CXXXIII. THE EFFECT OF HALOGEN SALTS ON THE CLOTTING OF MILK BY TRYPSIN.

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WHILST working on the effect of halogen salts on steaptic digestion [Clifford, 1934], it was noticed that many of them enabled trypsin to coagulate milk. Since such a function is generally ascribed to Ca alone, it was decided to carry out further experiments.

Mellanby [1912] investigated the coagulation of milk by trypsin but added CaCl_2 in nearly all his experiments; he also compared the action of the latter with those of MgCl_2 and BaCl_2 , finding that they also increased the milk clotting activity, though in a less degree than did the Ca salt.

In the same year Mellanby and Woolley [1912] stated that enzymes acting in an alkaline medium, *e.g.* trypsin, need extra Ca to produce typical coagula in milk.

This idea was followed up by Seth [1924] who used CaCl_2 -milk to investigate tryptic activity, and still later Kleiner and Tauber [1934, 1, 2] found the clotting of CaCl_2 -milk to be a function of low concentrations of crystalline trypsin. They did not try the effect of adding any other salt.

Experiments were therefore made to determine the effect of various halogen salts on tryptic clotting.

EXPERIMENTAL.

The method used was that described in a previous paper [Clifford, 1927] on the effects of halogen salts on peptic milk clotting.

10 ml. of milk and 2 ml. of water, or halogen salt solution of the requisite concentration, were warmed to 37° . To this mixture was added 1 ml. of a 2 % solution of pancreas substance. The tube was inverted to mix, and the time of clotting was determined by means of a stop-watch.

An average of the times of clotting of six tubes was taken for each concentration of salt used, and in no set of six was the difference greater than 10 seconds.

Three separate batches of commercial pancreas substance (2 powdered and 1 liquid) and at least two separate samples of each halogen salt were used in the experiments described.

The amount of halogen salt varied from 2 ml. of 3.24 *M* to 2 ml. of 0.05 *M*, each dilution being twice that of the previous one. Since the total volume in a tube was 13 ml. the concentrations of salt in the tubes varied from 0.5 to 0.008 *M*. With the alkaline earth halogen salts the dilutions were continued down to 0.001 *M*.

RESULTS.

No firm coagulum was ever obtained on the addition of 1 ml. of 2 % pancreas substance to 10 ml. of milk and 2 ml. of water, and in practically every case the appearance of the mixture remained unaltered. With three samples of milk there was a fine granulation in the tube after keeping it at 37° for 30 minutes but never a definite coagulum. Probably these three samples of milk had a high content of calcium, chloride, or both.

Fluorides (Table I). The fluorides of Na, K and NH_4 at higher concentrations of salt (0.5–0.06 *M*) gave a definite coagulum with trypsin and milk. The acidity of the ammonium salt was sufficient to curdle the milk, and therefore, before using it in an experiment it was neutralised with anhydrous sodium carbonate.

Table I. *Fluorides*.

Concentration <i>M</i>	Time to produce clot		
	Na	K	NH_4
0.5	—	1 min. 41 secs.	4 mins. 13 secs.
0.25	—	2 mins. 55	4 10
0.125	4 mins. 18 secs.	3 48	4 10
0.06	4 27	4 37	5 18
0.03	No clot in 45 mins.	No clot in 1 hr. 10 mins.	No clot in 1 hr. 5 mins.
0.015	"	"	"
0.008	"	"	"
Standard	No clot in 1½ hrs.	No clot in 1 hr. 18 mins.	No clot in 2 hrs.

Below a concentration of 0.06 *M* the three fluorides did not give rise to a clot with milk and trypsin.

Chlorides (Table II). Li, Na, K and NH_4 chlorides all cause trypsin to coagulate milk, when added in concentrations of 0.5–0.008 *M*. With the chlorides of Mg, Ca and Ba, similar results were obtained, but the clotting time was shortest between 0.06 and 0.015 *M*; above and below these limits the action was marked, but less powerful.

Table II. *Chlorides*.

Concentration <i>M</i>	Time to produce clot			
	Li	Na	K	NH_4
0.5	1 min. 59 secs.	1 min. 26 secs.	1 min. 7 secs.	1 min. 1 sec.
0.25	1 54	1 17	1 8	0 53 secs.
0.125	1 53	1 25	1 13	0 57
0.06	2 mins. 14	1 44	1 29	1 12
0.03	2 53	2 mins. 15	2 mins. 8	1 33
0.015	3 52	2 57	2 59	2 mins. 22
0.008	5 2	4 27	4 29	4 38
Standard	No clot in 1 hr. 30 mins.	No clot in 2 hrs.	No clot in 1 hr. 30 mins.	No clot in 1 hr. 20 mins.

Concentration <i>M</i>	Time to produce clot		
	Mg	Ca	Ba
0.5	1 min. 38 secs.	1 min. 17 secs.	—
0.25	1 4	0 55	—
0.125	0 48	0 34	0 min. 53 secs.
0.06	0 38	0 26	0 37
0.03	0 38	0 26	0 29
0.015	0 49	0 36	0 37
0.008	1 8	0 57	0 58
0.004	1 37	1 24	1 25
0.002	2 mins. 25	2 mins. 5	2 mins. 20
0.001	4 10	3 40	4 21
Standard	No clot in 2 hrs.	No clot in 1 hr. 30 mins.	No clot in 1 hr. 45 mins.

The alkaline earth chlorides showed greater power to aid coagulation than did those of Li, Na, K and NH_4 , but not to the extent previously noted with peptic clotting [Clifford, 1927].

There was no great difference between members of either group of chlorides in their capacity to aid tryptic coagulation, though MgCl_2 was rather less powerful in its action than BaCl_2 or CaCl_2 .

 Table III. *Bromides.*

Concentration <i>M</i>	Time to produce clot			
	Li	Na	K	NH_4
0.5	1 min. 2 secs.	1 min. 12 secs.	2 mins. 0 sec.	2 mins. 33 secs.
0.25	0 59	1 24	1 min. 59 secs.	2 5
0.125	1 10	1 34	2 mins. 23	2 3
0.06	1 25	1 53	2 52	2 25
0.03	1 56	2 mins. 29	3 54	2 59
0.015	2 mins. 58	4 37	5 1 sec.	3 48
0.008	No clot in 43 mins.	No clot in 40 mins.	6 3 secs.	4 45
Standard	No clot in 3 hrs.	No clot in 1 hr. 30 mins.	No clot in 2 hrs.	No clot in 1 hr. 15 mins.

Concentration <i>M</i>	Time to produce clot		
	Mg	Ca	Ba
0.5	5 mins. 4 secs.	1 min. 44 secs.	—
0.25	2 6	1 10	1 min. 10 secs.
0.125	1 min. 26	0 46	0 54
0.06	1 9	0 33	0 40
0.03	1 10	0 32	0 35
0.015	1 23	0 42	0 44
0.008	1 55	1 9	1 0 sec.
0.004	2 mins. 35	1 41	1 34 secs.
0.002	3 55	2 mins. 31	2 mins. 17
0.001	6 8	8 1 sec.	3 51
Standard	No clot in 2 hrs.	No clot in 2 hrs. 45 mins.	No clot in 2 hrs.

 Table IV. *Iodides.*

Concentration <i>M</i>	Time to produce clot			
	Li	Na	K	NH_4
0.5	2 mins. 49 secs.	1 min. 30 secs.	1 min. 12 secs.	2 mins. 8 secs.
0.25	2 0 sec.	1 11	1 13	1 min. 17
0.125	2 17 secs.	1 22	1 24	1 30
0.06	2 47	1 42	1 48	1 49
0.03	3 22	2 mins. 19	2 mins. 37	2 mins. 16
0.015	4 25	3 28	4 15	3 15
0.008	No clot in 50 mins.	No clot in 1 hr.	No clot in 1 hr. 30 mins.	No clot in 35 mins.
Standard	No clot in 1 hr. 15 mins.	No clot in 2 hrs. 30 mins.	No clot in 2 hrs. 30 mins.	No clot in 1 hr. 30 mins.

Concentration <i>M</i>	Time to produce clot		
	Mg	Ca	Ba
0.5	1 min. 58 secs.	1 min. 32 secs.	1 min. 57 secs.
0.25	1 51	1 2	1 3
0.125	0 46	0 44	0 46
0.06	0 49	0 36	0 40
0.03	1 3	0 39	0 38
0.015	1 26	0 52	0 47
0.008	2 mins. 11	1 15	1 2
0.004	3 0 sec.	1 56	1 38
0.002	4 23 secs.	3 mins. 10	2 mins. 40
0.001	No clot in 30 mins.	No clot in 45 mins.	No clot in 1 hr. 25 mins.
Standard	No clot in 1 hr. 8 mins.	No clot in 2 hrs. 40 mins.	No clot in 1 hr. 5 mins.

Bromides (Table III). The results are similar to those obtained with chlorides, except that Li and Na bromides were inactive at a concentration of 0.008 *M*.

Again the salts of Mg, Ca and Ba were less powerful at the highest concentration (0.5 *M*) and at the lower concentrations (0.008–0.001 *M*), and MgBr₂ gave a less marked effect than CaBr₂ or BaBr₂.

Iodides (Table IV). Iodides also show a marked effect in enabling trypsin to clot milk. This action is not quite so powerful as that of the chlorides and bromides, since the salts of Li, Na, K and NH₄ show no effect at 0.008 *M*, and those of Mg, Ca and Ba are inactive at 0.001 *M*.

The slight inhibitory action of the high concentrations of the Mg, Ca and Ba salts is again marked, together with the less effective action of MgI₂ as compared with the other two iodides.

DISCUSSION.

All the halogen salts investigated enable trypsin to coagulate milk, irrespective of the cation. The reaction was least marked with the fluorides which showed no effect at concentrations below 0.06 *M*. The chlorides and bromides of the alkaline earths caused trypsin and milk to form a coagulum in a concentration as low as 0.001 *M*, but the iodides needed a concentration of 0.002 *M*. At the highest concentrations used (0.5 *M*) the coagulating aid of the Li, Na, K and NH₄ halogen salts was as powerful as that of Mg, Ca and Ba, but the latter were more effective at concentrations below 0.125 *M*.

From these results it seems that the acceleration of tryptic activity in clotting milk so often noticed when CaCl₂ is added is quite as much a function of the Cl as of the Ca, and that the addition of any other soluble halogen salt would be equally effective over a wide range of concentrations.

This accords with the theory of Kleiner and Tauber [1934, 2] who state that the clotting of milk by trypsin is due to a depression of proteolytic action, thus allowing the casein stage to show, since in a previous paper [Clifford, 1933] it has been shown that the halogen salts of Li, Na and K tend to depress the proteolytic action of trypsin.

This is not, however, a full explanation, since the fluorides are the greatest depressors of proteolytic action and are at the same time the least active in promoting milk coagulation, and the concentration of bromides at which no appreciable effect on proteolytic activity can be seen is considerably higher than that at which no effect on milk coagulation is demonstrable.

SUMMARY.

1. Milk does not give a firm clot with trypsin.
2. If the halogen salts of Li, Na, K, NH₄, Mg, Ca or Ba be added to milk it gives a firm clot with trypsin.
3. This coagulating action is least with fluorides (ceases at 0.03 *M*).
4. The halogen salts of the alkaline earths are only slightly more efficient than those of Li, Na, K and NH₄.
5. At concentrations above 0.25 *M* the halogen salts of Mg, Ca and Ba are less effective than at concentrations between 0.125 and 0.015 *M*.
6. The Mg salts are less powerful than those of Ca or Ba in promoting the clotting of milk by trypsin.

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REFERENCES.

- Clifford (1927). *Biochem. J.* **21**, 544.
 — (1933). *Biochem. J.* **27**, 326.
 — (1934). *Biochem. J.* **28**, 418.
 Kleiner and Tauber (1934, 1). *J. Biol. Chem.* **104**, 266.
 — — (1934, 2). *J. Biol. Chem.* **104**, 271.
 Mellanby (1912). *J. Physiol.* **45**, 345.
 — and Woolley (1912). *J. Physiol.* **45**, 370.
 Seth (1924). *Biochem. J.* **18**, 1403.