AN EXPERIMENTAL STUDY OF THE COMPARATIVE EFFECT OF RAPID AND SLOW FREEZING UPON THE CHEMICAL COMPOSITION AND FOOD VALUES OF CERTAIN PROTEIN SUBSTANCES.

D. B. Finn.

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AN EXPERIMENTAL STUDY OF THE COMPARATIVE EFFECT OF RAPID AND SLOW FREEZING UPON THE CHEMICAL COMPOSITION AND FOOD VALUE OF CERTAIN PROTEIN SUBSTANCES

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D.B.Finn

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AN EXPERIMENTAL STUDY OF THE COMPARATIVE EFFECT OF RAPID AND SLOW FREEZING UPON THE CHEMICAL COMPOSITION AND FOOD VALUES OF CERTAIN PROTEIN SUBSTANCES.

One of the most widely used methods for the preservation of food material is that of freezing. Treatment by freezing not only permits storage of food materials for long periods of time, but also leaves the frozen substances, after having been thawed out, in a state which very closely resembles that of unfrozen material. Certain changes can take place in frozen material which render it distinctly inferior to the unfrozen product. Some of these changes may take place in the initial freezing, and the others during the period of storage which follows.

This paper will concern itself with the changes occurring during the initial freezing.

Physical changes are easily observed in frozen tissue, and have been studied in some detail by various workers (1)(2). The mechanical damage which a tissue undergoes is due to the formation of ice crystals, and to increasing internal pressure. When the freezing takes place slowly, the number of crystalization centres seem to be small, with the result that large ice crystals grow within the cell, and eventually bring about the rupture of the containing membrane with a consequent escape of juices which freezes subsequently in the intracellular spaces. When on the other hand the tissue is frozen quickly there are a great number of crystalisation centres formed which result in the occurrence of similar numbers of small crystals. These

small crystals do not rupture the cell membrane, and therefore bring about far less mechanical damage to the tissue. One might expect the phenomenon of freezing which is really one of dehydration to be accompanied by slight changes in the protein materials themselves, since it is almost impossible to cool a bulky tissue down to the eutectic point instantaneously.

In practice the rate of cooling is such that water first freezes as pure ice crystals leaving a solution of salts in the muscle substance which becomes more and more concentrated as the freezing process goes on until an equilibrium is reached at the final temperature. The state of this equilibrium will vary with the final temperature. Thus at a temperature of -2^{0} C. one might expect the following phases to appear,- pure ice, salt in solution, protein in the colloidal state and 'dehydrated' protein. As the temperature was lowered one would expect an increase in pure ice, the appearance of salt crystals once an increase in dehydrated protein.

Upon thawing the series of changes would be reversed but not entirely so (3). Ice would disappear, salts would go into solution and the proteins imbibe a certain amount of water. The extent of this imbibition by proteins is undetermined and would be influenced by the degree of dehydration and the changes which had been brought about by the action of the salt solutions.

The present investigation was undertaken as a preliminary study of the effect of various rates of freezing and thawing upon muscle proteins. This effect has been studied under the following heads:

1. The influence of rates of freezing upon digestion by Trypsin .
2. The effect of the rate of freezing and thawing upon autolytic decomposition.
3. The effect of the rate of freezing and thawing upon the water soluble proteins.
4. The effect of freezing upon some of the extractives.

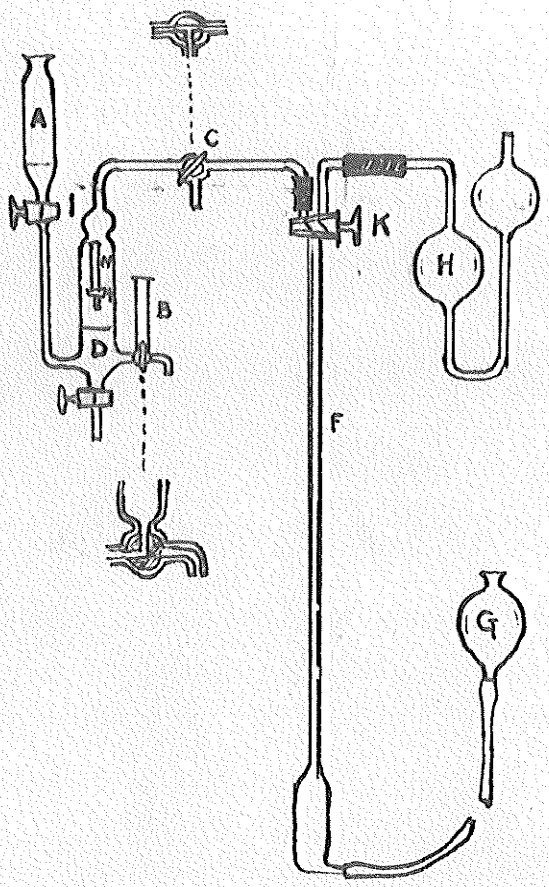
(1) THE INFLUENCE OF RATES OF FREEZING UPON DIGESTION BY TRYPSIN

In this part of the work it was planned to study the effect of rapid and slow freezing upon the digestibility of the protein. The protein used in this instance was muscle from newly killed dog since it was found impossible to obtain absolutely fresh protein in large enough amounts from other sources. Trypsin was used as an enzyme for the reason that greater amounts of amino acid nitrogen would be set free from the digestion, the course of which was followed by means of Van Slyke's Micro Amino Acid Nitrogen apparatus. In preliminary trials with this apparatus it was found difficult to obtain consistent blank determinations.

METHOD:

Water from F (See Plate 1A) fills the capillary leading to the Hempel pippette H and also the other capillary as far as C. Into A one pours a volume of glacial acetic acid sufficient to fill one-fifth of D. For convenience A is etched with a mark to measure this amount. The acid is run into M, Cock C being turned so as to let air escape from D. Through A one now pours sodium nitrite solution (20 gms of $NaNO_2$ to 100 c.c. water) until D is full and enough excess present to rise a little above the cock into A.

PLATE I-A



- FIG I -

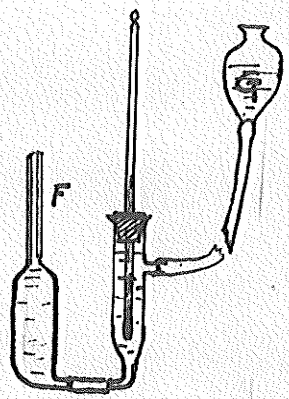


FIG II

-VAN SLYKE AMINO ACID APPARATUS (MICRO)-

D. DEAMINIZING CHAMBER.
F. BURETTE
H. HEMPEL PIPETTE
G. LEVELLING BULB

It is convenient to mark A for the measuring of this amount also. The gas exit from D is now closed at C and, A, being open, D is shaken for a few seconds. The nitric oxide which instantly collects is let out at C and the shaking repeated. The second crop which washes out the last portion of air is also let out at C. D is now connected with a motor and shaken until all but 4 c c of the solution have been displaced by nitric oxide and driven back into A. A mark on D indicates the 4 c c point. One then closes I and turns C and K so that D and F are connected,

The amino acid solution to be analyzed is measured off in B and the desired amount run into D. An antifoam such as N. Heptyl Alcohol is now added from N. D is now shaken until the reaction between the amino acids and nitrous acid is complete (from 5 to 10 minutes), the gas generated collecting in F.

The reaction being complete, all the gas in D is displaced into F by liquid from A and the mixture of nitrogen and nitrous oxide is driven from F into H which contains alkiline permanganate. H is now shaken by means of a motor and the nitrous oxide absorbed. The pure nitrogen is then driven into F and measured.

A modification to the apparatus (Fig. 2, Plate 1A), was made in order to obtain accurate temperature readings of the gas.

Table I shows blanks which were obtained using Kahlbaum $NaNO_2$ and Merck's Glacial Acetic Acid. The sodium nitrite was of Pre-War Stock and is considered to be of high purity. Secondary Capryl Alcohol was used to prevent foaming.

TABLE I.

$NaNO_2$ 30% solution.

Room Temp.	Barom.	Temp. $NaNO_2$,	Time of Agitation: Mins.	Burette reading: c.c.	Mg Amino N.	c.c.	
21°C.	735.2	23°C.	5	0.21	0.115	1	dist.H_2O
21	735.2	23°C	5	0.14	0.076	1	"
21.5	735.2	23°C.	5	0.19	0.104	1	"
21.5	735.2	23°C.	5	0.21	0.115	1	"
21.5	735.2	23°C.	5	0.17	0.093	1	"

Table II shows blank determinations made with a 30% KNO_2 solution, also Kahlbaum. In these blank determinations the acid nitrite mixture was shaken back three times before adding 1 c.c. H_2O for the blank determination.

TABLE II.

KNO_2 30% solution

Room Temp.	Barom.	Temp. KNO_2 sln.	Time of Deaminization: Mins.	Burette reading: c.c.	Mg Amino N	c.c.	
20	743.6	25.7	5	0.15	0.083	1.0	H_2O
19.5	"	25.0	5	0.155	0.086	1.0	"
19.0	"	24.0	5	0.14	0.078	1.0	"
19.0	"	23.5	5	0.155	0.086	1.0	"

In order to examine the effect of variation in the number of crops of NO which were discarded, the determinations reported in Table III. were made:

TABLE III.

30% KNO_2 solution

Room Temp.	Barom.	Temp. of KNO_2 sln.	No. of crops of NO discarded:	Time of Deam. Mins.	Burette: c.c.	Mg Amino N.
19.0°	743.8	21.5	2	10	0.18	0.100
19.0	"	22.	3	10	0.12	0.067
19.0	"	22.	2	10	0.19	0.106
19.0	"	22.	4	10	0.15	0.084

From Table III. it can be seen that there is a slight drop in the blank with increasing numbers of discarded crops.

It was decided to try the method as outlined by Van Slyke (4) in which acid nitrite is shaken back once, and the apparatus arranged so that all gases generated for the next two minutes in the deaminizing chamber were allowed to escape.

TABLE IV.

Room Temp:	Barom:	KNO_2 Time of pre-lim. shaking: mins.	30% solution Time of Deam-inization: mins.	Burette: c.c.	Mg Amino N.	c.c.	
20.	742.6	2	10	0.16	0.088	1	H_2O
20.	"	2	10	0.16	0.088	1	"
20.	"	2	10	0.16	0.088	1	"
19.5	744.4	2	10	0.155	0.086	1	"

Results obtained in this manner are given in Table IV. Seem these results seem fairly consistent, it was decided to check the accuracy of the apparatus by analyzing a 1% solution of alanine.

Table V. gives results which were obtained by using this new procedure of Van Slyke's, and a 1% (approx) solution of Kahlbaum alanine. Considerable variation is evident, and though none of the values come very close to the calculated value, they are not consistent in themselves. For this reason it was decided to study the apparatus until consistent results could be obtained.

TABLE V.

Temp.	Barom:	Time of Deamin. Mins.	Burette Reading: c.c.	Mg.N.	Corrected Mg.N.	Calculated Mg.N	c.c.	
17.5°	733.8	5	1.775	0.98	0.90	0.84		
17.0	"	5	1.685	0.94	0.86	0.84	0.5	Alanine sol
17.0	"	10	1.895	1.05	0.94	0.84	0.5	" "
18.0	"	10	1.795	0.98	0.89	0.84	0.5	" "
18.0	"	10	0.125	0.069	0.069	Blank	0.5	H_2O ~~soln~~
18.0	"	10	0.120	0.066	0.066	"	0.5	" "
18.0	"	10	1.78	0.987	0.92	0.84	0.5	Alanine"
18.5	"	10	1.81	1.00	0.93	0.84	0.5	" soln.
18.0	"	10				Spoiled		
18.0	"	10	1.78	0.987	0.92	0.84	0.5	alanin
18.0	734.8	10	1.78	0.987	0.92	0.84	0.5	" soln.
23.0	"	10	1.97	1.067	0.98	0.84	0.5	"
23.0	"	10	0.185	0.102	0.102	Blank	0.5	H_2O ~~soln.~~
24.0	"	10	0.16	0.086	0.086	"	0.5	" "
23.5	"	10	0.16	0.086	0.087	"	0.5	" "
24.0	"	10	2.1	1.133	1.046	0.84	0.5	alanin
21.0	745.7	10	1.68	0.935	0.903	0.84	0.5	" soln.
21.0	"	10	1.67	0.928	0.897	0.84	0.5	" "
21.0	740.2	10	1.685	0.93	0.905	0.84	0.5	" "

The nature of the inert gas which is collected in a blank determination

The apparatus illustrated in Plate I was constructed for the purpose of recording the volume of gas generated, and to furnish a means for its analysis.

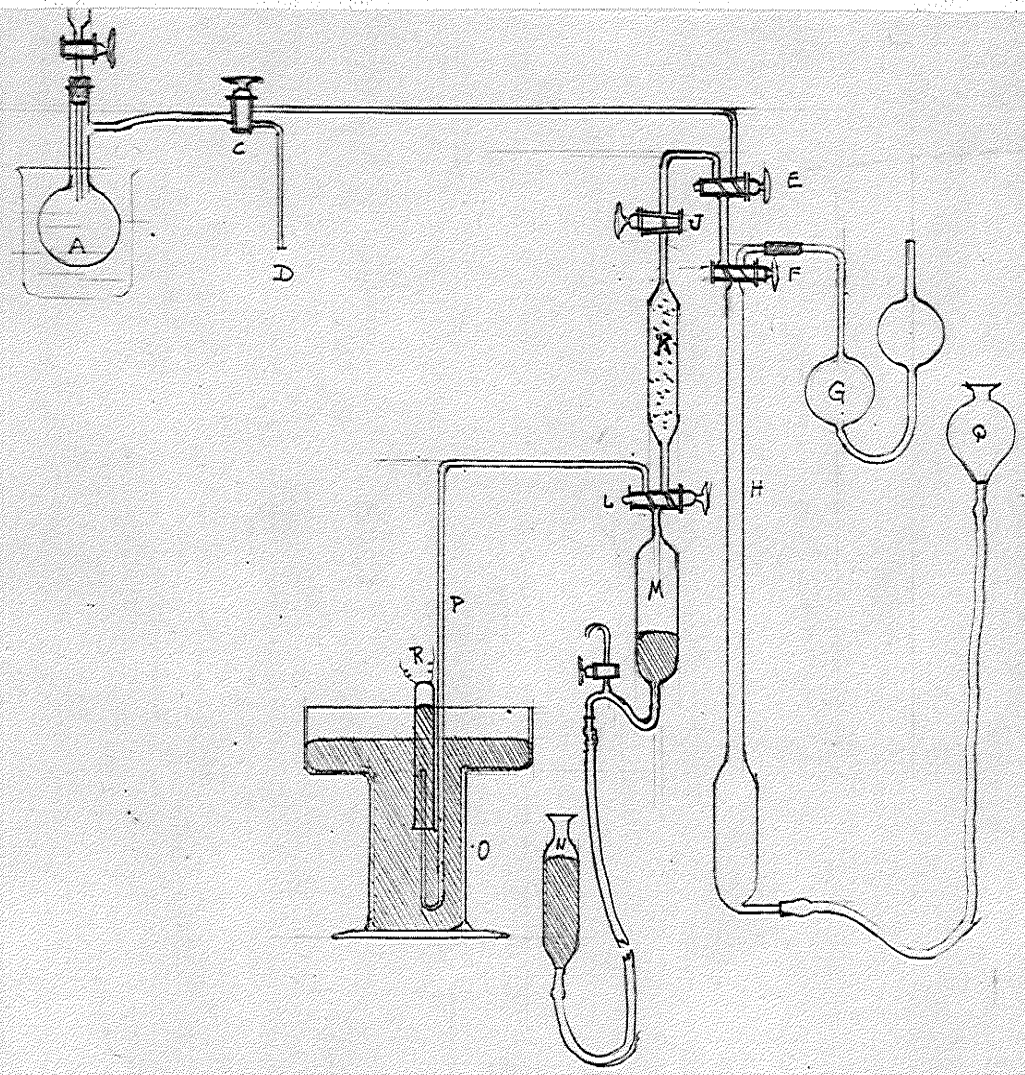


PLATE I

DESCRIPTION OF METHOD:

The Nitrite to be examined eas placed in Flask A until it was completely full, the excess escaping through D. Pipette B was now filled with the glacial acetic acid, and allowed to run into A, excess nitrite escaping from D. The evolved gas was first measured in the burette E, after which it was passed into the Hempel pipette G, which contained alkaline permanganate. The pipette G was now agitated until all the NO has been absorbed. G was then changed for a pipette containing alkaline pyrogallol. The gas meantime being stored in K was then passed into G, and left for a considerable period of time with frequent agitation, after which its volume was again measured. By means of the mercury pump K.M.N. the gas was dried, and placed in the eudiometer in the trough O. The eudiometer R was equipped with platinum electrodes, and connected to an induction coil. Several c.c. of pure O_2 were now placed in R, and a few c.c. of nitrate-free 5 per cent KOH run into the mixed gases. An electric arc was now caused to flow between the electrodes, until there was no further reduction in the volume of the gas. The KOH solution was now drawn from the eudiometer and tested for nitrates. Alkaline pyrogallol was injected into the eudiometer and excess oxygen abrorbed.

Experiments of this nature showed that with a 30 per cent Na NO_2 (Kahlbaum) solution, and glacial acetic acid (Merck B.L.), the gas consisted of a large fraction NO which was absorbed by alkaline permanganate, and a smaller fraction which was oxidized to HNO_3 by means of the electric spark and oxygen, no absorption having taken place in alkaline pyrogallol. Thus the inert gas

which collects during a blank determination is N_2.

Table VI. shows the rate of evolution of successive crops of gas and the proportion of this volume which was inert (N_2).

TABLE VI.
$NaNO_2$ + Glacial CH_3COOH

Room temp. 25°		Temp. of $NaNO_2$ 25°
Time taken to evolve 150 c.c.	Volume of NO + N_2	Vol. of N_2 per 150 c.c.
		c.c.
23 mins.	150 c.c.	1.1
30 mins.	150 c.c.	0.4
95 mins.	150 c.c.	0.4
255 mins.	150 c.c.	0.4

It was thought that the high value of the N_2 from the first 150 c.c. of NO may have been due to air held in solution in the reagents. To overcome this possibility the nitrite and the acetic acid were boiled before mixing and the determination carried out over air free water.

Table VII shows the results obtained with boiled nitrite which was stored under oil and of acetic acid which was boiled, cooled and used immediately.

TABLE VII.

$NaNO_2$ + Acetic Acid, Air Free

Room temperature 24

Temperature of $Na\ NO_2$ + Acetic Acid rising from 24° to 30°

Time for evolution of 150 c.c.	Volume of NO + N_2	Volume of N_2 per 150 c.c.
		c.c.
65 mins.	150 c.c.	1.2
20 mins,	150 c.c.	0.4
10 mins,	150 c.c.	0.3

Volumes of reagents approximately 100 c.c. $NaNO_2$ + 15 c.c. CH_3COOH

From the results shown in the above table it seems unlikely that the 1.2 c.c. is due to air in ~~circulation~~ solution.

In order to determine the period or maximum evolution of N_2 it was decided to analyze successive crops of 50 c.c. each. Table VIII shows results obtained.

TABLE VIII.

Room temperature 25°

Temperature of $Na\ NO_2$ 25°

Time of Evolution:	Volume of NO - N_2	Volume of N_2 per 50 c.c.
	c.c.	c.c.
	50	0.55
	50	0.35
	50	0.15
	50	0.15
	50	0.20
	50	0.20

Volumes of Reagents approximately 100 c.c. $NaNO_2$ + 15 c.c. CH_3COOH

From the preceding it appears that the major evolution of inert gas takes place during the evolution of the first 100 cc of NO and the subsequent crops contain a constant quantity of N_2.

In order to determine the responsibility of acetic acid in the reaction, 7 c.c. $C_2H_4O_2$ were added to approximately 100 c.c. Na NO_2 solution, and the gas allowed to evolve, until the N_2 evolution became constant. 5 additional cc of acetic acid were then added to the acid nitrite,

TABLE IX.

Room temperature 25°
Temperature $NaNO_2$ 25°

Volume of NO - N_2	Volume of N_2 per 50 cc, cc
150 cc.	0.1
150 cc.	0.3
5 cc. Acetic acid added	
150 cc.	0.45

Thus there is a slight increase in the rate of evolution of N_2 when acetic acid is added. This would indicate that the acid may be responsible for the appearance of N_2.

At this point attention was drawn by Doctor Ingvaldsen to the consideration that the secondary capryl alcohol which had been used to prevent foaming in the Van Slyke apparatus may have been responsible for the variation in the values of the blank determination.

In order to determine its role the following procedure was adopted:

The apparatus was thoroughly cleaned and a series of blanks determined without the addition of any antifoam.

TABLE X.

Blanks - No Alcohol - 1 c.c. H_2O (KNO_2 30% soln.)

Temp.	Barom.	Burette Reading. c.c.	Time of Deam.
21°	724	0.085	5 mins.
21°	"	0.083	5 mins.
21°	"	0.085	5 mins.

The results are fairly constant.

Next a series of blanks were determined using one drop (approx. .1 c.c.) of secondary capryl alcohol.

TABLE XI.

Blanks -.1cc secondary Capryl Alcohol - 1 cc H_2O - (KNO_2 30% soln)

Temp.	Barom.	Burette; c.c.	Time of Deam.
21	724	0.16	5 mins.
21	"	0.27	5 mins.

Considerable variation is evident

The apparatus was again thoroughly cleaned and N. Heptyl alcohol substituted for the secondary caprilic. A 30% solution of KNO_2 being used.

TABLE XII.

KNO_2 + Acetic Acid + 1 drop N. Heptyl alcohol + 1 cc. H_2O

Temp.	Barom.	Burette. c.c.	Deam time
20°	724	0.07	5 mins.
21	"	0.073	5 mins.
21	"	0.8	5 mins.

TABLE XIII

30% Na NO_2 + Acetic Acid + 1 drop E. Heptyl alcohol + 1 cc. H_2O

Temp.	Barom.	Doam time.	Burette.
23°	741.6	5 mins.	c.c. 0.11
23°	"	5 mins.	0.135
23°	"	5 mins.	0.125

From the above data it will be seen that secondary capryl alcohol is responsible for considerable variation in the amount of inert gas evolved and that normal heptyl alcohol does not have this effect, and that consistent blanks can be obtained.

Comparison of the Rate of Digestion between Frozen and Unfrozen Egg Albumin.

The whites of two newly laid eggs were separated from the yolks and mixed by gentle whipping.

One half of this albumin was placed in a clean flask, which was immersed in a freezing mixture and the albumin allowed to freeze solid.

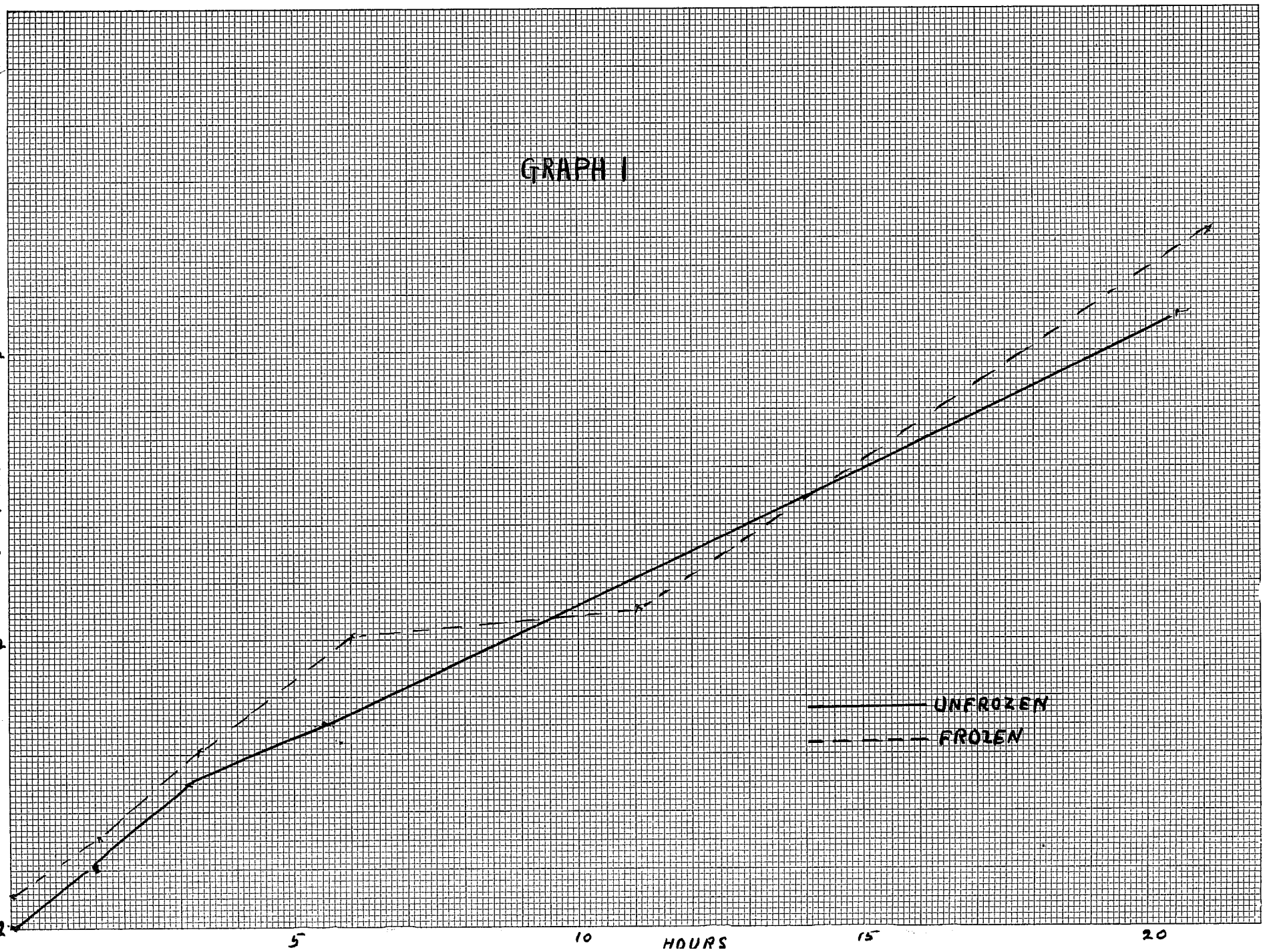
20 c.c. of the remaining albumin were made up to 50 c.c. with 0.5% Na_2CO_3 solution and mixed thoroughly. Five c.c. of this mixture were placed in each of 6 test tubes. To each of the test tubes was added 10 c.c. 0.5% Na_2CO_3. 2 c.c. glycerol ext. of hog pancreas and 2 c.c. toluene, the tubes being stoppered with rubber stoppers to prevent evaporation.

Five of the tubes were placed in an incubator at 37.5°C. the remaining tube being analyzed immediately.

THIS MARGIN RESERVED FOR BINDING.

IF SHEET IS READ THIS WAY (HORIZONTALLY), THIS MUST BE TOP.

IF SHEET IS READ THE OTHER WAY (VERTICALLY), THIS MUST BE LEFT-HAND SIDE.



After having been frozen overnight (x) the frozen portion was treated in exactly the same manner.

The tubes containing the protein were taken from the incubator at intervals of 1, 3, 5 and 21 hours.

TABLE XIV

Shows the results of this experiment.

Temp O°C.	Barom:	Time of Deam. Mins:	Burette Read.	Amino N.	Amino N corrected blank:	N per cc. of a lb.	Time Incubator Hours:	Tube:	Blank used = 0.07 mg N Unfrozen
22	725.8	10	.615	.331	.261	.22	0	A	
22	"	10	.620	.333	.263	.23	0	A	
22	"	10	.66	.355	.285	.24	1-1/3hr	B	
22	"	10	.66	.355	.285	.24	1-1/3	B	
22	"	10	.73	.395	.325	.27	3¼	C	
22	726.4	10	.715	.386	.316	.26	3¼	C	
22	"	10	.705	.376	.306	.26	3¼	C	
24	"	10	.82	.437	.367	.31	5⅓	D	
24	"	10	.82	.437	.367	.31	5⅓	D	
23	739.0	10	1.04	.578	.508	.43	21⅓	E	
23	"	10	1.03	.572	.502	.42	21⅓	E	
									Frozen
25	740.5	10	.65	.351	.281	.23	0	I	
25	"	10	.65	.351	.281	.23	0	I	
25	"	10	.68	.367	.297	.25	1-1/3	II.	
24	"	10	.75	.408	.338	.28	3-1/3	III.	
24	"	10	.76	.412	.342	.29	3-1/3	III	
25	"	10	.81	.438	.368	.31	6	IV.	
25	"	10	.83	.448	.378	.32	6	IV.	
23	745.5	10	.84	.460	.390	.33	11	V.	
23	"	10	.835	.460	.390	.33	11	V.	
22	747.8	10	1.11	.616	.546	.46	21	VI.	
22	"	10	1.10	.610	.540	.44	21	VI	

These results expressed graphically, are shown in Graph 1. From which it will be seen that freezing does not appreciably alter the rate of digestion of egg albumin.

x. The albumin had thawed during the night, the temperature next morning being 5° C.

Examination of the Effect upon the rates of Tryptic digestion upon frozen and unfrozen muscle.

In order that the meat used should have undergone as little post mortum change as possible, it was decided to use muscle from a freshly killed dog.

A dog was killed in anesthesia, and the muscles of the fore and hind limb dissected out and freed as far as possible from tendons and fats, after which they were minced.

Four grams of this minced material were placed in each of three series of seven test tubes, the meat being weighed upon a small piece of white paper which with its contents was then placed into the tube.

Series A to G was not frozen.

Series 1 to 7 was frozen slowly by placing the tubes in between the inner and the storm windows of the laboratory, where, though there was no uniformity of temperature, it was judged that the meat would freeze slowly. The temperature outside was approximately -10° F. and the room temperature 75°F.

Series I to VII were frozen quickly by immersing the tubes into a liquid freezing mixture of $CaCl_2$ and snow which was at a temperature of -15°F.

Series 1 to 7 and I to VII were kept frozen until the tubeswere required for digestion. About 4 hours elapsed between the time that the dog was killed and the minced meat was placed in the freezinf media.

The digestion was carried out in the following manner:

To each tube was added 25 c.c. 0.5% Na_2CO_3, 2 c.c. of activated glycerol extract of hog pancreas and 5 c.c. of toluene. The tube was now stoppered and thoroughly agitated, and placed in an incubator at 37.5° C. Digestion was arrested at various intervals by placing the tube in boiling water for 5 minutes. In this manner the temperature of the contents rose to 95°C. which was maintained for about three minutes. The tube was now cooled and the contents filtered through cheese cloth. The filtrate was centrifuged and the supernatant liquid used for analysis.

The results are shown in Table XV and GraphII from which it can be seen that slowly frozen protein is digested more rapidly by trypsin than is quickly frozen protein. The latter more nearly approaching the rate of digestion of unfrozen muscle.

TABLE XV - Unfrozen Muscle

Temp.	Barom.	c.c. material used for analysis:	Time of Deam:	Burette Reading c.c.	Mg Amino Acid N.	Amino A. N. corrected blank 0.052:	Amino A N per c.c.	Time incubated hours:	Tube:	Mean Amino N per c.c. digest
22	749.7	1.0	10	1.02	.566	.516	.516	0	A	.51
22	"	1.	10	1.05	.573	.521	.521	0	A	
22	"	1.	10	1.32	.724	.682	.682	1	B	.67
21	"	1.	10	1.29	.721	.669	.669	1	B	
21	"	1.	10	2.2	1.23	1.178	1.178	3	C	1.17
21	"	1.	10	2.2	1.23	1.178	1.176	3	C	
21	"	0.75	10	2.23	1.247	1.195	1.593	6	D	1.56
21	"	0.75	10	2.17	1.215	1.163	1.549	6	D	
18	750.	1.0	10	2.78	1.55	1.498	1.49	6	D	
19	751.4	0.5	10	1.89	1.07	1.016	2.02	12	E	2.04
19	"	0.55	10	2.10	1.19	1.138	2.07	12	E	
22	"	0.70	10	2.85	1.59	1.536	2.20	20¾	F	2.20

TABLE XVI - Rapidly Frozen Muscle

22	741.2	1.	10	0.895	.492	.440	.440	0	I	
22	"	1.	10	0.915	.503	.451	.451	0	I	.44
22	"	1.	10	0.895	.492	.440	.440	0	I	
22	"	1.	10	1.33	.730	.678	.678	1	II	.67
22	"	1.	10	1.33	.730	.678	.678	1	II	
22	"	1.	10	1.84	1.013	.961	.961	2	III	
22	"	1.	10	1.76	.968	.916	.916	2	III	.91
22	"	1.	10	1.76	.968	.916	.916	2	III	
22.5	740.4	1.	10	2.47	1.350	1.298	1.298	4	IV	
22	"	1.	10	2.45	1.335	1.283	1.283	4	IV	1.28
22	"	1.	10	2.45	1.335	1.283	1.283	4	IV	
21	"	0.5	10	1.80	0.99	.938	1.876	8½	V	2.03
21	"	0.5	10	1.84	1.15	1.098	2.196	8½	V	
19	748.3	0.5	10	2.20	1.24	1.188	2.376	22½	VI	2.37

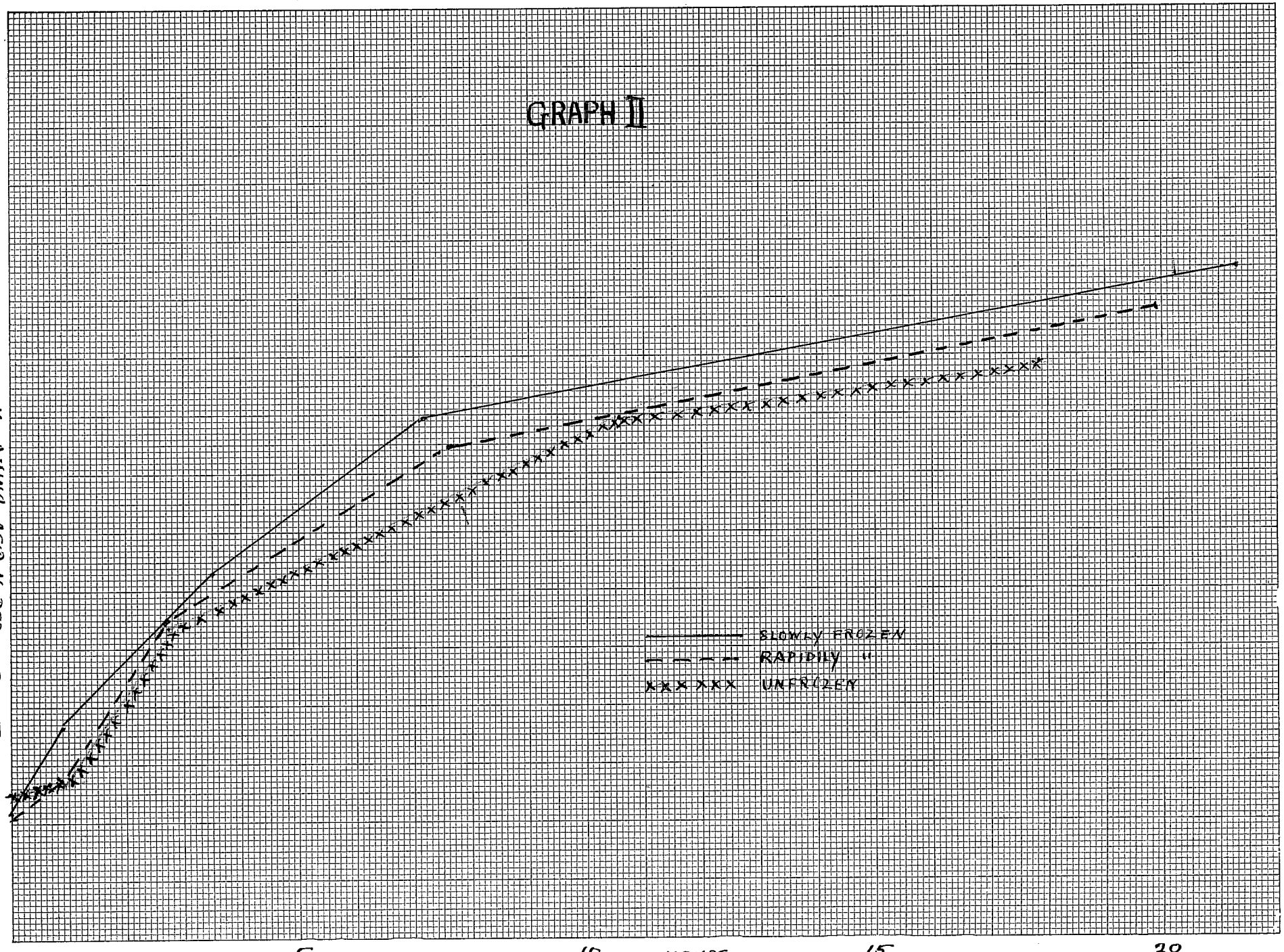
TABLE XVII - Slowly Frozen Muscle

19	748.6	1.	10	0.91	0.514	.462	.462	0	1	.46
19	"	1.	10	0.905	0.511	.459	.459	0	1	
20	"	1.	10	1.51	0.847	.795	.795	1	2	.78
20	"	1.	10	1.47	0.825	.773	.773	1	2	
20	"	1.	10	1.91	1.072	1.020	1.020	3	3	1.03
20	"	1.	10	1.97	1.105	1.053	1.053	3	3	
20	"	1.	10	2.575	1.445	1.393	1.393	4	4	1.39
18	744.	0.5	10	1.84	1.037	.985	1.970	6	5	
18	"	0.5	10	1.78	1.003	.951	1.902	6	5	1.97
19	"	0.5	10	1.86	1.040	.988	1.976	6	5	
19	746	0.5	10	2.37	1.330	1.278	2.556	24	6	2.55

These results are shown graphically in Graph II.

MG AMINO ACID N PER CC DIGEST

GRAPH II



Mg AMINO ACID N PER cc DIGEST

(2) The Effect of the Rate if Freezing and Thawing upon autolytic decomposition.

A dog was anesthetized and bled from the heart. The hind legs were quickly skinned and the muscles removed. Portions of about 10 gms. were freed of fat and tendon, weighed in tiered stoppered glass vessels and covered with toluene. This operation was complete within 40 minutes of the death of the animal.

One series of samples were placed in an atmosphere of -15°C. Freezing was complete in 3 to 4 hours. After being left in the frozen state for 5 hours the material was allowed to remain in the room temperature (22°C) for 70 minutes when thawing was complete. It was now placed in an incubator for 24 hours at 37°C. after which it was minced and extracted for two hours with 50 c.c. of distilled water and 5 c.c. of 3 per cent acetic acid on a boiling water bath. This extraction was repeated foro one hour with a similar quantity of acidulated water. After being filtered the extractives and washings were made to a known volume. Nitrogen was estimated in the solid and liquid portions by the Kjeldahl method.

Another series of samples were treated in exactly the same manner with the omission of freezing.

The results obtained are recorded in Table XVIII. from which it will be seen that freezing does not effect the rate of autolysis as judged by the non-coagulable water soluble nitrogen.

TABLE XVIII.

Soluble non-coagulable nitrogen in per cent of total nitrogen of incubated frozen and non-frozen muscle.

Lab. No.	Frozen:	Lab. No.	Unfrozen.
11 F	16.2	11	16.45
12 F	16.4	12	16.45
13 F	16.5	13	16.2

The effect of rates of freezing and thawing upon the coagulable and non-coagulable water soluble nitrogen was determined in the following manner:

From 1 to 2 grams were weighed into tared centrifugal tubes. The tubes were treated as follows:

Series (1) - Not frozen.

Series (2) - Frozen rapidly, thawed rapidly. (Referred to as F.R.T.R.)

Series (3) - Frozen rapidly, thawed slowly. (Referred to as F.R.T.S.)

Series (4) - Frozen slowly, thawed rapidly. (Referred to as F.S.T.R.)

Series (5) - Frozen slowly, thawed slowly. (Referred to as F.S.T.S.)

The rapid freezing was accomplished by immersing the tubes in a freezing mixture at -27°C. Freezing was complete in about 10 minutes. Slow freezing was obtained by leaving the tubes in an atmosphere of -3° C.(approx). Freezing was complete in from two to three hours. Rapid thawing was brought about by immersing the tubes in water at 60°C. Slow thawing was produced by leaving the tubes in an atmosphere at + 2°C. Thawing was complete in from 2 to 3 hours.

All frozen material was brought to a temperature of approximately -25°C. before thawing.

After this treatment 10 c.c. of distilled water and 2 c.c of toluene were added. The tubes were then incubated for 24 hours at 37°C. after which the material was extracted three times with cold distilled water, each extraction occupying about 20 minutes. The results are summarized in Table XIX.

TABLE XIX.

Water soluble, coagulable and non-coagulable, Nitrogen in per cent of total nitrogen.

Series	Period of incubation. hrs.	Detn. 1.	Detn. 2.	Mean.
Unfrozen	24	28.6	27.5	28.0
F.R.T.R.	24	29.5	29.0	29.25
F.R.T.S.	24	28.2	27.0	27.6
F.S.T.R.	24	28.0	29.1	28.5
F.S.T.S.	24	24.7	--	24.7

No significant difference is evident.

(3) The Effect of Rates of Freezing upon the Water Soluble Proteins.

The effect of freezing upon the non-coagulable nitrogen in muscle tissue is shown in Table XX. The technique employed is exactly as is described on Page except that the materials are not incubated.

TABLE XX.

Water soluble non-coagulable nitrogen in per cent of total nitrogen of frozen and unfrozen muscle tissue.

Lab. No.	Frozen:	Lab. No.	Unfrozen.
14 F	13.39	14	13.5
15 F	14.1	15	12.6
16 F	13.4	16	12.8

Freezing therefore does not effect the non-coagulable N.

The effect of various rates of freezing and thawing upon the coagulable and non-coagulable water soluble nitrogen was determined by cold water extraction of the frozen material, the technique being exactly the same as that described on Page incubation being omitted.

The results are shown in Table XXI.

TABLE XXI.

Water soluble coagulable and non-coagulable nitrogen in per cent of total nitrogen in rapidly and slowly frozen muscle tissue.

Series	Detn. 1	Detn. 2	Mean
Unfrozen	19.	17.	18.5
F.R.T.R.	19.3	18.2	18.7
F.R.T.S.	18.3	--	18.3
F.S.T.R.	22.9	22.6	22.8
F.S.T.S.	25.2	22.0	23.6

Slow freezing increases the coagulable N.

(4) The Effects of Freezing upon some of the extractives of Muscle Tissue.

A. Creatinine:

Creatinine was determined upon a water extract of muscle tissue which had been treated in the following manner:

(1) Unfrozen.

(2) Unfrozen incubated for 24 hours at 27°

(3) Frozen

(4) Frozen incubated for 24 hours at 27°

About 10 grams of material were used for each experiment. The technique of freezing and extractions were exactly as described on Page

The results are summarized in Table XXII.

TABLE XXII.

Creatinine in mg. per gram of tissue.

Unfrozen	- 0.22
Unfrozen incubated	- 0.36
Frozen	- 0.06
Frozen incubated	- 0.18

The creatinine determination was carried out by using the technique described by Cameron for use on blood.

B. Lactic Acid:

A dog was anesthetized and bled from the heart. Similar muscles were removed from each of the hind legs, freed as far as possible from tendon and fat, and split longitudinally. The four samples which were obtained were weighed into glass vessels and covered with toluene. Two were frozen in an atmosphere of -10°C. after which they were thawed by exposure to room temperature. One of these was now incubated at 37°C. for two hours. Of the two samples which were not frozen, one was incubated for 2 hours at 37°C. and the other extracted immediately after weighing.

The following materials were thus obtained:

(1) Unfrozen
(2) Unfrozen incubated
(3) Frozen
(4) Frozen incubated

Extraction was carried out in the following manner. The tissue was minced with 95 per cent ethyl alcohol at a temperature of -5°C. The minced muscle was then washed into a flask with 50 c.c. of cooled 95 per cent alcohol and left in an ice chest for 24 hours. The alcohol was then filtered off and evaporated on a water bath. The residue was treated with saturated ammonium sulphate to remove lipins and proteins. The last traces of alcohol were removed by adding benzene, when the low boiling tertiary mixture alcohol-benzene-water quickly boiled off. The precipitated lipins and proteinsw were filtered off through asbestos. The filtrate and washings were made to a known volume from which aliquot parts were taken for estimation of lactic acid.

Lactic acid was determined by the oxidation method of Freidman Cotonio and Shaffer (10). The accuracy of this method could not be established since neither pure lactates or lactic acid were available. Determinations made upon merk. U.S.P. lactic acid which was stated to contain approximately 85 per cent lactic acid, indicated that the results obtained were if anything too high.

TABLE XXIII

Mg. of Lactic Acid per gram of Tissue.

	Detn. 1	Detn. 2	Mean
Unfrozen	1.4	1.4	1.4
Unfrozen incubated	4.7	4.7	4.7
Frozen	4.0	4.1	4.0
Frozen incubated	5.2	5.1	5.1

C. Glycogen:

The glycogen content of the muscles used in the lactic acid determination was examined.

The method used is described by Boyland[5] and consists of variations on the procedure described by Pflugen[6]. Improvements in the method have been made by Evans[7].

The residue of the tissue insoluble in alcohol is treated for 3 hours on a water bath with 0.25 c.c. 60 per cent KOH for each gram of original material. When cool the digest is diluted with water to the extent of three times the volume of KOH used. Alcohol is now added until the total volume is seven times that of the original volume of KOH. After standing over night the precipitated glycogen is filtered on a Gooch crucible and well washed with 60 per cent alcohol. The glycogen and asbestos are transferred to a flask and digested on a water bath with 2.2 per cent HCl for 5 hours. The solution is then filtered and made to a known volume. Aliquot part of this are taken for the estimation of glucose. Glucose is determined by oxidation with excess alkaline ferricyanide, the excess is measured iodometrically, Hagedoon and Jensen[8], Isserkutz and Both[9].

Twenty-five c.c. of the glucose solution is heated for 15 minutes on a boiling water bath with 20 c.c. of a solution containing 0.33 per cent $K_3Fe(CN)_6$ and 2.12 per cent Na_2CO_3. When cooled 10 c.c. of a solution containing 7.5 per cent $ZnSO_4$, 25 per cent NaCl and 5 per cent KI, followed by 10 c.c. of 3 per cent CH_3COOH, are added. The liberated iodine is titrated with N/100 $Na_2S_2O_3$. A blank is carried out on 20 c.c. of the ferri-

cyanide solution without glucose. It was found easier to obtain consistent results if the acid hydrolysate was first neutralized with NaH CO_3 solution, which procedure was adopted.

Results are summarized in Table XXIV.

TABLE XXIV

Mg. of Glycogen containing 96.2 per cent $(C_6 H_{12} O_6)_N$ per gram of tissue.

	Detn. 1	Detn. 2	Mean
Unfrozen	0.5	0.48	0.49
Unfrozen incubated 2 hours at 37°C	0.	0.	0.
Frozen	0.2	0.3	0.25
Frozen incubated 2 hours at 37°C	0.	0.	0.

DISCUSSION:

The work described must be regarded as a preliminary study of some of the changes which occur in muscle tissue due to freezing and of methods by which these changes can be followed.

It is evident that the use of secondary capryl alcohol in the Van Slyke amino acid apparatus causes variations in the amounts of inert gas which are evolved during a determination. Normal heptyl alcohol does not have this effect. The evolution of the inert gas, which has been shown to be nitrogen, is greatest in the early stages of the reaction and takes place in the absence of any "antifoam" substance. In the later stages the amount of gas evolved becomes constant. This points to the necessity of discarding a sufficient number of the early crops of gas to be

evolved before using the apparatus for an amico acid determination. The nitrogen held in solution by the reagents is of no significance. Impurities such as formates or ~~prussic~~ FORMIC acid in the acetic acid may be responsible for the evolution of nitrogen which theoretically should not occur in the reaction between acetic acid and a nitrite.

Freezing does not affect the rate of tryptic digestion of egg albumin to any extent. From Graph I it will be seen that the rates of digestion are practically the same. This is not surprising since the protein is non-cellular and consequently does not undergo the mechanical damage which increases the contact of the substrate with the engyme, that a cellular material would have undergone during freezing.

Slowly frozen muscle proteins are digested more rapidly by trypsin than quickly frozen proteins, the latter nearly approaching the rate of digestion in unfrozen muscle proteins. That this is due to mechanical damage seems likely since autolysis does not vary in rate in non-frozen and frozen proteins. This is shown in Table XVIII, 16.3 mg. of soluble nitrogen appearing in both the frozen and unfrozen tissue which was free from bacterial activity.

The effect of freezing alone upon the water soluble proteins is shown in Tables XX and XXI, from which it can be seen that there is veryl little effect upon the non coagulable nitrogen. The coagulable water soluble nitrogen seems to be increased by slow freezing, rapid freezing having no influence in its production. Further investigation of this would be of interest.

Lack of time prevented a more thorough examination of the effect of freezing upon the muscle extractives. This work is in

need of extension and verification before conclusions can be drawn.

With regard to the lactic acid determinations it was found that special care was necessary in the removal of the alcohol from the extracted lactic acid. The addition of benzene to the ammonium sulphate solution of the residue from the alcoholic extract was only effective when more traces of alcohol were present. The evaporation of the alcohol extract upon the water bath was therefore carried out until no trace of alcohol could be detected by odour. This procedure might be attended by losses of lactic acid and is in need of investigation. The effect of aldehydes in the alcohol used should also be examined.

S U M M A R Y.

Secondary caprYl alcohol is found to cause a variation in the amount of inert gas evolved during blank determinations carried out in the Van Slyke amino acid apparatus. Normal heptyl alcohol does not have this effect.

The inert gas is shown to be nitrogen and its evolution was found to become constant after an initial period.

Impurities in the acetic acid used are probably responsible for a considerable part of the nitrogen evolved.

Freezing does not affect the rate of tryptic digestion of egg albumin to any extent.

Slowly frozen muscle proteins are more quickly digested by trypsin than are rapidly frozen muscle proteins, the latter more nearly approaching the rate of digestion of unfrozen proteins.

Analysis is not enhanced by freezing.

Slow freezing increases the amount of coagulable water soluble nitrogen in the tissue.

A preliminary examination of the effect of freezing upon some of the muscle extractives is required.

This work was undertaken in an endeavor to find a chemical basis for the unpalatibility of frozen food material with special reference to fish. Since it was impossible to obtain sufficient quantities of fish in which post mortum changes were not well advanced, meat was substituted. The results so far obtained do not seem to shed much light on the problem of unpalatability and it is evident that other means for its solution will have to be found.

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