THE EFFECT OF CALCIUM AND OTHER IONS ON THE AUTOCATALYTIC FORMATION OF TRYPSIN FROM TRYPSINOGEN

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The effect of salts upon the formation of trypsin from its inactive precursor, trypsinogen, has been extensively studied. Delezenne (1905), Zunz (1906, 1907), Ayrton (1909), Meyer (1910), and Wohlgemuth (1912) showed that the spontaneous activation of fresh pancreatic juice or extracts of fresh pancreas was hastened upon the addition of calcium or other alkaline earth salts, the former being most effective. De Sonza (1908) and Mellanby and Woolley (1913) considered that the accelerating action of calcium salts was due to neutralization since the amount of calcium chloride required corresponded closely with that necessary to precipitate the carbonate of the pancreatic juice. They found that barium and strontium chloride or neutralization with hydrochloric acid was equally as effective as calcium chloride. Waldschmidt-Leitz (1924), on the other hand, was unable to show any increase in spontaneous activation with calcium salts. More recently Farber and Wynne (1935) found that the activity of impure pancreatic proteinase was definitely stimulated by calcium salts but thought it possible that this increase in activity was due to the removal of inhibitors. The contradictory results found in the literature are undoubtedly due both to the impure materials and to the diversity of the conditions used. This paper is a summary of an extensive study[1] of the effect of salts on the autocatalytic formation of trypsin from purified crystalline trypsinogen.

Crystalline trypsinogen and trypsin have been isolated from beef pancreas by Kunitz and Northrop (1934, 1936). They found that a solution of crystalline trypsinogen containing a trace of trypsin was gradually changed into trypsin in the pH range of 5.0 to 9.0. The reaction, however, was incomplete.

[1] Dissertation submitted by Margaret R. McDonald to the Graduate Faculty of Rutgers University, June, 1940, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

This was found by Kunitz (1939) to be due to another reaction occurring during the process of formation of trypsin, namely the transformation of part of the trypsinogen into an inert protein which could not be changed into trypsin by any of the known means. The experimental curves for the rate of formation of trypsin as well as for the rate of formation of inert protein were found to be symmetrical S-shaped curves closely resembling those of simple autocatalytic reactions. The kinetics of the formation of trypsin and of inert protein from trypsinogen could be explained quantitatively on the theoretical assumptions that both reactions were of the simple unimolecular type, that in each case the reaction was catalyzed by trypsin, and that the rate of formation of each of the products was proportional to the concentration of trypsin and to the concentration of trypsinogen in solution. During the process of formation of trypsin and inert protein the ratio of the concentrations of the two products in any given reaction mixture remained constant, was equal to the ratio of the velocity constants of the two reactions, and was independent of the original concentration of trypsinogen.

The present studies show that salts influence the transformation of trypsinogen into trypsin in one of the following ways.

(*a*) Increasing both the rate of formation of trypsin and the rate of formation of inert protein. (*b*) Decreasing both the rate of formation of trypsin and the rate of formation of inert protein. (*c*) Increasing the rate of formation of trypsin and decreasing the rate of formation of inert protein. (*d*) Decreasing the rate of formation of trypsin and increasing the rate of formation of inert protein.

The effect of salts upon the ultimate amounts of trypsin and inert protein formed depends upon the relative influence of the various ions on the rate of both reactions since the final amounts of the products formed depend on the ratio of the velocity constant, K_1, for the formation of trypsin to the velocity constant, K_2, for the formation of inert protein. The influence of salts is due to both the cation and the anion; the chemical nature of the ions is more important than their valency.

The behavior of the calcium ion is particularly striking since it inhibits completely the formation of inert protein even in concentrations as low as 0.02 M with the result that the trypsinogen is converted quantitatively into trypsin. In the presence of calcium salts, therefore, the transformation of trypsinogen into trypsin by means of trypsin follows the course of a simple unimolecular autocatalytic reaction.

The ions studied may be arranged in the following approximate series, the first members in each group having an increasing and the last members a decreasing effect.

A. Effect on the Rate of Formation of Trypsin

Anions	Cations
Sulfate, citrate, oxalate, tartrate, acetate	Calcium
Fluoride, chloride	Strontium
Bromide	Barium, magnesium
Nitrate	Sodium
Iodide	Lithium
	Potassium, ammonium
	Rubidium
	Caesium

B. Effect on the Rate of Formation of Inert Protein

Anions	Cations
Iodide	Barium
Nitrate	Magnesium
Tartrate, citrate, bromide	Lithium
Oxalate, acetate, sulfate	Potassium
Chloride	Sodium
Fluoride	Ammonium, rubidium, caesium
	Strontium
	Calcium

C. Effect on the Ultimate Percentage of Trypsinogen Changed into Trypsin

Anions	Cations
Acetate, sulfate, oxalate, citrate, tartrate, fluoride, chloride	Calcium
	Strontium
Bromide	Magnesium, sodium
Nitrate	Rubidium, ammonium, lithium, potassium
Iodide	Caesium, barium

EXPERIMENTAL

General Procedures

The methods used for the study of the formation of trypsin and of inert protein from trypsinogen were those previously described (Kunitz, 1939). Crystalline trypsinogen was prepared from extracts of fresh beef pancreas by the method of Kunitz and Northrop (1936), further purified and freed from inhibitor by precipitation with trichloracetic acid, and dialyzed against 0.005 M phosphoric acid. Complications due to protein hydrolysis were avoided by keeping all reaction mixtures at 5°C. The solutions were kept sterile by the addition of 0.1 ml. 1 per cent merthiolate in 1.4 per cent borax to each 100 ml. solution. Salts of Merck's reagent grade were employed.

The pH of each activation mixture was adjusted when necessary with phosphoric acid or potassium hydroxide. The measurements were made by means of a low re-

sistance glass electrode of the Mouquin and Garman type (1937). In the range of pH used the salt error was negligible (Gardiner and Sanders, 1937; Cranston and Brown, 1937; Dole and Wiener, 1937). In a comparison of measurements with the glass and hydrogen electrodes the maximum variation in pH observed was 0.04 with an average deviation of 0.02.

EXPERIMENTAL RESULTS

1. Effect of Ammonium Salts on the Autocatalytic Formation of Trypsin.— The experimental data for the transformation of trypsinogen into trypsin in various concentrations of ammonium salts are given in Figs. 1 *a*, 1 *b*, 1 *c*, 1 *d*. Fig. 1 *a* shows that not only does the rate of formation of trypsin increase with increasing concentration of ammonium sulfate but that the final amount of trypsin formed is also greater. In the case of ammonium chloride (Fig. 1 *b*) the rate of formation of trypsin decreases with increasing concentration of salt while the final amount of trypsin formed is practically the same. Increasing the concentration of ammonium nitrate (Fig. 1 *c*) decreases both the rate of formation of trypsin and the amount of trypsin formed. The rate of formation of trypsin decreases with increasing concentration of ammonium acetate (Fig. 1 *d*) while the final amount of trypsin formed increases.

The diversity in the results is due to the difference in the effect of the various ammonium salts not only on the velocity constant, K_1, for the formation of trypsin but also on K_2, the velocity constant for the formation of inert protein. The initial slopes of the curves are proportional to K_1 while the percentage trypsinogen ultimately changed into trypsin is equal to $\frac{r}{1+r} \times 100$, where $r = \frac{K_1}{K_2}$. The observed values for the percentage trypsinogen finally changed into trypsin and the calculated values[2] of K_1, K_2, and $\frac{K_1}{K_2}$ are given in Table I. In the presence of ammonium sulfate both velocity constants at first decrease slightly and then increase rapidly as the concentration of salt increases. The

[2] The velocity constants K_1 and K_2 for the formation of trypsin and inert protein respectively were calculated by means of the equations derived by Kunitz (1939), namely

$$K_1 + K_2 = 2.3\, m$$

and

$$K_1 = \frac{2.3\, m}{G_0} \times \frac{A_e - A_0}{A_e}$$

where m is the slope of the straight line resulting when the values of $\log \frac{A}{A_e - A}$ are plotted against t.

G_0, the original concentration of trypsinogen

A_0, the original concentration of trypsin

A, the concentration of trypsin at any time t

and A_e, the final concentration of trypsin.

effect is much greater on K_1, however, so that the ratio $\frac{K_1}{K_2}$ and hence the final amount of trypsin formed increases with increasing concentration of ammonium sulfate.

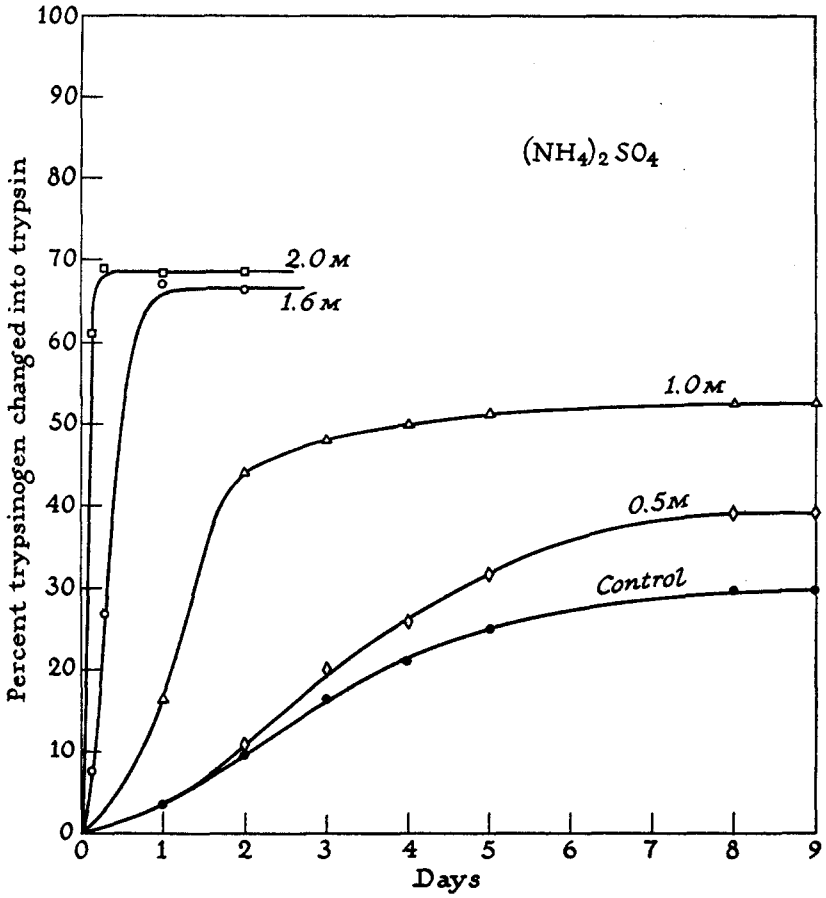

FIG. 1 *a*

FIGS. 1 *a*, 1 *b*, 1 *c*, 1 *d*. Effect of various concentrations of ammonium salts on the formation of trypsin. Activation mixtures: various concentrations of ammonium salts in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml.

The velocity constants, K_1, and K_2, decrease with increasing concentration of ammonium chloride, the rate of decrease being the same for both reactions. The value of $\frac{K_1}{K_2}$ is therefore unchanged and the percentage trypsinogen changed into trypsin is the same regardless of the concentration of ammonium chloride.

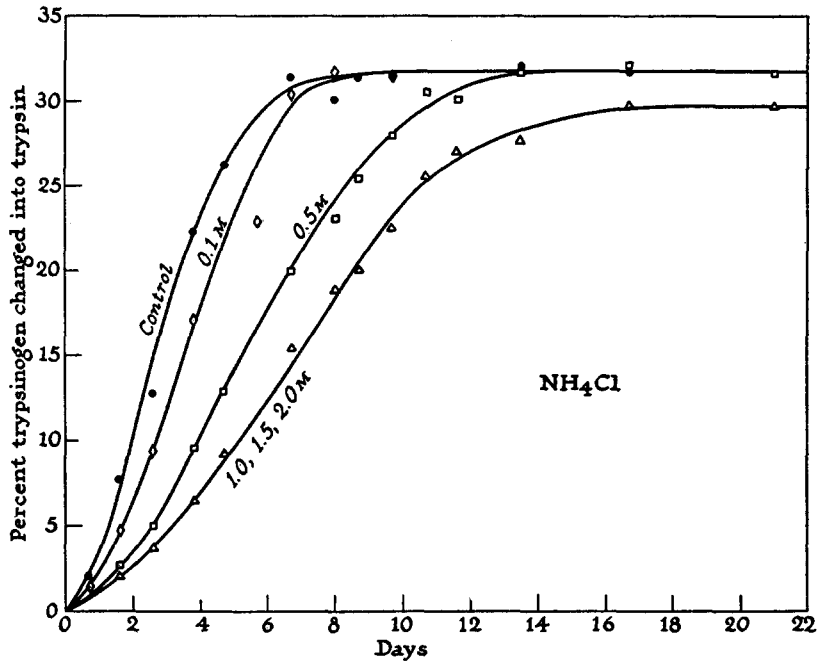

FIG. 1 *b*

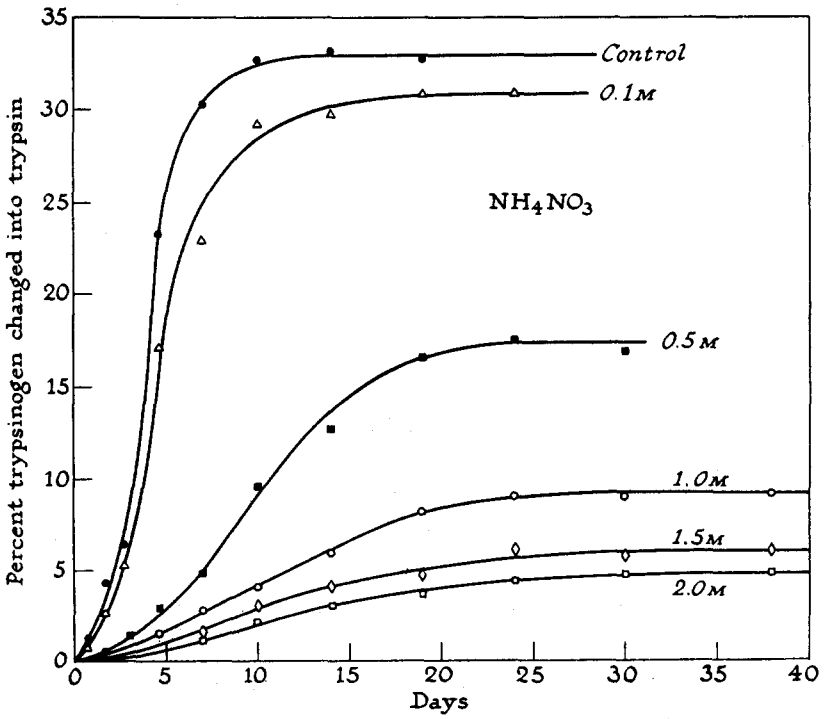

FIG. 1 *c*

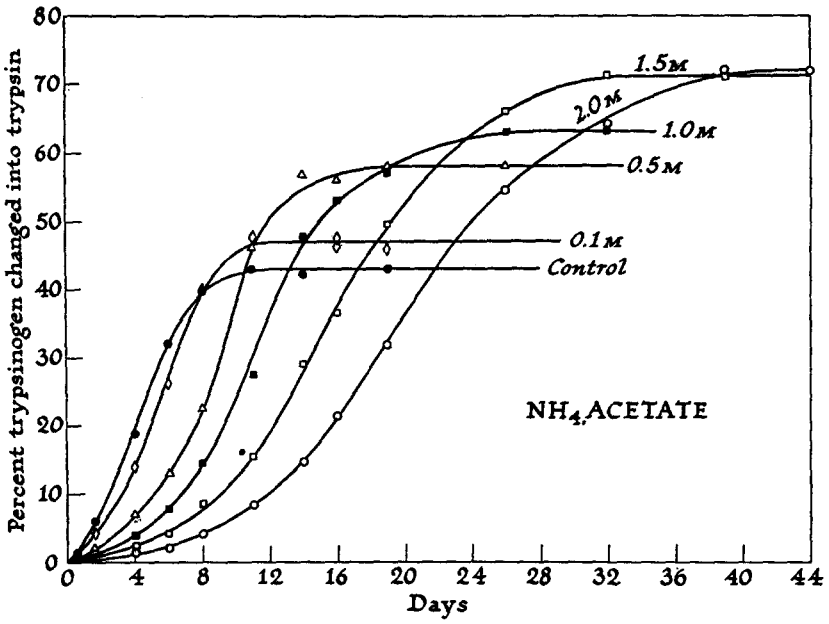

FIG. 1*d*

TABLE I

Effect of Ammonium Salts

Molar concentration		0	0.1	0.5	1.0	1.5	2.0
Salt	pH	Per cent trypsinogen changed into trypsin (observed)					
Ammonium sulfate	7.3	29	30	39	53	66	68
" chloride	7.2	31	31	32	30	30	30
" nitrate	7.3	33	31	16	9	6	5
" acetate	7.0	42	48	59	63	71	71
		K_1 (per mg. P.N. per day)					
" sulfate	7.3	89	83	92	235	1450	4850
" chloride	7.2	103	85	60	52	52	52
" nitrate	7.3	87	63	26	16	13	10
" acetate	7.0	83	60	43	32	24	17
		K_2 (per mg. P.N. per day)					
" sulfate	7.3	225	194	148	219	740	2210
" chloride	7.2	230	187	131	120	120	124
" nitrate	7.3	177	142	135	153	195	197
" acetate	7.0	114	66	33	18	10	7
		$\frac{K_1}{K_2}$					
" sulfate	7.3	0.40	0.43	0.62	1.07	1.96	2.19
" chloride	7.2	0.45	0.46	0.46	0.43	0.43	0.42
" nitrate	7.3	0.49	0.44	0.19	0.10	0.07	0.05
" acetate	7.0	0.73	0.91	1.30	1.78	2.40	2.42

The final amount of trypsin formed from trypsinogen in the presence of ammonium nitrate decreases rapidly with increasing concentration of the salt since the velocity constant for the formation of trypsin decreases while the velocity constant for the formation of inert protein remains practically unchanged.

Increasing the concentration of ammonium acetate increases the percentage trypsinogen changed into trypsin by decreasing K_2 to a much greater extent than K_1.

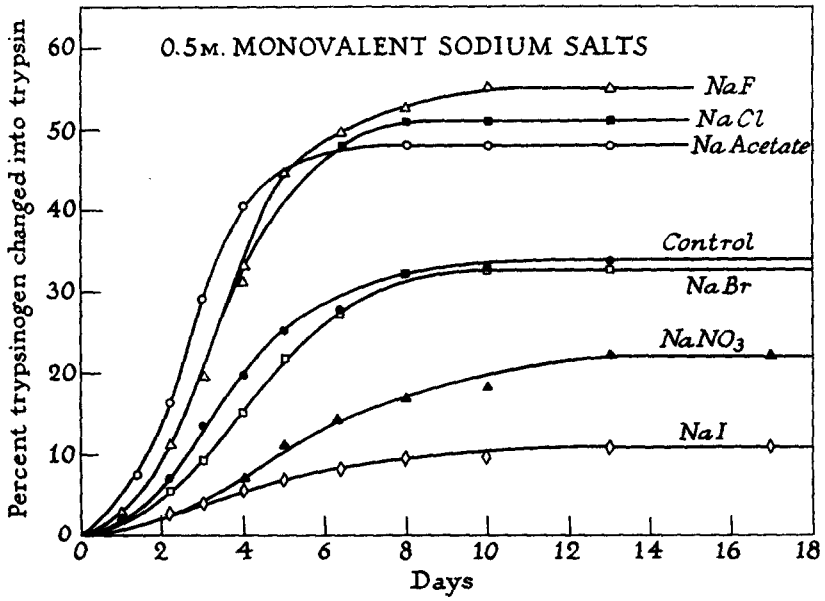

FIG. 2*a*

FIGS. 2 *a*, 2 *b*, 2 *c*. Effect of various anions on the formation of trypsin. Activation mixtures: various sodium salt solutions in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

2. Effect of Various Anions on the Autocatalytic Formation of Trypsin.— The striking differences in the effects of various anions on the transformation of trypsinogen into trypsin and inert protein found with ammonium salts were obtained also with sodium salts, as is illustrated in Figs. 2 *a*, 2 *b*, 2 *c*, and Table II. These show, in general, that the sulfate, citrate, oxalate, tartrate, acetate, fluoride, and chloride ions accelerate the rate of formation of trypsin, the effect decreasing in the order given,[3] while the iodide, nitrate, and bromide ions decrease its rate of formation. The rate of formation of inert protein is accelerated by iodide, nitrate, tartrate, and citrate and decreased by fluoride, chloride, sulfate, acetate, oxalate, and bromide ions. In the presence of

[3] All series in this paper are arranged in order of decreasing effect of the ions.

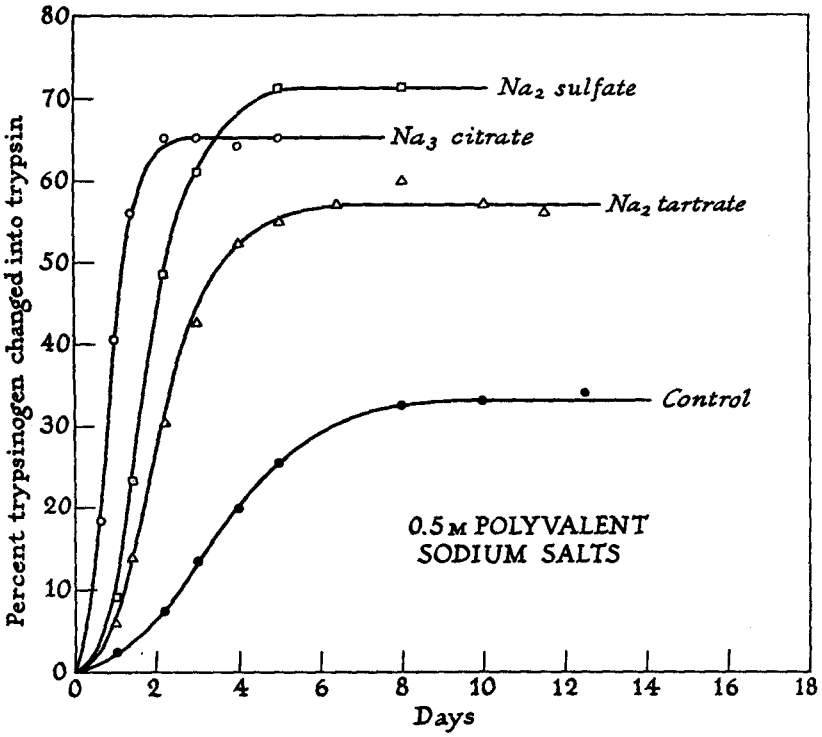

FIG. 2*b*

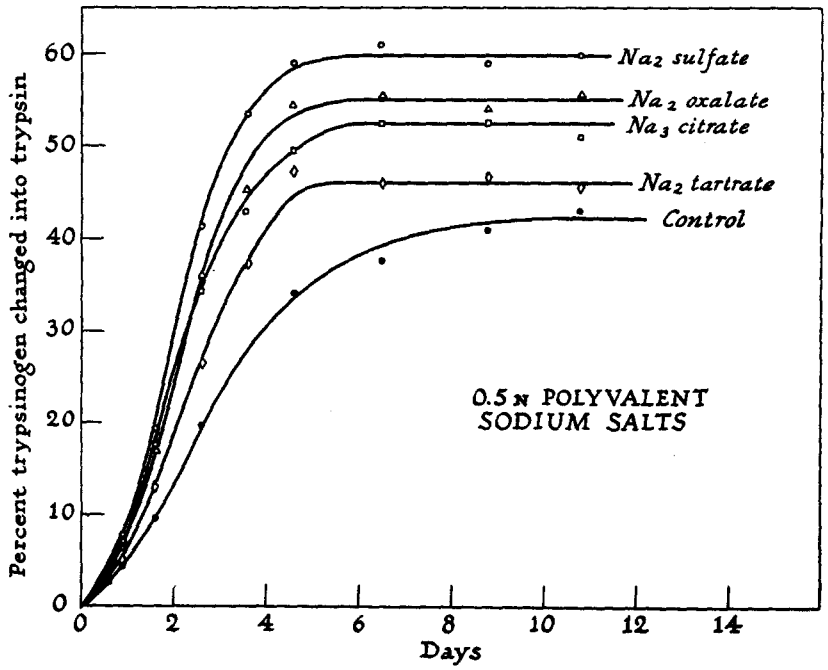

FIG. 2*c*

sulfate, oxalate, citrate, tartrate, fluoride, chloride, and acetate the ultimate amount of trypsin formed is greater than without the salt while with iodide, nitrate, and bromide less trypsin is formed. Although there is in general a greater tendency for the polyvalent anions to increase the rate of formation of trypsin, the valency of the anion is not the controlling factor since much greater differences were observed between the halides, for example, than between the acetate, citrate, and tartrate.

3. Effect of Various Cations on the Autocatalytic Formation of Trypsin.— Figs. *3 a* and *3 b*, show the experimental data for the formation of trypsin from trypsinogen under the influence of various salts of the alkali metals. The

TABLE II

Effect of Sodium Salts

Salt	Molar concentration	Per cent trypsinogen changed into trypsin (observed)	K_1	K_2	$\frac{K_1}{K_2}$
			per mg. P.N. per day		
Control		33	87	176	0.49
Sodium citrate	0.5	65	455	244	1.87
" sulfate	0.5	68	231	107	2.16
" tartrate	0.5	57	214	161	1.33
" acetate	0.5	48	131	139	0.94
" fluoride	0.5	55	104	84	1.24
" chloride	0.5	51	104	100	1.04
" bromide	0.5	32	81	171	0.47
" nitrate	0.5	22	59	203	0.29
" iodide	0.5	11	47	380	0.12
Control		42	92	125	0.74
Sodium sulfate	0.25	60	141	93	1.52
" citrate	0.17	52	135	122	1.11
" oxalate	0.25	56	131	102	1.29
" tartrate	0.25	46	129	140	0.92

calculated values of K_1 and K_2 are given in Table III. The salts of sodium and lithium were found to increase the rate of formation of trypsin while those of caesium, rubidium, and potassium were found to decrease it. The rate of formation of inert protein was decreased by the salts of rubidium, caesium, sodium, potassium, and lithium, the latter having the least effect. The largest ultimate amount of trypsin formed was found with the sodium salts, followed in order by lithium, potassium, rubidium, and caesium.

The experimental data for the formation of trypsin from trypsinogen in the presence of salts of the alkaline earths are given in Fig. 4. The velocity constant for the formation of trypsin (Table IV) increases with the addition of calcium, strontium, barium, and magnesium. Barium increases the velocity constant for the formation of inert protein while calcium, strontium, and mag-

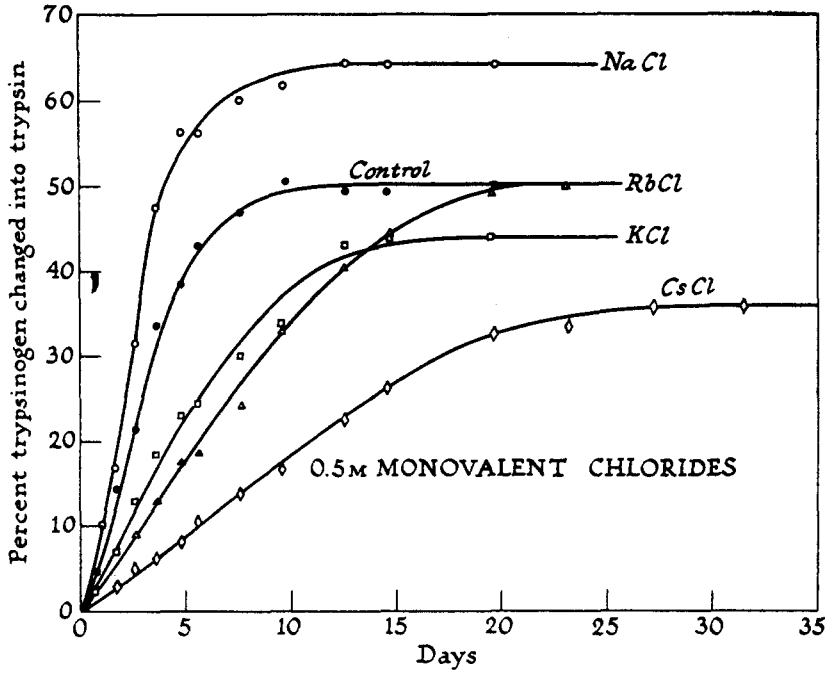

FIG. 3*a*

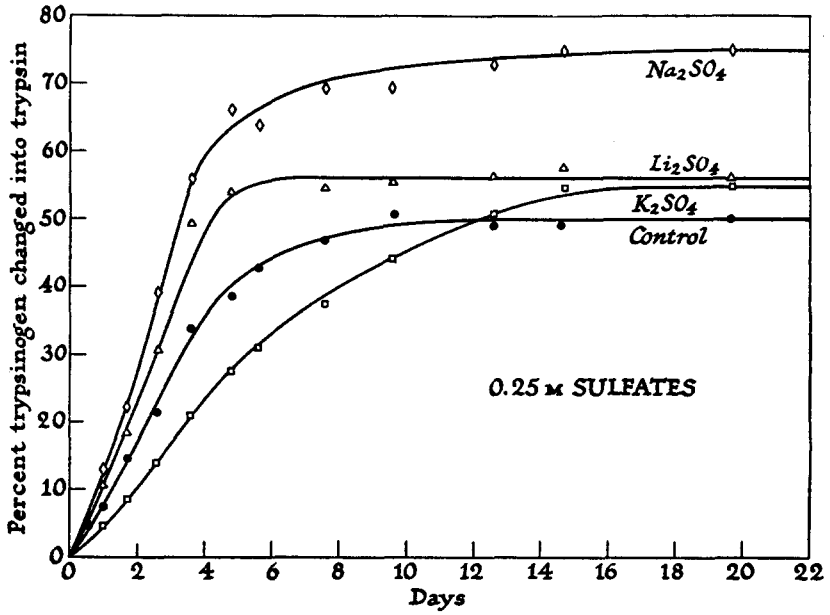

FIG. 3*b*

FIGS. 3 *a* and 3 *b*. Effect of monovalent cations on the formation of trypsin. Activation mixtures: solutions of salts of the alkali metals in 0.1 M phosphate buffer pH 7.6 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

TABLE III

Effect of Salts of the Alkali Metals

Salt	Molar concentration	Per cent trypsinogen changed into trypsin (observed)	K_1	K_2	$\frac{K_1}{K_2}$
			per mg. P.N. per day		
Control		50	80	79	1.01
Lithium sulfate	0.25	57	99	75	1.32
Sodium "	0.25	75	115	37	3.1
Potassium "	0.25	55	53	45	1.18
Sodium chloride	0.5	64	97	55	1.76
Potassium "	0.5	44	52	67	0.78
Rubidium "	0.5	50	40	40	1.00
Caesium "	0.5	36	26	47	0.55

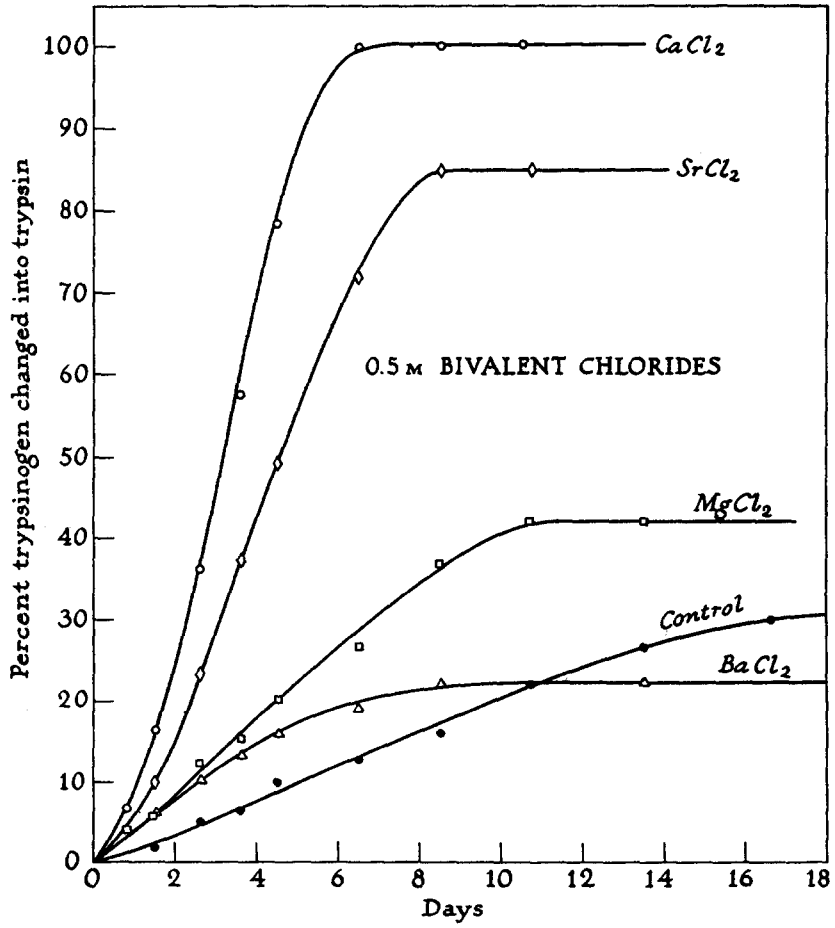

FIG. 4. Effect of bivalent cations on the formation of trypsin. Activation mixtures: solutions of the chlorides of the alkaline earths in 0.1 M borate buffer pH 8.0 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.1.

nesium decrease it. The ultimate amount of trypsin formed is therefore greater in the presence of calcium, strontium, and magnesium chloride than in the control and smaller in the presence of barium chloride. It is evident that the different cations produce as wide a variation in the results as do the different anions.

4. Effect of Calcium Ion on the Formation of Trypsin.—The action of calcium salts on the transformation of trypsinogen into trypsin is unique in that of all the ions studied only calcium completely inhibited the formation of inert protein. In the presence of calcium salts, even in concentrations as low as 0.02 M, trypsinogen is converted quantitatively into trypsin[4] and the formation follows the course of a pure autocatalytic reaction.

(*a*) *Effect of Varying the Concentration of Calcium Chloride.*—The effect of various concentrations of calcium chloride on the transformation of trypsinogen into trypsin and inert protein is shown in Fig. 5 and Table V. The smooth

TABLE IV

Effect of 0.5 M Alkaline Earth Chlorides

Salt	Per cent trypsinogen changed into trypsin (observed)	K_1	K_2	$\frac{K_1}{K_2}$
		per mg. P.N. per day		
Control	31	26	57	0.46
Magnesium chloride	42	40	56	0.71
Calcium "	100	90	0	∞
Strontium "	85	65	11	5.90
Barium "	22	42	145	0.29

curves in Fig. 5 are drawn through theoretical values of A calculated by means of the equation[5]

$$A = A_e \frac{\frac{A_0}{A_e - A_0} e^{K_1 G_0 t}}{1 + \frac{A_0}{A_e - A_0} e^{K_1 G_0 t}}$$

The rate of formation of trypsin increases gradually with increasing concentration of calcium chloride while the rate of formation of inert protein decreases rapidly. The value of K_2 in 0.0008 M calcium chloride is only one-fifth of the value of K_2 in the control. The fraction of trypsinogen changed into trypsin increases from 25 per cent in the control to 65 per cent in the presence of 0.0008 M and to 99 per cent in 0.02 M calcium chloride.

[4] The trypsin formed can be easily crystallized; the procedure will be described in a future publication.

[5] e is the Naperian constant: the other symbols are defined in footnote 2.

(*b*) *Effect of Varying the Concentration of Trypsinogen.*—Fig. 6 *a* shows the curves for the formation of trypsin from various concentrations of trypsinogen in the presence of 0.1 M calcium chloride. In each case all of the trypsinogen is changed into trypsin and no inert protein is formed. Within the range of

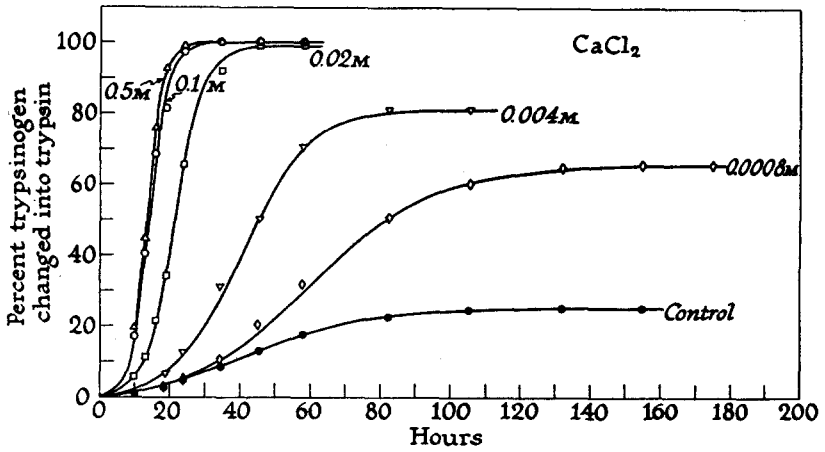

FIG. 5. Effect of concentration of calcium chloride on the formation of trypsin. Activation mixtures: various concentrations of calcium chloride in 0.1 M borate buffer pH 8.0 and containing 0.1 mg. protein nitrogen per ml. Final pH = 7.2. Smooth curves drawn through calculated points.

TABLE V

Effect of Calcium Chloride

Molar concentration calcium chloride	Per cent trypsinogen changed into trypsin (observed)	K_1	K_2	$\frac{K_1}{K_2}$
		per mg. P.N. per day		
0	25	12	37	0.33
0.0008	65	13	7	1.86
0.004	81	25	6	4.17
0.02	99	58	0.6	97.0
0.1	100	90	0	∞
0.5	100	104	0	∞

concentrations of trypsinogen used, doubling the concentration of trypsinogen doubles the rate of formation of trypsin. The values of $\log \frac{A}{G_0 - A}$ *vs.* t, for the same series are plotted in Fig. 6 *b*. The experimental points lie on straight lines, the slopes of which are proportional to the initial concentrations of trypsinogen used in agreement with the theory of the kinetics of a simple unimolecular autocatalytic reaction.

The curves for the rate of formation of trypsin from various concentrations of trypsinogen in the presence of 0.02 M and 0.001 M calcium chloride are given

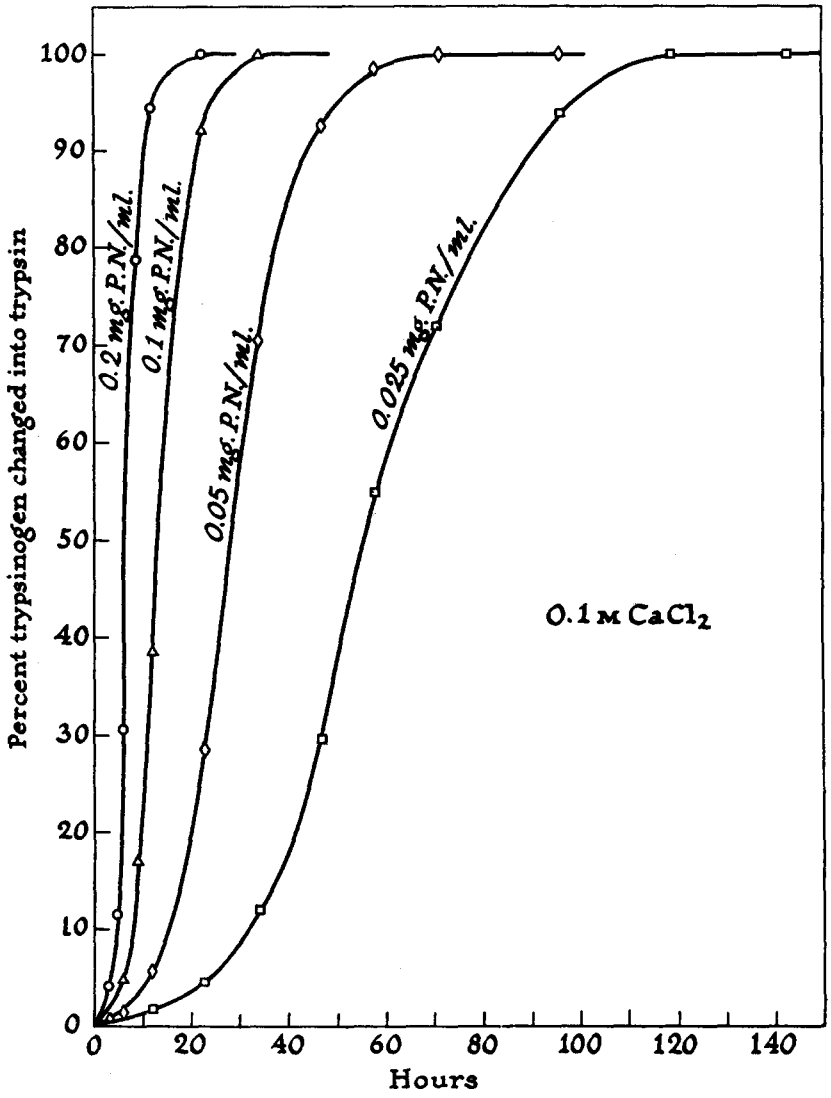

FIG. 6 *a*

FIGS. 6 *a*, 6 *b*, 6 *c*, 6 *d*. Effect of concentration of trypsinogen on the formation of trypsin. Activation mixtures: calcium chloride solutions in 0.1 M borate buffer pH 8.0 and containing various amounts of trypsinogen per ml. Final pH = 7.2.

in Figs. 6 *c* and 6 *d*. Although here the concentrations of the salt are insufficient to inhibit completely the formation of inert protein, the percentage of trypsinogen changed into trypsin is independent of its initial concentration in

the case of 0.02 M calcium chloride and even increases with increasing concentration of trypsinogen in 0.001 M. It appears from these results that the effect of calcium in preventing the formation of inert protein is not due to the formation of an undissociated stoichiometric compound between it and the

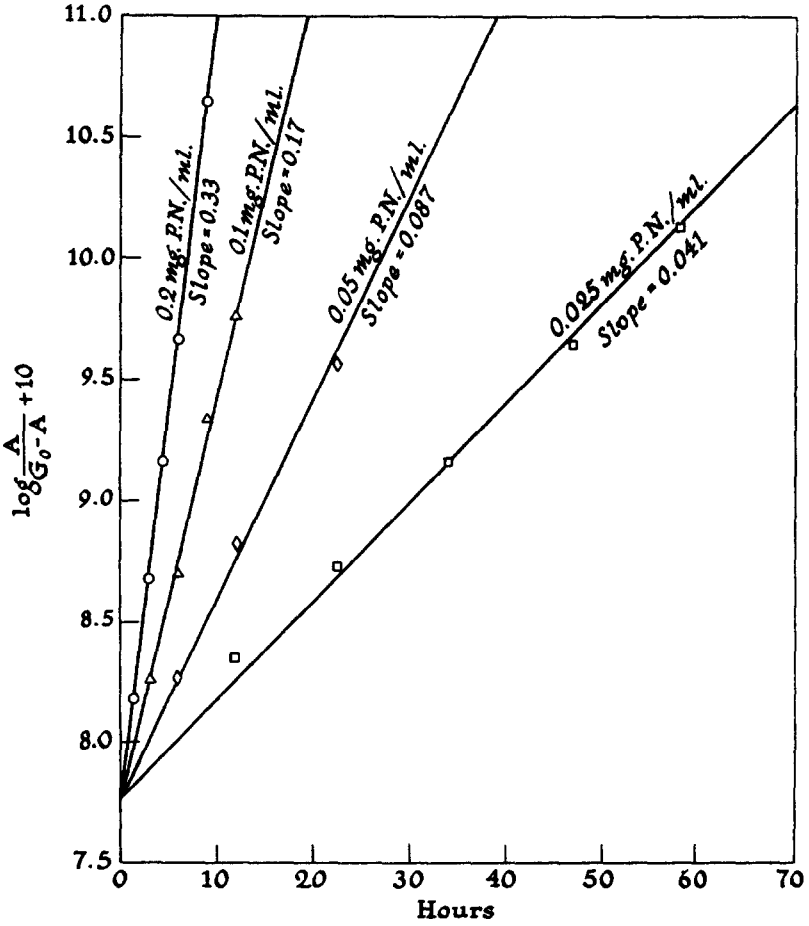

FIG. 6*b*

trypsinogen since in that case the percentage of trypsinogen changed into trypsin would decrease as its concentration increased.

(*c*) *Effect of Calcium Chloride at Various pH.*—Figs. 7 *a* and 7 *b* give the curves for the formation of trypsin from trypsinogen in the presence and absence of calcium chloride at various pH. In both cases the rate of formation of trypsin increases with increasing pH. In the absence of calcium chloride, however, the ultimate amount of trypsin formed decreases rapidly with increase

in pH due to the larger amounts of inert protein formed in the alkaline solutions (Kunitz, 1939), whereas in the presence of calcium chloride the transformation of trypsinogen into trypsin is complete over the whole range of pH used.

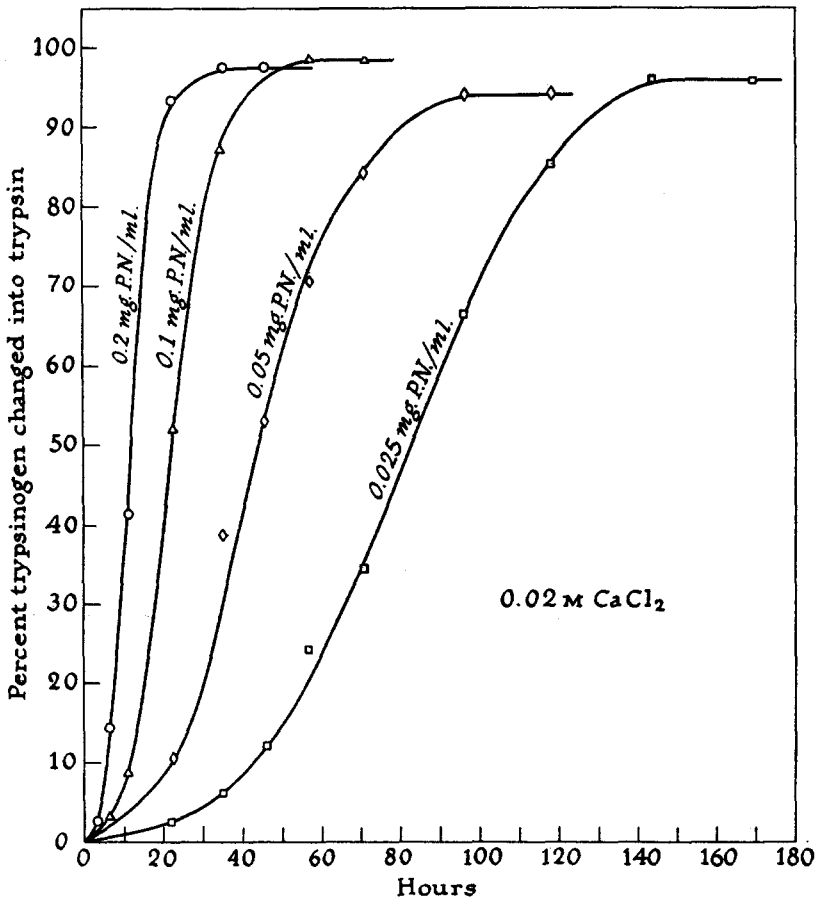

FIG. 6*c*

(*d*) *Effect of Various Calcium Salts.*—Fig. 8 gives the experimental data for the formation of trypsin in the presence of various calcium salts. The trypsinogen is converted quantitatively into trypsin in all cases and the rate of formation of trypsin is approximately the same regardless of the anion used. Evidently the action of the calcium ion predominates, masking the action of the anion.

(*e*) *Effect of Calcium Chloride on the Action of Trypsin.*—Calcium chloride

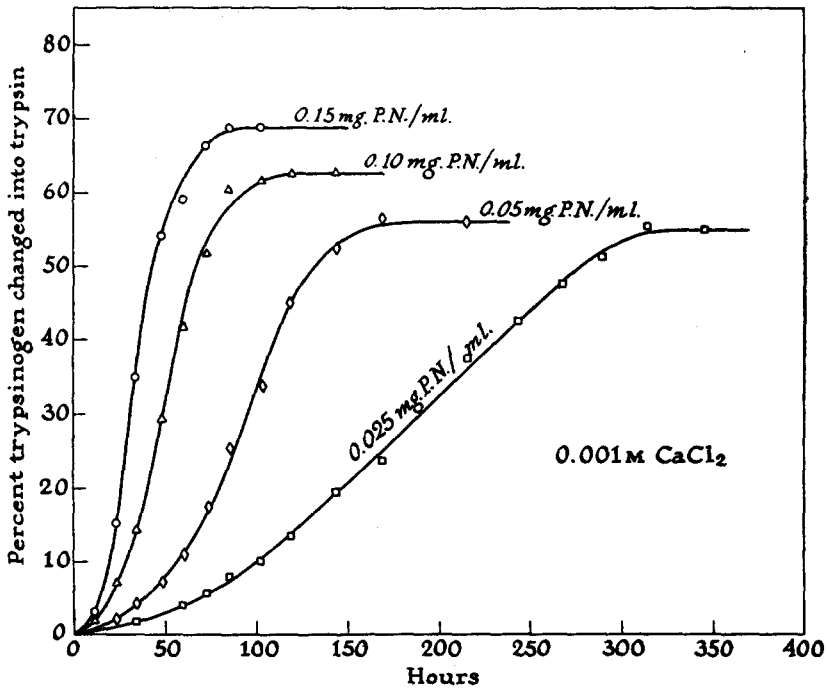

FIG. 6*d*

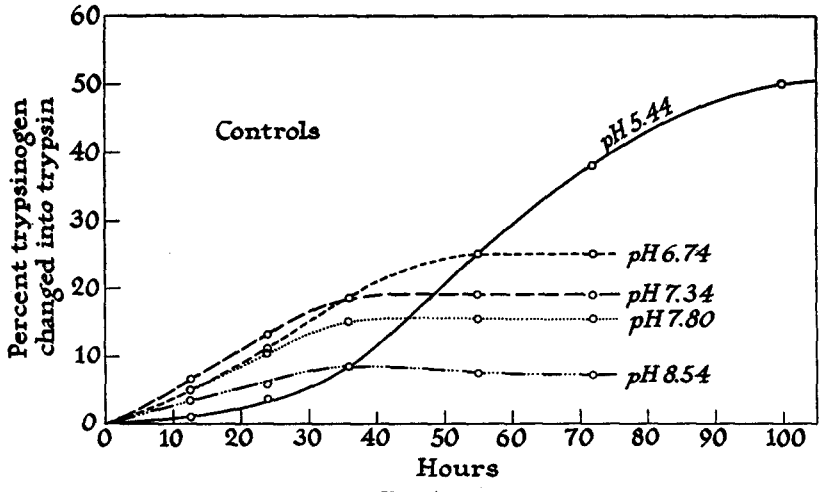

FIG. 7 *a*

FIGS. 7 *a* and 7 *b*. Effect of pH on the formation of trypsin. Activation mixtures: 0.02 M acetate-barbiturate buffers of various pH in 0.02 M calcium chloride or water and containing 0.1 mg. trypsinogen protein nitrogen per ml.

in concentrations sufficient to inhibit completely the formation of inert protein and to accelerate the rate of formation of trypsin was found to have practically

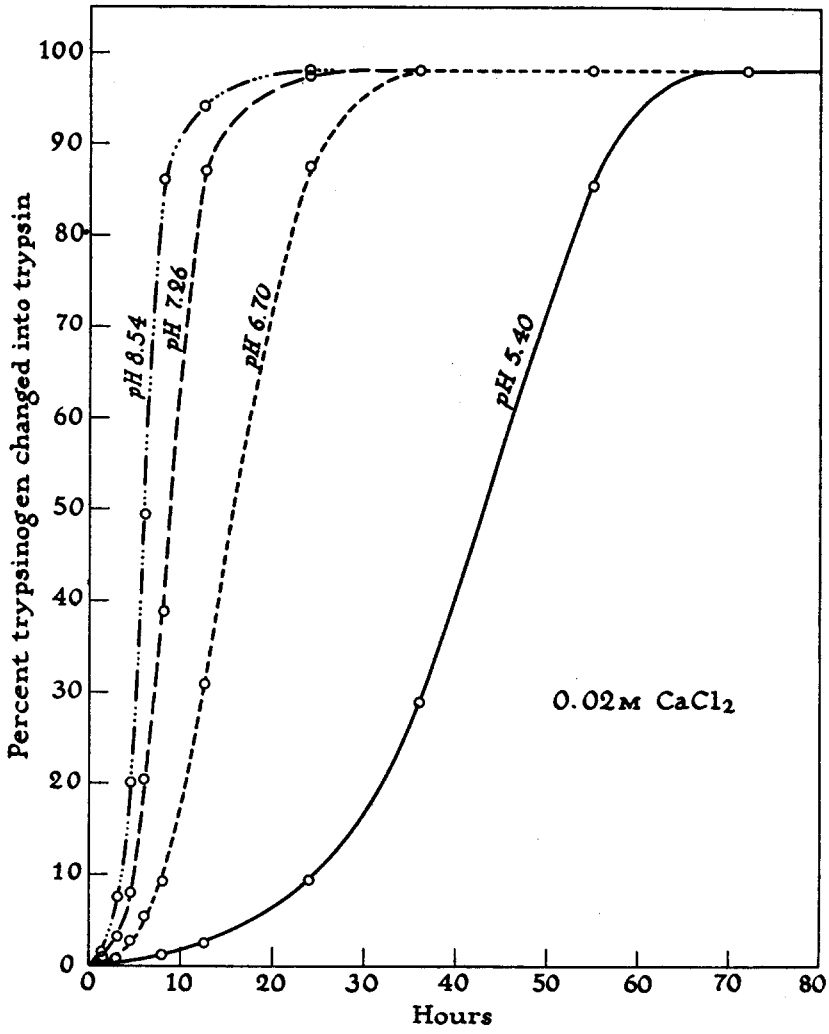

FIG. 7*b*

no effect on the tryptic digestion of hemoglobin or of benzoyl-*l*-arginine amide nor on the formation of chymotrypsin from chymotrypsinogen by means of trypsin. This together with the fact that the influence of calcium chloride on the formation of trypsin from trypsinogen is exactly the opposite of its

influence on the formation of inert protein, although both reactions are catalyzed by trypsin, leads one to assume that the action of calcium chloride is on the trypsinogen rather than on the trypsin, or else that the various actions of trypsin are quite different in character.

(*f*) *Effect of Calcium Chloride on Enterokinase and Mold Kinase.*—The rate of formation of trypsin from trypsinogen as catalyzed by enterokinase (Kunitz,

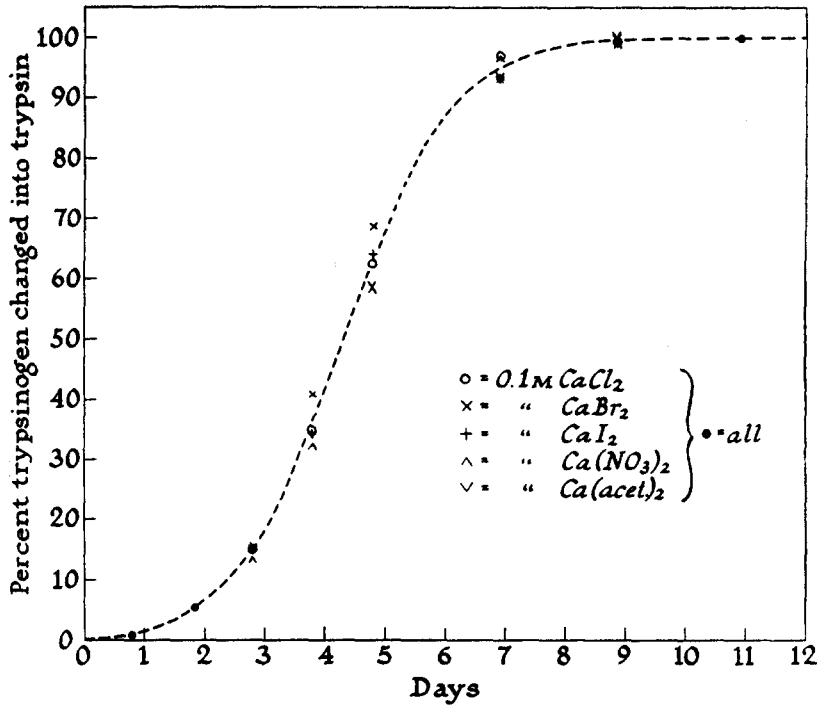

FIG. 8. Effect of various calcium salts on the formation of trypsin. Activation mixtures: 0.1 M solutions of various calcium salts in 0.1 M borate buffer pH 8.0 and containing 0.01 mg. trypsinogen protein nitrogen per ml. Final pH = 7.5.

1939 *a*) and by mold kinase (Kunitz, 1938) was found to be depressed in the presence of 0.1 M calcium chloride.

SUMMARY

Crystalline trypsinogen is completely transformed into trypsin by means of trypsin in the presence of calcium salts. The process follows the course of a pure autocatalytic unimolecular reaction.

In the absence of calcium salts, the autocatalytic formation of trypsin from trypsinogen is complicated by the transformation of part of the trypsinogen

into an inert protein which cannot be changed into trypsin by any known means.

Salts increase or decrease the rate of both reactions so that the ultimate amount of trypsin formed varies with the nature and concentration of the salt used. With equivalent concentrations of salt the percentage of trypsinogen changed into trypsin is greatest in the presence of calcium ion followed in order by strontium; magnesium and sodium; rubidium, ammonium, lithium, and potassium; caesium and barium. With the anions the largest percentage of trypsinogen transformed into trypsin was found with the acetate, sulfate, oxalate, citrate, tartrate, fluoride, and chloride ions followed in order by bromide, nitrate, and iodide.

The formation of inert protein is completely suppressed by concentrations of calcium ion greater than 0.02 M.

REFERENCES

Ayrton, B., 1909, *Quart. J. Exp. Physiol.*, **2**, 201.

Cranston, J. A., and Brown, H. F., 1937, *J. Roy. Tech. Coll.*, Glasgow, **4**, 46.

Delezenne, M. C., 1905, *Compt. rend. Soc. biol.*, **59**, 476.

de Sonza, 1908, Inaugural thesis, London.

Dole, M., and Wiener, B. Z., 1937, *Tr. Electrochem. Soc.*, **72**, 107.

Farber, L., and Wynne, A. M., 1935, *Biochem. J.*, London, **29**, 2323.

Gardiner, W. C., and Sanders, H. L., 1937, *Ind. and Eng. Chem., Analytical Edition*, **9**, 274.

Kunitz, M., 1938, *J. Gen. Physiol.*, **21**, 601.

Kunitz, M., 1939, *J. Gen. Physiol.*, **22**, 293.

Kunitz, M., 1939*a*, *J. Gen. Physiol.*, **22**, 429.

Kunitz, M., and Northrop, J. H., 1934, *Science*, **80**, 505.

Kunitz, M., and Northrop, J. H., 1936, *J. Gen. Physiol.*, **19**, 991.

Mellanby, J., and Woolley, V. J., 1913, *J. Physiol.*, **46**, 159.

Meyer, K., 1910, *Biochem. Z.*, Berlin, **23**, 68.

Mouquin, H., and Garman, R. L., 1937, *Ind. and Eng. Chem., Analytical Edition*, **9**, 287.

Waldschmidt-Leitz, E. W., 1924, *Z. phys. Chem.*, **132**, 181.

Wohlgemuth, J., 1912, *Biochem. Z.*, Berlin, **39**, 302.

Zunz, E., 1906, *Bull. Soc. roy. sc. méd. et nat. Bruxelles*, **64**, 28, 98.

Zunz, E., 1907, *Ann. Soc. roy. sc. méd. et nat. Bruxelles*, **16**, 63.