The Effect of Sodium Fluoride on the Digestion and Metabolism of Protein

by

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THE EFFECT OF SODIUM FLUORIDE ON THE DIGESTION AND METABOLISM OF PROTEIN

INTRODUCTION

Sodium fluoride in the diet is known to cause a mottling effect on teeth. Unpublished data[1] from this laboratory show that sodium fluoride in the diet of white rats not only causes mottled enamel but also produces a stunting of growth of the animal. The average gain in six weeks for rats on diets which contain no fluorine was 158 grams while litter-mates on a similar diet containing 0.1% sodium fluoride gained only 63 grams. The animals receiving sodium fluoride were less efficient in the utilization of their food. The average gain per gram of food in the control rats was 0.339 grams as compared with a gain per gram of food of 0.199 grams in white rats receiving 0.1% sodium fluoride.

Other workers have also observed the stunting effect of fluorides. Sollman, Schettler, and Wetzel[2] performed experiments to determine whether decrease in food consumption and the resultant decrease in growth were due to the taste of fluorine in the food. The rats had equal access to the diets with and without fluorine and they ate one food as readily as the other. Thus it was concluded that the

diminished food consumption was not due to a distaste for the fluorine containing food. Only with the maximum concentration of 0.23% sodium fluoride was there a distinct discrimination against fluorine diets.

Sodium fluoride has been known for some time to have an inhibiting action on many enzymes, including some digestive enzymes. Pierce and Lovenhart[3] found sodium fluoride to have a pronounced inhibiting action on animal lipases. Clifford[4] has shown that it brings about an inhibition of salivary amylase. However, Vandevelde and Poppe[5] found the digestion of egg white, blood, and protein of cows' milk by trypsin and pepsin was not influenced by the presence of fluorides, and Lang and Lang[6] stated that although fluorine does not injure trypsin and pepsin it does injure rennet. More recent work of Clifford[7,8] has shown that the clotting time of milk under the influence of pepsin is accelerated by very low fluorine concentrations, retarded by higher concentrations, and unaffected by intermediary concentrations. The inhibition of coagulation occurred suddenly at a definite molarity of added fluoride and a difference of concentration of 0.0036 M changed the clotting time of milk from a few minutes to two to six hours.

Since sodium fluoride does have an effect on the digestive enzymes, and since protein digestion and metabolism is closely linked with growth, a possible explana-

tion of the stunting effect and the poor utilization of food of rats on diets containing sodium fluoride was suggested. The influence of sodium fluoride on the digestion and the metabolism of proteins in white rats was therefore investigated.

The first part of this paper reports a study of the effect of sodium fluoride on the proteolytic enzyme pepsin in vitro using egg white and milk as the substrates. The second part deals with the effect of sodium fluoride on the proteolytic enzymes as shown by a study of the coefficient of digestibility of protein in white rats on diets containing sodium fluoride as compared with those on diets without this salt. Since the stunting of growth might be due either to interference with the digestion or the metabolism of protein, the third part of the paper deals with the influence of sodium fluoride on the protein metabolism as observed through a series of balance studies on white rats.

EXPERIMENTAL

PART I. THE EFFECT OF SODIUM FLUORIDE ON THE PROTEOLYTIC ENZYME PEPSIN IN VITRO

A. The Influence of Sodium Fluoride on the Proteolytic Enzyme Pepsin Using Milk as the Substrate.

Methods and Materials:

To determine the influence of sodium fluoride on the proteolytic enzyme pepsin, a modification of the method described by Clifford[7,8] was used. To a series of test tubes containing a definite amount of milk and varying concentrations of sodium fluoride, pepsin solution was added and the time of coagulation of milk determined.

Fresh, raw milk from the University Dairy was used. The action of pepsin on raw milk was found to be more uniform than on the pasteurized milks tried. Experimentation showed that the end point given by the raw milk could be more easily distinguished and hence was more satisfactory to use in these experiments.

The standard solutions of sodium fluoride were made up as follows from a 0.5 M solution of sodium fluoride:

Sol. #1	contained	10cc	of	0.5 M	NaF	&	190cc	of	distilled	water.
" #2	"	20cc	"	"	"	"	180cc	"	"	"
" #3	"	30cc	"	"	"	"	170cc	"	"	"
" #4	"	40cc	"	"	"	"	160cc	"	"	"
" #5	"	50cc	"	"	"	"	150cc	"	"	"
" #6	"	60cc	"	"	"	"	140cc	"	"	"
" #7	"	70cc	"	"	"	"	130cc	"	"	"

These standard solutions when mixed with the milk and pepsin had the following molarities: Sol. #1, 0.0036 M; sol. #2, 0.0072 M; sol. #3, 0.0108 M; sol. #4, 0.0144 M; sol. #5, 0.0180 M, sol. #6, 0.0216 M; and sol. #7, 0.0252 M.

The optimum hydrogen ion concentration for the part

of the pepsin enzyme that causes coagulation of milk is about pH 6. Although sodium fluoride hydrolyzed to a considerable extent, due to the buffer action of milk, no change took place in the pH of any of these milk-pepsin-fluorine mixtures. The milk used was found to have a pH 6 and hence these experiments were carried on at the optimum pH of the enzyme.

A series of test tubes containing the standard sodium fluoride solutions and a tube of distilled water were stoppered to prevent evaporation and placed in a water-bath at 40° C. Ten cc. of milk was pipetted out and allowed to stand in the water bath exactly five minutes in order to bring the milk to the temperature of the bath with the smallest amount of change to the coagulation power of the milk itself. A fresh 0.2 to 0.3% pepsin solution was prepared each time and brought to temperature. The strength of the pepsin solution was adjusted until 10 cc. of milk, 2 cc. of pepsin solution and 2 cc. of distilled water, clotted in two to three minutes. After the milk had come to temperature, 2 cc. of a standard sodium fluoride solution and 2 cc. of the pepsin solution were added from Ostwald pipettes as rapidly as possible. A control tube was made up in which the sodium fluoride solution was replaced by distilled water. After the pepsin was added the tube was quickly inverted to mix the contents and immediately replaced in the bath, at the same time a stop watch being

started.

The clotting time was determined as follows: The test tubes were removed from the water bath about every 30 seconds, observed for the first signs of flocculent precipitate and then immediately returned to the bath. At the first signs of precipitate the watch was stopped; a few seconds later a clot appeared. This gave a very definite end point. In all cases three or more measurements were made at each concentration of sodium fluoride in each of the five different experiments. From the difference in coagulation time in the series of varying sodium fluoride concentrations as compared with the control tube the effect of the salt on the enzyme could be studied.

Results:

Table I shows the coagulation time of milk by pepsin

TABLE 1
THE EFFECT OF SODIUM FLUORIDE ON THE COAGULATION TIME OF MILK BY PEPSIN AT 40° C.

Conc. of NaF Mols/Liter	Time of Coagulation in Minutes (Average of 3 Measurements.)					
	Exp't. 1	Exp't. 2	Exp't. 3	Exp't. 4	Exp't.5	Average
.0000	2.33	2.41	2.27	2.44	2.25	2.34
.0036	2.08	2.16	1.96	2.01	1.95	2.03
.0072	2.01	1.92	1.80	1.87	1.82	1.88
.0108	2.10	2.11	1.93	1.94	1.98	2.01
.0144	2.12	2.13	2.25	2.51	2.51	2.30
.0180	2.55	3.16	3.40	3.2	3.35	3.13
.0216	about 8	about 4	4.3	6.0	about 7	about 6
.0252	none in 3 hours		about 10	From 27 min. to none in $3\frac{1}{2}$ hours		From 10 min. to none in $3\frac{1}{2}$ hours

is influenced by sodium fluoride. The effect varies with the concentration of sodium fluoride. Sodium fluoride in concentrations below 0.0144 M has a slightly accelerating effect upon the pepsin as shown by an average coagulation time of 2.03 minutes for 0.0036 M, 1.88 minutes for 0.0072 M, and 2.01 for 0.0108 M as compared with 2.34 minutes for the control tube which contained no sodium fluoride. At 0.0144 M, sodium fluoride has neither an accelerating nor an inhibiting effect; the average coagulation time was 2.30 minutes for the control tube, which was practically the same as the coagulation time with the absence of sodium fluoride. Above 0.0180 M sodium fluoride the coagulation time was inhibited; between 0.0216 M and 0.0252 M the coagulation time of milk increased from about six minutes to several hours in most cases.

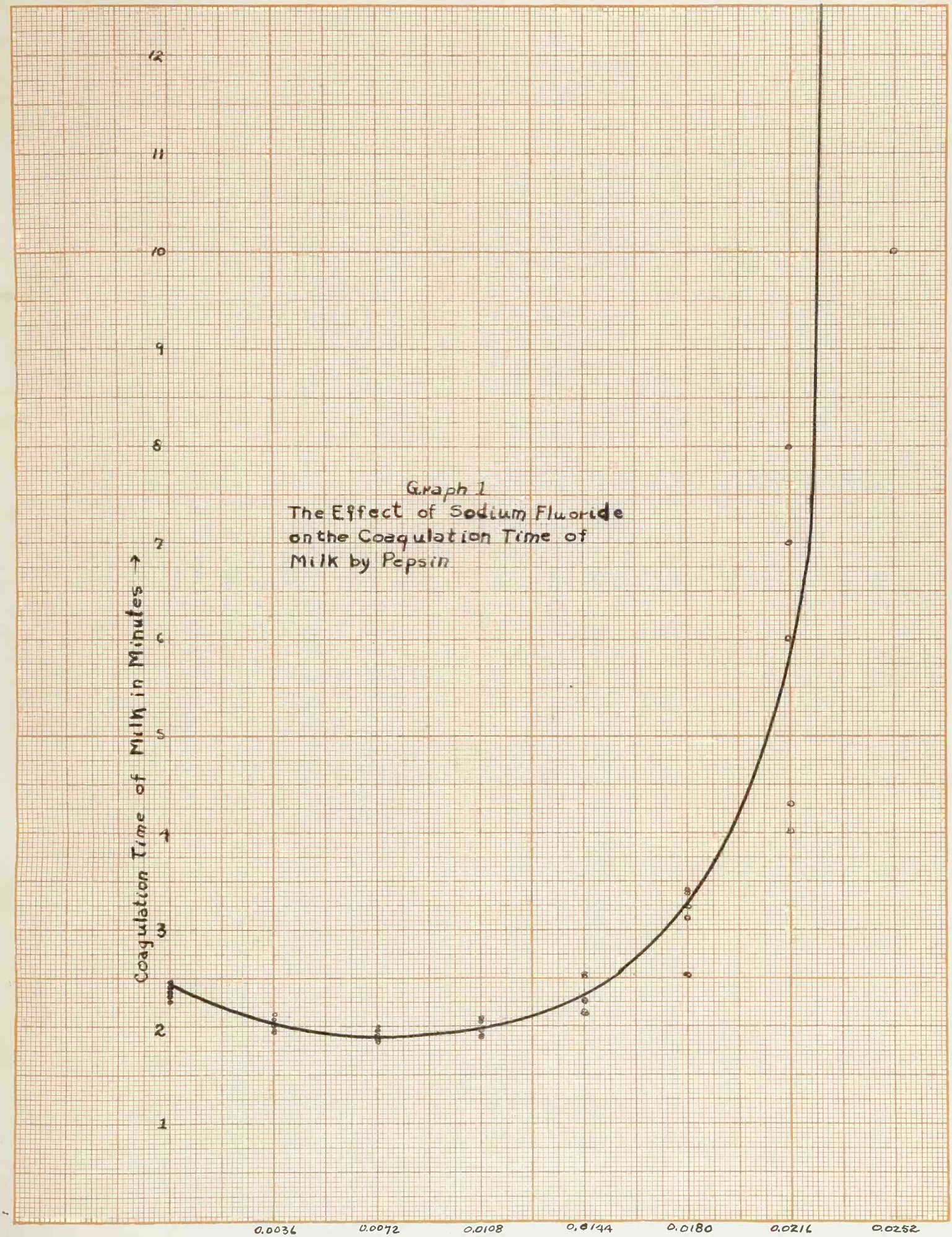
At high concentrations it was very difficult to get end points which checked closely enough so that you could determine an exact end point. A definite coagulation did not occur in a few seconds after the flocculent precipitate was noted and the precipitate itself seemed to form slowly. At the concentration 0.0252 M a precipitate was observed in some cases in about 15 minutes as compared with several hours for its duplicate. At low concentrations very good checks were obtained, e.g., at zero concentration of sodium fluoride the time to bring about coagulation was 2.43, 2.40, 2.38, 2.45, and 2.39 minutes.

These results are also expressed in Graph I, which shows the average coagulation time of milk by pepsin as influenced by the seven different concentrations of sodium fluoride.

In previous work done in this laboratory it was found that no concentration of sodium fluoride below 0.05% of the diet had an effect on the growth of the animal. The concentration of sodium fluoride in the diet which caused the first stunting effect was 500 parts per million. In the solution used in the milk clotting experiment with approximately the same concentration of sodium fluoride, namely, 454 parts per million, the enzyme pepsin was slightly accelerated.

When 0.1% sodium fluoride or 1000 parts per million was included in the diet a very marked inhibition of growth was noted. A similar concentration of 1058 parts of sodium fluoride per million in the milk clotting experiment resulted in a very large inhibition of the enzyme pepsin. At this concentration the time to bring about the coagulation of milk changed from a few minutes to several hours.

A possible relationship between great inhibition of the digestive enzyme pepsin in vitro at the high concentration of sodium fluoride and the decrease in growth of animals on diets containing approximately the same concentration is suggested by these results.



Graph 1
The Effect of Sodium Fluoride on the Coagulation Time of Milk by Pepsin

B. The Effect of Sodium Fluoride on the Dissolution of Egg White by Pepsin.

Authorities differ in opinion as to whether pepsin is made up of two separate enzymes or whether it is one enzyme with two distinct properties which brings about the coagulation of milk and also hydrolyzes protein. The effect of sodium fluoride on the coagulation property has been determined and next a determination of its influence upon the part of the enzyme which hydrolyzes protein was made. For this work the Mett's method[9] was selected as a basis. This method measures the effect of sodium fluoride on the activity of pepsin by determining the amount of egg white digested.

Methods and Materials:

Small glass tubes containing egg white were placed in test tubes which contained varying concentrations of sodium fluoride. Pepsin was added and the test tubes were allowed to remain in the water bath at 38° C. for from 48 to 76 hours, while the digestion of the egg white took place. At the end of the period the amount of digestion was found by measuring, with a vernier caliper, the amount of egg white remaining in the tubes. Thus the effect of sodium fluoride on the pepsin could be determined.

The tubes containing the egg white were prepared in the following manner: Glass tubing 1 to 1½ mm. in diameter

was cut into 20 cm. lengths, carefully cleaned and the ends drawn out into capillaries. The fresh, uncooked white of an egg was cut into pieces, filtered through cheese cloth and placed in a vacuum for one hour to prevent the subsequent formation of air bubbles. The egg white was then aspirated into the prepared tubes and coagulated by placing the tubes in water heated to 85° C. and allowing the water to cool slowly. The end of the tubes were sealed by dipping into melted paraffin. The tubes of coagulated egg white were cut into pieces about one and one-half centimeters in length with squared ends just before placing in the solution.

The set of seven standard sodium fluoride solutions of varying concentrations used in the previous experiment were also adopted for this work.

The Mett's tubes of egg white were placed in a test tube containing 10 cc. of hydrochloric acid with a pH of 2 (which is the optimum pH of the enzyme pepsin). Two cc. of varying concentrations of sodium fluoride solution or distilled water (in the control tube) and 2 cc. of a freshly prepared 0.3 to 0.4% pepsin solution were added, making a total of 14 cc. The test tubes were placed in a water bath at 40° C. and kept there for about 60 hours or until approximately half of the egg white was digested. The tubes were then removed and measured. The following results were obtained:

Molarity of sodium fluoride solution	mm. of egg white dissolved
0.0000	6.5
0.0036	4.4
0.0072	3.5
0.0108	2.5
0.0144	1.0
0.0180	1.5
0.0216	1.0
0.0252	1.0

In this experiment a great difference was found in the amount of egg white digested in the control tube and those tubes containing sodium fluoride. It may be seen that the amount of egg white digested varied with the concentration of sodium fluoride. For example, in the control tube 6.5 mm. of egg white are dissolved as compared with 4.4 mm. for the lowest concentration of sodium fluoride and 1.0 mm. for the highest.

A measurement of the pH at the end of the experiment showed that as the concentration of sodium fluoride increased so did the pH of the solution. This was due to the hydrolysis of sodium fluoride which is the salt of a strong base and a weak acid. The highest concentration of sodium fluoride increased the pH of the mixture from 2.0 to 3.7. Northrop[10] found that hydroxyl ions have a strong inactivating effect upon pepsin. It was necessary to find whether or not this decrease in the amount of egg white dissolved in the sodium fluoride containing tubes was only an effect of hydroxyl ions on pepsin. To do this a control series was made up by replacing the 2 cc. of sodium fluoride

solution by 2 cc. of sodium hydroxide of such concentration that it produced in the mixture the same pH as the corresponding sodium fluoride solution. The pH of all these solutions was determined colorimetrically by means of the La Motte colorimeter. When these two series were tested out there appeared to be no difference in the amount of egg white dissolved at a given pH regardless of the presence or absence of the fluoride ion.

Sol. No.	Molarity of NaF in expt'l. tubes	pH of mixture	mm. of egg white dissolved in NaF tubes	mm. of egg white dissolved in control tube made up with NaOH
(0)	0.0000	2.0	6.5	—
1	0.0036	2.1	4.3	4.8
2	0.0072	2.3	3.5	3.5
3	0.0108	2.6	2.5	1.8
4	0.0144	3.0	1.0	1.3
5	0.0180	3.4	1.5	1.0
6	0.0216	3.7	1.0	1.3
7	0.0252	3.7	1.0	1.0

Thus it seemed that the amount of egg white dissolved depended upon the pH of the solution rather than upon the concentration of sodium fluoride. For example, at a pH of 2.3, 3.5 mm. of egg white were dissolved in both the tubes regardless of whether this pH was brought about by the hydrolysis of sodium fluoride or the addition of sodium hydroxide.

Pepsin is practically inactivated at pH 4[10] and since the highest concentration of sodium fluoride produced a pH of 3.7 the conditions were so far from the optimum

condition for the enzyme that the results might well be questioned.

In order to overcome the objection of making measurements at pH which was so far from optimum, another series of tubes were prepared by adding such amounts of hydrochloric acid to the sodium fluoride tubes that a constant pH of 2 was obtained for the entire series. The following results were obtained:

Sol. No.	Molarity of NaF in expt'l. tubes	Mm. of egg white dissolved when pH of solution varies on hydrolysis of NaF	Mm. of egg white dissolved when pH is held constant by add'n. of HCl
1	0.0036	4.5 at pH 2.1	4.5 at pH 2.0
2	0.0072	3.8 at pH 2.3	4.6 at pH 2.0
3	0.0108	2.4 at pH 2.6	4.8 at pH 2.0
4	0.0144	1.1 at pH 3.0	5.5 at pH 2.0
5	0.0180	1.3 at pH 3.4	6.1 at pH 2.0
6	0.0216	0.8 at pH 3.7	5.5 at pH 2.0
7	0.0252	0.8 at pH 3.7	6.3 at pH 2.0

From this work it appears that the chloride ion has an accelerating effect on pepsin, for as the amount of this ion increased so also did the amount of egg white dissolved. To the lowest concentration of sodium fluoride it was necessary to add only a small amount of hydrochloric acid to keep the pH at 2, and the amount of egg white digested in this tube was 4.5 mm. But as the amount of hydrochloric acid increased in order to overcome the hydroxyl ions produced by the highest concentration of sodium fluoride the amount of egg white dissolved also increased, namely, to 6.3 mm.

This work was repeated using as a control for this series another series to which sodium chloride was added in such concentrations that the chloride content was matched. By this arrangement both of these series have the optimum pH of pepsin, both have the same amount of chloride ion and the only difference was in the fluoride ion of the one series. The following results were obtained:

Sol. No.	Molarity of NaF in expt'l. tubes.	Mm. of egg white dissolved at pH 2 in NaF tubes.	Mm. of egg white dissolved at pH 2 in control tubes of same chloride content.
1	0.0036	5.0	5.0
2	0.0072	6.0	6.0
3	0.0108	6.0	6.3
4	0.0144	5.5	6.0
5	0.0180	6.5	6.5
6	0.0216	6.0	7.0
7	0.0252	6.5	7.0

For this work no difference was noted that was within the limits of error of the experiment which could be attributed to the fluoride ion. The effect of chloride ion was found to be so great in proportion to any small effect of the fluoride that no safe conclusions could be drawn. When, for example, the amount of chloride ion was the same in the tube containing 0.0036 M sodium fluoride and in the control tube without sodium fluoride, the amount of egg white dissolved was 5.0 mm. in both cases. At a higher concentration of 0.0252 M sodium fluoride the amount dissolved was 6.5 mm. as compared with 7.0 mm. in the

control.

Since the introduction of various ions influenced peptic activity the next step was to try to buffer the solution so that the pH and at the same time the amount of foreign ions could be kept constant. It was very difficult to find a buffer which would produce a pH 2 and at the same time give sufficient buffering action so that the addition of sodium fluoride did not markedly increase the pH. The Sorensen[11] glycine-hydrochloric acid buffer proved to be the most efficient. Although it was not possible to keep the pH constant at 2, it was possible to very markedly limit the range of pH so that it did not vary so greatly from the optimum pH of pepsin as it did in the tubes previously used without a buffer. Without a buffer the addition of the highest concentration of sodium fluoride changed the pH from 2.0 to 3.7. When the Sorensen buffer was used the addition of the highest concentration of sodium fluoride only changed the pH from 1.90 to 2.35.

This buffer solution is made up of one liter of exactly tenth normal solution of hydrochloric acid and one liter of a tenth molecular glycocoll solution (7.505 gms.) containing sodium chloride (5.85 gms.). The tubes were made up as follows: ten cc. of the buffer solution was pipetted into a test tube, 2 cc. of a 0.3 to 0.4% pepsin made up in buffer solution, 2 cc. of sodium fluoride or sodium hydroxide solution and two Mett's tubes were added.

In this latter work every possible precaution was taken in order to make the method as accurate as possible. The salts were recrystallized at least three times and all the solutions were made up in redistilled water. The Mett's tubes were carefully prepared and checked for defects before using. The pH of all the solutions were taken electrometrically using the quinhydrone electrode. Since the buffering action was not sufficient to keep the hydrogen ion concentration at exactly pH 2 and since even a small increase in the hydroxyl ions has a marked effect upon peptic activity the experiment was most carefully controlled. To accomplish this a series of tubes were made up with the same amount of buffer and enzyme solution plus sodium hydroxide of such a concentration as to equalize the pH to that of the corresponding sodium fluoride tubes.

Results:

The effect of sodium fluoride on the digestion of egg white is shown in Table 2. As the concentration of

TABLE 2
THE EFFECT OF SODIUM FLUORIDE ON THE DISSOLUTION OF EGG WHITE BY PEPSIN AT 38° C.

Sol. No.	pH	Molarity of NaF in fluorine series	Mm. of Egg White Dissolved (Average of 2 trials.)									
			Expt. 1: NaF	Control	Expt. 2: NaF	Control	Expt. 3: NaF	Control	Expt. 4: NaF	Control	Expt. 5: NaF	Control
0	1.90	.0000	5.5	-	7.8	-	8.0	-	8.0	-	10.0	-
1	2.00	.0036	6.0	4.5	7.3	7.3	6.9	7.0	7.0	7.1	8.2	8.3
2	2.05	.0072	6.2	6.9	7.2	7.3	6.3	6.3	7.0	6.8	7.5	7.2
3	2.12	.0108	6.0	5.4	5.5	5.9	5.5	5.1	5.8	5.0	8.0	9.0
4	2.17	.0144	4.7	4.6	5.7	5.3	5.0	4.7	5.5	5.2	5.7	6.3
5	2.23	.0180	4.4	4.6	4.8	5.0	4.2	4.3	5.1	4.5	5.5	5.3
6	2.30	.0216	4.1	3.8	4.3	4.1	4.1	3.5	4.5	4.2	5.3	6.3
7	2.35	.0252	3.7	3.6	3.2	3.9	3.3	3.7	4.1	4.0	4.7	4.8

sodium fluoride increases there was a decrease in the amount of egg white dissolved. However, by a comparison with the corresponding control tube made up to the same pH with sodium hydroxide, it may be seen that this difference is due to the increase in hydroxyl ions. For example, in experiment 4, the amount of egg white dissolved decreased from 8.0 mm. in the tube containing no sodium fluoride to 4.1 mm. in the tube containing 0.0252 M sodium fluoride but the pH of these tubes was 1.90 and 2.35 respectively. A comparison of the amount of egg white dissolved in a tube of the same pH, namely, 2.35, without fluoride shows that 4.0 mm. were dissolved. Likewise, in the same experiment in the tube containing 0.0036 M sodium fluoride with a pH of 2.00 the amount of egg white dissolved was 7.0 mm. as compared with 7.1 mm. in the sodium hydroxide tube of the same pH.

Thus it is shown that sodium fluoride did not influence the digestion of egg white by pepsin in any of the concentrations used. Any effect recorded is believed to be due to the influence of the hydroxyl rather than the fluoride ion.

PART 2. THE EFFECT OF SODIUM FLUORIDE ON THE COEFFICIENT OF DIGESTIBILITY OF PROTEIN IN WHITE RATS

The effect of sodium fluoride on the proteolytic enzymes in vivo was determined by a study of the percentage of protein digested by white rats on a diet containing sodium fluoride as compared with those on diets without this salt. The results are expressed in terms of coefficient of digestibility which may be defined as follows:

$$\text{Coefficient of Digestibility} = \frac{\text{Food nitrogen} - \text{Fecal nitrogen}}{\text{Food nitrogen}} \times 100$$

Methods and Materials:

The coefficients of digestibility were obtained for young growing rats and for young adult animals. The control animals received diet No. 13 consisting of one third whole milk powder, two thirds whole wheat and 1.33% sodium chloride. To this basal diet was added 0.05% and 0.1% sodium fluoride and these diets were numbered 215 and 214 respectively.

The rats were kept in individual round cages. Scattering of food was prevented by using a lid over the food jar which resembled a shallow inverted funnel with a hole in the middle just large enough for the rat's head. Distilled water was supplied from a bottle attached to the outside of the cage.

In the first set of experiments young rats were taken at weaning, separated and placed on the various diets for about two weeks in order that they become adjusted to these diets. At the end of this foreperiod the experiments were started. There were two five day experimental periods with three days intervening. Careful food records were kept and all the feces were collected each day and placed in acid or air dried in an oven at 110° F. These two methods of preservation were compared to make sure there was no loss of nitrogen in drying.

For the older animals a slightly different procedure of determining the coefficient of digestibility was adopted because they scatter food and because their feces do not go through the meshes of the cage easily, and hence may become contaminated by the food or consumed by the rat. In order to overcome these difficulties the Bergeim Method[12] was used, for this method does away with the necessity of keeping food records and making total collection of feces. In this method the ratio of iron to nitrogen in the food and feces is determined and compared, and from this the

ratio of nitrogen in the food to nitrogen in the feces and hence the coefficient of digestibility can be calculated.

The method is based upon the assumption that iron is 100% excreted by way of the feces and is not stored to any extent in normal metabolism. If the ratio of nitrogen to iron in the food and feces is known and if the iron is 100% excreted in the feces, we can calculate the retention of nitrogen and hence obtain the coefficient of digestibility. For example, if the ratio, nitrogen to iron, in the feces is 4 to 1 and in the food is 10 to 1, the unabsorbed nitrogen is 4 to 10 or 40% and the utilized nitrogen is 100-40 or 60%. The Bergeim method was tested by Gallup[13] and Heller, Breedlove and Likely[14] by comparing with the usual method and was found to be satisfactory.

The animals used in these latter experiments were young adults that had been on the diet for a period of a month or more. Although the cages used were galvanized iron the amount of contamination of feces with iron was not considered significant since the feces were collected many times during the day.

The nitrogen in the food and feces was determined by modifications of the Kjeldahl method. After the feces were dried and weighed they were ground in a mortar and samples taken for nitrogen determinations. For the feces thus treated the Gunning Hibbard[15] modification of the Kjeldahl method was used. When the feces were placed in

acid, all the feces from each rat for the entire five day period were digested by the Dyer-Kjeldahl[16] method. The latter method causes the oxidation to take place more quickly and hence the time required to digest this large amount of material was greatly decreased.

The iron was determined by Rose's[17] modification of the Zimmerman Reinhard method.

The food and feces were ashed, a small amount of Hcl (2 to 3 cc.) and 20 to 30 cc. of water was added. After boiling a few minutes, a 1 M solution of stannous chloride was added drop by drop until the yellow color disappeared and then exactly two drops in excess were added. The solution was cooled to about 20° C. and 10 cc. of 0.25 M mercuric chloride were added, stirring vigorously. After diluting to about 100 or 125 cc., 10 cc. of "preventive solution" (2M H_2SO_4, 2M H_3PO_4 and 0.3M $MnSO_4$) were added and the mixture titrated with potassium permanganate solution the strength of 4 mg. of iron per cubic centimeter (0.00223 grams per cc.).

Results:

The effect of sodium fluoride on the coefficient of digestibility of protein is shown in Tables 3 and 4. The

TABLE 3

THE EFFECT OF SODIUM FLUORIDE ON THE COEFFICIENT OF DIGESTIBILITY IN YOUNG RATS (FIVE DAY PERIOD)

Rat No.	Period No.	Fecal Nitrogen (grams)	Food Nitrogen (grams)	Coefficient of Digestibility	Average
Diet #13 containing 0.00% NaF					
9469 ♀	1	.01361	.12462	89.1	
	2	.01502	.17996	91.7	
9464 ♀	1	.01737	.15980	89.1	
	2	.01840	.16619	89.9	
9731 ♂	1	.01370	.12464	89.0	
	2	.01345	.10227	86.9	
9735 ♂	1	-	-	-	
	2	.01011	.08949	88.7	89.2 ± .37
Diet #215 containing 0.05% NaF					
9467 ♀	1	.01063	.10866	90.3	
	2	.00858	.11186	92.3	
9466 ♀	1	.01470	.14702	90.5	
	2	.00726	.08949	91.9	
9730 ♂	1	.00930	.08310	88.8	
	2	.01165	.11186	89.6	
9734 ♂	1	.00846	.07351	88.4	
	2	.01039	.09588	89.2	90.1 ± .25
Diet #214 containing 0.10% NaF					
9468 ♀	1	.00577	.06392	90.8	
	2	.00498	.03196	84.4	
9465 ♀	1	.00799	.07990	90.0	
	2	.00562	.07670	92.7	
9727 ♂	1	.00773	.07670	89.9	
	2	.01002	.09588	89.6	
9733 ♂	1	.00890	.07670	88.4	
	2	.00940	.08945	89.5	89.4 ± .57

TABLE 4

THE EFFECT OF SODIUM FLUORIDE ON THE COEFFICIENT OF DIGESTIBILITY OF PROTEIN IN ADULT RATS (BERGEIM METHOD)

Rat No.	Period No.	Ratio Protein/Fe in Food	Mgs. of Iron per gram of Feces	Mgs. of Protein per gram of Feces	Ratio Protein/Fe in Feces		Coefficient of Digestibility	Average
Diet #13 containing 0.00% NaF								
8860♂	1	7.23	1	.2392	.0201	8.42	1	88.4	
	2	"		.2910	.0194	6.67	1	90.8	
7246♂	1	"		.3335	.0242	7.22	1	90.0	
	2	"		.2664	.0226	8.47	1	88.3	
8857♂	1	"		.3117	.0200	6.41	1	91.1	
	2	"		.2650	.0221	8.34	1	88.5	89.5 ± .34
Diet #215 containing 0.05% NaF								
8859♂	1	7.23	1	.2190	.0217	9.92	1	86.3	
	2	"		.3142	.0192	6.10	1	91.6	
8060♂	1	"		.2700	.0219	8.11	1	88.8	
	2	"		.2965	.0226	7.61	1	89.5	
8072♂	1	"		.3251	.0232	7.13	1	90.1	89.3 ± .59
Diet #214 containing 0.10% NaF								
8209♂	1	7.23	1	.2597	.0204	7.88	1	89.2	
	2	"		.2548	.0242	9.50	1	86.9	
8858♂	1	"		.3108	.0237	7.45	1	89.7	
	2	"		.2687	.0205	7.65	1	89.5	88.8 ± .42

coefficient of digestibility of young rats on the control diet containing no sodium fluoride is 89.2 ± .37. Litter mates on the same basal diet to which 0.05 and 0.1% sodium fluoride had been added have coefficients of digestibility of 90.1 ± .25 and 89.4 ± .57 respectively. The adult rats on the control diet have an average coefficient of

digestibility of 89.5±.34 as compared with 89.3±.59 and 88.8±.42 on the fluorine diets. Hence sodium fluoride at this concentration in the diet did not affect the amount of protein digested. It may also be noted that there was no difference in ability to digest protein between the young and old rats.

PART 3. THE EFFECT OF SODIUM FLUORIDE ON THE METABOLISM OF PROTEIN IN WHITE RATS

The effect of sodium fluoride on the metabolism of protein in white rats was studied by means of a series of nitrogen balance experiments. White rats were taken at weaning and placed on the control diet number 13 and experimental diets numbers 215 and 214, which consist of the control diet plus 0.05 and 0.1% NaF respectively, a foreperiod of two weeks being allowed in order that the rats might become accustomed to the diet. Four experimental periods of five days each were adopted. There was an intervening period of three days between the first two and last two balances and a period of fourteen days between the second and third balances.

Methods and Materials:

The Mitchell[18] balance method was modified for this work. Individual round cages were used, distilled water was supplied from a bottle on the side of the cage, a

non-scatter top was used on the food cup, the cage was placed on a plate covered with two filter papers for collecting the urine and feces. Careful food records were kept. The filter paper used had been previously treated with dilute hydrochloric acid (10% by volume) and dried. Thus the urine could be collected on the filter paper without loss of ammonia.

Each day the upper filter paper was removed and a fresh paper put on the bottom. The paper which was removed was cut into strips and the urine washed out with dilute hydrochloric acid, about 200 to 300 cc. divided into six to eight washings being necessary to completely remove the urine from a filter paper. When the washings were colorless the paper was considered free from urine.

These washings were filtered and the composite washings for each rat were kept in a bottle under toluene. The total washings from the cage and papers were made up to two liters and 100 cc. aliquots taken for nitrogen analysis. The ordinary Kjeldahl method[15] was used for the nitrogen analysis for food and feces.

The feces were collected daily and placed in dilute hydrochloric acid to which toluene had been added. The total collection for each rat was digested by the Dyer-Kjeldahl[16] method and aliquots, usually one third or more of the total, were taken for nitrogen analysis.

The amount of nitrogen injested was determined by

analyzing the amount of nitrogen in a sample of food and multiplying the nitrogen in one gram by the grams of food eaten. The output in the feces and urine had also been determined by analysis. Hence the nitrogen balance could be computed.

Results:

The effect of sodium fluoride in the metabolism of protein in white rats is shown in Tables 5 and 6.

The total nitrogen retained per day varied markedly. In Balance 1 the amount retained by the control animal was 10.80 mgs. as compared with 4.10 and 5.93 mgs. for the animals receiving 0.1% sodium fluoride. This general trend also appears in Balances 3 and 4, though not to such a marked extent. However, this figure itself is not significant because the control animals were larger and hence ate more food, gained more weight, and therefore naturally stored more protein.

In order to eliminate these factors the following things were calculated: First, milligrams of nitrogen retained per gram of body weight. This column shows that there was no significant difference in the nitrogen retained in the control animals and those receiving sodium fluoride when the factor of weight was ruled out. The amount varied from 0.1 to 0.4 mgs. per gram of body weight in the individual animals. Second, milligrams retained per

TABLE 5
THE EFFECT OF SODIUM FLUORIDE ON THE METABOLISM OF PROTEIN IN WHITE RATS

Rat No.	Diet No.	% NaF	Wt. of Rat (Grams)	gain in wt. (grams)	food consumed (grams)	Fecal Nitrogen (grams)	Urinary Nitrogen (grams)	Food Nitrogen (grams)	Balance (grams)
Balance #1 Age 42-47 days									
9731	13	0.00	105-122	17	39	.01370	.05692	.12464	.05402
9735	13	0.00	102-114	12	36	-	-	-	-
9730	215	0.05	99-109	10	26	.00930	.04183	.08310	.03197
9734	215	0.05	76- 94	18	23	.00846	.02674	.07351	.03831
9729	214	0.10	87- 95	8	24	.00773	.04800	.07670	.02097
9733	214	0.10	69- 79	10	24	.00890	.03814	.07670	.02966
Balance #2 Age 50-55 days									
9731	13	0.00	131-136	6	32	.01345	.06016	.10227	.02866
9735	13	0.00	120-129	9	28	.01011	.05184	.08949	.02755
9730	215	0.05	111-126	15	35	.01165	.04661	.11186	.05360
9734	215	0.05	88- 96	8	30	.01039	.04607	.09588	.03943
9729	214	0.10	96-110	14	30	.01002	.05446	.09588	.03140
9733	214	0.10	83- 91	8	28	.00940	.04260	.08949	.03748
Balance #3 Age 69-74 days									
9731	13	0.00	145-179	24	65	.02143	.15034	.20092	.02916
9735	13	0.00	153-173	20	61	.01848	.10198	.18855	.06809
9730	215	0.05	153-171	18	52	.01917	.09135	.16073	.05021
9734	215	0.05	125-138	13	45	.01788	.08436	.13910	.03687
9729	214	0.10	121-130	9	37	.01335	.07555	.11437	.02547
9733	214	0.10	94-104	10	30	.01195	.06077	.09273	.02010
Balance #4 Age 77-82 days									
9731	13	0.00	178-203	20	61	.01891	.11333	.19782	.06558
9735	13	0.00	171-194	23	58	.01646	.10611	.17928	.05671
9730	215	0.05	168-181	13	48	.01895	.09921	.14368	.03052
9734	215	0.05	144-150	6	45	.01963	.10198	.13910	.01750
9729	214	0.10	140-145	5	40	.01442	.07504	.12364	.03418
9733	214	0.10	104-112	8	30	.01115	.06223	.09273	.01935

TABLE 6
SUMMARY OF THE EFFECT OF SODIUM FLUORIDE ON THE METABOLISM OF PROTEIN IN WHITE RATS

Rat No.	Diet No.	% of NaF	Total N_2 retained per day (mgs.)	N_2 retained per gm. body weight (mgs.)	N_2 retained per gm. gain in weight (mgs.)	% N_2 intake retained	Gain per gram of food (grams)	N_2 retained per gram of food (mgs.)
Balance #1 Age 42-47 days								
9731	13	0.00	10.80	.443	3.18	45.3	.435	1.385
9735	13	0.00	-	-	-	-	.333	-
9730	215	0.05	6.39	.293	3.20	38.5	.385	1.230
9734	215	0.05	7.66	.408	2.13	52.1	.782	1.666
9727	214	0.10	4.19	.221	2.62	27.3	.333	0.874
9733	214	0.10	5.93	.375	2.96	38.7	.416	1.236
Balance #2 Age 50-55 days								
9731	13	0.00	5.73	.211	4.78	28.0	.188	0.896
9735	13	0.00	5.51	.214	3.06	30.8	.321	0.984
9730	215	0.05	10.72	.425	3.57	45.2	.428	1.531
9734	215	0.05	7.89	.411	4.93	41.1	.267	1.314
9727	214	0.10	6.28	.285	2.24	32.7	.466	1.046
9733	214	0.10	7.50	.412	4.61	41.9	.286	1.335
Balance #3 Age 69-74 days								
9731	13	0.00	5.83	.163	1.22	14.5	.369	0.449
9735	13	0.00	13.62	.394	3.40	26.1	.328	1.162
9730	215	0.05	10.04	.293	2.79	33.2	.346	0.966
9734	215	0.05	7.38	.267	2.84	26.5	.289	0.819
9727	214	0.10	5.09	.196	2.83	22.3	.243	0.688
9733	214	0.10	4.02	.193	2.01	21.7	.333	0.287
Balance #4 Age 77-82 days								
9731	13	0.00	13.12	.323	2.62	33.2	.391	1.025
9735	13	0.00	11.34	.292	2.47	31.6	.397	0.978
9730	215	0.05	6.10	.169	1.58	21.2	.271	0.636
9734	215	0.05	3.50	.117	2.91	12.6	.133	0.389
9727	214	0.10	6.84	.240	6.84	28.6	.125	0.855
9733	214	0.10	4.87	.173	2.42	20.9	.267	0.645

gram gain in weight. This calculation rules out the difference of the increased gain in weight of the control animals. The results show that although animals receiving sodium fluoride do not retain so much they gain less and consequently the nitrogen per unit gain in weight does not vary from that of the controls. The average was about 3 mgs. retained per gram gain in weight. This figure varied from 1.6 to 6.8 mgs. in the individual animals irrespective of diet. Third, the percentage of food nitrogen retained also proved to be a matter of individual difference rather than a factor influenced by sodium fluoride in the diet. This ranged from 12 to 50%. Fourth, although previous work had shown that the grams gain per gram of food over a six weeks period in animals on sodium fluoride diets were much less than the controls and that they were much less efficient in their food utilization, the five day balances did not bring out any significant difference. Fifth, the milligrams of nitrogen retained per gram of food ruled out the influence in the different amounts of food consumed and in the subsequent gain in weight. This showed no difference in the first two balance periods but there appeared to be a slightly greater amount in the control animals in Balances 3 and 4. In Balance 4, for example, the control animals retained 1.025 and 0.978 mgs. as compared with 0.855 and 0.643 mgs. in the rats receiving 0.1% sodium fluoride.

Thus it may be seen that 0.05 and 0.1% sodium fluoride in the diet of white rats does not affect their ability to metabolize nitrogen.

SUMMARY

An investigation was carried out in order to find if the stunted growth and poor food utilization of rats on diets containing 0.1% sodium fluoride was due to an interference of this salt with the digestion or metabolism of protein.

Experiments were first performed in vitro to determine the effect of sodium fluoride on the proteolytic enzyme pepsin, when milk and egg white were used as the substrates. When milk was used as the substrate, the influence of sodium fluoride was determined by noting the coagulation time of milk in a series of tubes containing a definite amount of milk and pepsin solution and varying concentrations of sodium fluoride. The time of coagulation was found to vary with the concentration of sodium fluoride. Sodium fluoride in concentrations below 0.0144 M has a slight accelerating effect upon the pepsin, as shown by an average coagulation time of 2.03 minutes for 0.0036 M, 1.88 minutes for 0.0072 M, and 2.01 minutes for 0.0108 M as compared with 2.34 minutes in the control tube which contained no sodium fluoride. At 0.0144 M, the average

coagulation time was 2.30 minutes, which was practically the same as the coagulation time in the tube containing no sodium fluoride. Above 0.0180 M sodium fluoride the coagulation was inhibited. Between 0.0216 and 0.0252 M the coagulation time of milk by pepsin increased from about six minutes to several hours. The buffering action of milk was sufficient to keep the pH constant at 6 in all of these tubes.

When egg white was used as the substrate the Mett's method was adopted. Small glass tubes containing egg white were placed in test tubes which contained varying concentrations of sodium fluoride. Pepsin was added and the tubes allowed to remain in the water bath at 38° C. for from 48 to 76 hours, while the digestion of egg white took place. At the end of the period the amount of digestion was found by measuring the amount of egg white remaining in the tubes. Thus the effect of sodium fluoride on the hydrolyzing property of pepsin could be noted.

As the concentration of sodium fluoride increased there was a decrease in the amount of egg white dissolved. However, by a comparison with the corresponding control tube made up to the same pH with sodium hydroxide, it was evident that this difference was due to the increase in hydroxyl ions liberated from the hydrolysis of sodium fluoride. For example, in Experiment 4 the amount of egg white dissolved decreased from 8.0 mm. in the tube

containing no sodium fluoride to 4.1 mm. in the tube containing 0.0252 M sodium fluoride but the pH of these tubes was 1.90 and 2.35 respectively. A comparison of the amount of egg white dissolved in a tube of the same pH, namely, 2.35 without fluoride shows that 4.0 mm. were dissolved. Likewise in the same experiment in the tube containing 0.0036 M sodium fluoride with a pH of 2.0 the amount of egg white dissolved was 7.0 mm. as compared with 7.1 mm. in the sodium hydroxide tube of the same pH.

After the effect of sodium fluoride had been studied in vitro experiments were next carried out to find the effect of sodium fluoride on the digestion of protein in vivo. This was accomplished by means of a study of the coefficient of digestibility of protein in rats on a basal diet containing no sodium fluoride as compared with animals receiving the same basal diet plus 0.05 and 0.1% sodium fluoride.

The standard method was used for young rats. The total amount of nitrogen injested for a five day period was determined from carefully kept food records of analyzed diets. The total amount of feces was collected. From the nitrogen injested and the fecal nitrogen the percentage of protein digested was calculated. For the older rats the Bergeim modification of this method was adopted in which it was not necessary to make a total collection of feces. In

this method the ratio of iron to nitrogen in the food and feces was determined and from this the ratio of nitrogen in the food to nitrogen in the feces and hence the coefficient of digestibility was calculated.

The coefficient of digestibility of young rats on the control diets containing no sodium fluoride was found to be 89.2±.37. Litter-mates on the same basal diet to which 0.05 and 0.1% sodium fluoride had been added had coefficients of digestibility of 90.1±.25 and 89.4±.57 respectively. The adult rats on the control diet had an average coefficient of digestibility of 89.5±.34 as compared with 89.3±.59 and 88.8±.42 on the fluorine diets.

Since the stunting of growth might be due either to the interference of sodium fluoride with the digestion or the metabolism of protein, another study was made to find its influence on protein metabolism. This was determined by means of a series of nitrogen balance studies on young growing animals which were placed on experimental diets with and without sodium fluoride. Four five day balance periods were used. These studies showed that while the nitrogen retained per day varied markedly, this figure itself was not significant because the control animals were larger and hence ate more food, gained more weight and naturally stored more protein. For example, in Balance 1 the control animals retained 10.80 mgs. per day as

compared with 4.19 and 5.93 mgs. in animals receiving 0.1% sodium fluoride. When these factors were eliminated, however, there appeared to be no differences in the nitrogen retained on the diets with and without sodium fluoride.

CONCLUSIONS

(1) At low concentrations sodium fluoride accelerated the coagulation of milk by pepsin in vitro while at high concentrations it had an inhibiting effect. A difference in molarity of 0.0036 changed the coagulation time from a few minutes to several hours.

(2) Sodium fluoride did not influence the digestion of egg white by pepsin in vitro in any of the concentrations used. The inhibiting effect recorded was proved to be due to the influence of hydroxyl ions liberated by the hydrolysis of sodium fluoride, rather than the fluoride ions.

(3) Sodium fluoride did not affect the amount of protein digested in white rats as measured by the coefficient of digestibility.

(4) The ability of white rats to metabolize protein was not affected by addition of sodium fluoride to their diet.

(5) It may be concluded that the retarded growth and less efficient utilization of food in rats on diets

containing sodium fluoride is not due to any interference of sodium fluoride with the digestion or metabolism of protein.

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