more uncertain. As regards the lower temperature, 20 minutes heating at 60° C. would make no perceptible impression, since, as we have seen, 4 hours at 65° C. makes hardly any difference to the peroxidase content of the milk. It is then evident that the peroxidase reaction cannot be applied at all as a test for pasteurisation. It may, however, serve as an indication whether "pasteurised" milk has been overheated.

TABLE V.

Temperature	Constant	Time in mins. necessary to bring peroxidase down to 10 %	Time in mins. necessary to bring peroxidase down to 50 %
70° C.	0.01333	75	22.5
71	0.02973	33	10.3
72	0.06634	15	4.6
73	0.14802	6.8	2
74	0.33022	3	under a minute
75	0.73673	1.3	" "
80	40.72004	under a minute	" "

Table V gives the periods of time for which the milk must be heated at different temperatures between 70° C. and 80° C. in order to bring down the peroxidase content to 10 % and 50 % respectively. The calculations are made on the assumption that the temperature coefficient remains the same as the temperature rises and the constants in the table are obtained on that assumption. It is clear from the figures that at temperatures beyond 71° C. very short periods of heating produce well marked impressions on the peroxidase content of milk.

INFLUENCE OF ACIDITY AND ALKALINITY ON THE RATE OF INACTIVATION OF PEROXIDASE IN MILK.

Experiments were instituted with the object of studying the effect of acidity and alkalinity on the rate of inactivation of peroxidase in milk. The H ion concentration of the milk was determined by the gas chain method. Unfortunately the determination of H ion concentration in a medium like milk is fraught with many technical difficulties, and the results obtained, though definitely showing the general effect of acidity and alkalinity, are not exact enough for publication in detail. The result arrived at is that a small increase in acidity retards and in alkalinity accelerates the rate of inactivation. Thus the addition of such a quantity of lactic acid as changed the P_H^+ of the milk from about 6.57 to 5.75 brought down the constant at 70° C. from 0.01333 to 0.01086, while the addition of potash which changed the P_H^+ to about 8.26 increased the rate of inactivation at the same temperature to such an extent that the peroxidase content of the milk came down to 2 % in the preliminary heating.

INFLUENCE OF SALTS ON THE RATE OF INACTIVATION OF PEROXIDASE IN MILK.

It was observed by Chick and Martin [1911] that the addition of sodium chloride and ammonium sulphate retarded the rate of coagulation of egg-albumin by heat. It was therefore desirable to ascertain the influence of salts on the rate of inactivation of peroxidase in milk by the same agency. From preliminary experiments it was observed that the addition of sodium chloride greatly retarded the rate of inactivation. In milk made up to the concentration of a N/1 NaCl solution the inactivation of the peroxidase at 70° C. was brought down to a rate too slow for convenient observation, and therefore N/10 and N/20 solutions were used in the actual experiments. The addition of salts alters the tone of the colouration produced by the

peroxidase reaction and salt was therefore added to the scale tubes in the same proportion as to the sample under investigation. Tables VI, VII and VIII illustrate the action of sodium chloride and Fig. 2 shows the same results graphically.

TABLE VI.

(N/10 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.002063$
0	69.9° C.	72.5	72.5
30	69.9	59	62.9
90	69.9	48	47.4
120	69.9	42	41.1
150	69.9	36	35.6
180	69.9	32	30.9

TABLE VII.

(N/20 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.003995$
0	70.2	72.5	72.5
31	70.15	50	54.5
60	70.1	41	41.8
90	70	31	31.7
120	70	25	24.1
150	70	20	18.25

TABLE VIII.

(Normal Milk, no salt added.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.01721$
0	69.9	62.5	62.5
5	70.0	50	51.3
10	70.0	40	42
25	70.1	24	23.2
40	70.1	14	12.8
55	70.2	8	7
70	70.2	4	3.9

Calculating $K = \frac{1}{t} \log \frac{C_1}{C_2}$ from different points on the curves in Fig. 2 we get the following mean coefficients for the corresponding concentrations:

N/10 NaCl, $K=0.002063$.N/20 NaCl, $K=0.003995$.Normal Milk, $K=0.01721$.

It is seen from the above figures that the concentration of N/20 salt reduces the rate to about one quarter and N/10 to about one eighth. The constant for the normal milk is rather higher than the one usually obtained for a similar temperature, but the same sample of milk was used for the experiments with the N/10 NaCl and N/20 NaCl and, consequently, these results would also be proportionately higher.

Fig. 2.

The next thing to ascertain was whether other salts had a retarding effect on the rate of inactivation, and if so whether that influence was specific or otherwise. The number of different salts used had to be restricted as some, even in such dilutions as N/20, bring down the caseinogen at 70° C. immediately, or after some time. In all the cases tried the addition of the salts, in concentration of N/20, had a marked retarding effect, although the extent of that influence varied in most cases. The influence of some of the salts is shown by the results in the following tables. Fig. 3 gives curves expressing these results as well as those in Tables VII and VIII.

TABLE IX.

(N/20 KCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00526$
0	70.4° C.	65	65
15	70.5	56	54.2
30	70.5	51	45.2
45	70.5	38	37.7
60	70.5	29	31.4
75	70.5	24	26.2
90	70.5	20	21.8

TABLE X.

(N/20 Na_2SO_4 .)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00546$
0	70.35° C.	77.5	75.5
15	70.32	62.5	62.5
30	70.35	53	51.8
45	70.3	42	42.9
60	70.3	33	35.5
75	70.3	28	29.4
90	70.3	20	24.3

TABLE XI.

(N/20 KNO_3 .)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00981$
0	70.2° C.	72.5	82.8
15	70.4	59	59
30	70.35	40	42
45	70.4	28	30
60	70.4	20	21.35
75	70.35	16	15.2
90	70.4	10	10.8

Calculating the constants from the curves of Fig. 3 we get the following figures:

N/20 NaCl $K=0.00399$.

N/20 KCl $K=0.00526$.

N/20 Na_2SO_4 $K=0.00546$.

N/20 KNO_3 $K=0.00981$.

Normal milk $K=0.0225$.

The temperature for the normal milk and the N/20 NaCl was slightly lower than for the remainder. The constant of the normal milk has been calculated for the right temperature with the help of the temperature coefficient. The temperature coefficient for N/20 NaCl has not been deter-

Fig. 3.

mined and consequently no correction could be made for that constant. The results seem to point to the fact that the influence is specific and is not dependent solely on the valency of the ions. KNO_3 and KCl contain monovalent kations and anions, but their constants differ considerably. On the other hand, KCl and Na_2SO_4 contain anions of different valency—the former monovalent and the latter divalent—yet their constants are practically the same.

THE INFLUENCE OF SALTS ON THE RATE OF INACTIVATION OF
PEROXIDASE IN WHEY.

The possibility that the salts act on the caseinogen, which then in its altered state influences the rate of inactivation of the peroxidase, was not excluded by the foregoing results and accordingly the effect of the addition of salts to whey was investigated. The whey was prepared by curdling the milk with rennet and filtering it through a cheese-cloth. The experiments were then carried out in the same way as with the milk. The inactivation of the peroxidase in the whey was also observed to proceed as a reaction of the first order but more rapidly than in the milk at the same temperature. The intensity of the peroxidase reaction in the whey was stronger than in the milk, and it was found convenient to dilute it (1 part whey with 3 parts boiled distilled water).

TABLE XII.

(Diluted Whey, no addition of Salt.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.01485$
0	69.2° C.	72.5	72.5
15	69.35	47	43.4
30	69.3	28	26
45	69.3	14	15.6
60	69.3	9	9.3
75	69.3	5	5.5

TABLE XIII.

(Diluted Whey and N/20 NaCl.)

Time of heating in minutes	Temperature of milk	Percent. of peroxidase	Percent. of peroxidase calculated from $K=0.00344$
0	69.3° C.	72.5	72.5
15	69.3	62.5	64.3
30	69.3	55	57.2
45	69.3	50	50.8
60	69.3	42	45.1
75	69.3	40	40
90	69.3	38	35.5

Tables XII and XIII and Fig. 4 show the influence of N/20 NaCl on the diluted whey. As with milk, the retarding effect is well marked. N/20 KNO₃ was also found to produce a retarding effect.

Whatever the nature of the action of the salts may be, it is thus seen to be independent of the presence of the caseinogen and fat. Further investigation, which is now in progress, is needed in order to ascertain the nature of that action, and whether the enzyme is influenced directly or indirectly by the presence of the salts.

Fig. 4.

SUMMARY.

1. The temperature coefficient for the inactivation of peroxidase in milk by heat is found to be 2.23 per degree centigrade, a value of about the same order as the temperature coefficients for the inactivation by heat of principles like tetanolysin and vibriolysin nasik and for the rate of coagulation of egg-albumin.

2. The rate of inactivation of peroxidase in milk below 70° C. is so small that it does not afford the opportunity of utilising the peroxidase reaction

as a test for pasteurisation. The reaction may, however, indicate whether "pasteurised" milk has been overheated.

3. Small additions of acid retard and of alkali accelerate the rate of inactivation by heat of peroxidase in milk.

4. The presence of salts retards in a pronounced way the inactivation rate. This retardation varies with different salts and is independent of the valencies of their ions.

5. As in milk, the inactivation of peroxidase in whey proceeds as a reaction of the first order.

6. In whey also the presence of salts has a retarding influence on the rate of the enzyme.

In conclusion I wish to express my indebtedness to Professor Harden, F.R.S., for the valuable advice and assistance rendered during this investigation, and to Dr Onodera for helping me with some of the latter part of the experimental work.

REFERENCES.

- Chick and Martin (1910). *J. Physiol.*, **40**, 404.
— (1911). *J. Physiol.*, **43**, 1.
Van Eck (1911). *Zeitsch. Nahr. Genussm.*, **22**, 393.
Famulener and Madsen (1908). *Biochem. Zeitsch.*, **11**, 186.
Lane-Claypon, J. (1913). *Report to Local Gov. Board upon the Biological Properties of Milk*, etc. (where the literature is quoted).
Meyer, J. (1910). *Arbeit. kaiserl. Gesundheitsamt*, **34**, 115.