

**THE CONDITIONS OF ACTION OF "TRYPSIN" ON FIBRIN.** BY H. M. VERNON, M.A., M.D., *Fellow of Magdalen College, Oxford.* (Two Figures in text.)

*(From the Physiological Laboratory, Oxford.)*

OF previous methods for the estimation of the proteolytic power of pancreatic secretion and extracts, that suggested by Mette appears to be the most accurate. This method consists in subjecting short lengths of narrow bore glass tubing containing coagulated egg-albumin to the action of the ferment, and measuring the amount dissolved. Borissoff<sup>1</sup>, and subsequently Walther<sup>2</sup>, have shown that this amount varies as the square root of the relative quantity of ferment present; hence this quantity can be readily calculated. Coagulated egg-albumin, however, is so indigestible that even pure pancreatic juice as a rule dissolves only about 2 mm. in ten hours' digestion at 38° C. The method is therefore useless for measuring the activity of comparatively weak extracts; and apart from this, the necessity of prolonging the digestion for so many hours is, as we shall see later, a potent source of error.

Recently Rachford<sup>3</sup> has determined the effect of bile and of variations of acidity and alkalinity on pancreatic digestion by drying and weighing the fibrin remaining after digestion of known quantities under the different conditions. This method is doubtless an accurate one, but it is somewhat laborious; and as Rachford did not work out the relationship between quantity of ferment and amount of fibrin dissolved, it would be necessary to considerably extend his observations before founding a method of ferment estimation on them.

It should be mentioned that the method of Roberts<sup>4</sup>, which depends on the so-called metacasein reaction, or the rendering of milk

<sup>1</sup> Thesis (in Russian). St Petersburg. 1891.

<sup>2</sup> *Arch. d. Sci. Biol.* VII. p. 15. 1899.

<sup>3</sup> *This Journal*, XXV. p. 165. 1900.

<sup>4</sup> *Proc. Roy. Soc.* XXXII. p. 145. 1881.

coagulable on boiling, is not available for trypsin estimation. As has already been suggested by more than one observer, and as will be proved in detail in a subsequent paper, the reaction is undoubtedly due to a rennet ferment.

The method of trypsin determination to be described depends upon fibrin digestion, like most methods previously used, but in contradistinction to these, it aims at affording an accurate quantitative measure of the relative amount of ferment present. Raw fibrin, previously kept a few weeks in 50 % glycerin, was squeezed as free of glycerin as possible, and then *very* finely chopped. The amount of fibrin used, and the amount left after digestion, was determined volumetrically, after centrifugalisation. The fibrin was measured, and the digestions carried out, in small graduated centrifuge tubes holding 10 c.c. Roughly about 1·8 c.c. of the chopped fibrin having been introduced into the tubes, they were filled up with water and then centrifuged in a small hand centrifuge for two minutes. The fibrin, now compressed to about 1 c.c., had not attained quite a constant volume, as an additional two minutes' centrifugalisation was found on an average to diminish it by about 3 % more. As the physical labour of such long centrifugalisation is somewhat considerable, however, the two minutes' period was kept to throughout. 5 c.c. of water were now withdrawn from each tube, and 5 c.c. of 2 % sodium carbonate solution run in. The fibrin was shaken up, and the tubes kept in a water-bath at 38° C. for about an hour, so as to allow the fibrin to swell. If the fibrin be not swollen previous to the addition of the ferment the results obtained are misleading. The following figures show the times of digestion of 80 % of the volume of fibrin taken, on addition of a weak alcoholic extract of ox pancreas to fresh fibrin, and to fibrin previously swollen in 1 % sodium carbonate for 90 minutes.

Ferment added	Fibrin unswollen	Fibrin swollen	Difference in time
2 c.c.	64 mins.	33·3 mins.	30·7 mins.
1 „	83 „	52 „	31 „
·5 „	143 „	128 „	15 „

The ferment was in both cases acting in a 1 % sodium carbonate solution, but with 2 c.c. of the extract, the digestion time of the swollen fibrin was not much more than half as great as of the unswollen. With half as much ferment, the difference in times was absolutely about as great, but relatively much less. Finally with a quarter as much ferment, the difference was still less, absolutely as well as

relatively. One must assume, therefore, that the ferment can act very little upon the fibrin until it is swollen, and so, whatever the amount of ferment present, there is a certain more or less constant period of delay, during which the fibrin is undergoing its necessary swelling.

On another occasion, it was found that, using 1 c.c. of alcohol extract of ox pancreas, fibrin which had been previously swollen for 31 minutes had 80 % digested in 66 minutes; that swollen for 58 minutes, in 57 minutes; and that swollen for 2 hours, in 63 minutes. An hour seems to be the most favourable time for swelling, therefore, and hence in all subsequent experiments this was adopted as the normal time, it seldom being varied by more than 10 minutes in either direction.

It will have been noticed that the times of digestion of 80 % of the fibrin have been given. It was difficult to decide what particular period in the digestion should be chosen as the standard, and this was eventually adopted as a compromise. It is impossible to determine the time of complete solution, as there is always a small quantity of sediment remaining undigested. Again, if the time of solution of a lesser amount of the fibrin—such as 50 %—be chosen, an error is introduced owing to the fact that the longer the fibrin is exposed to the sodium carbonate the more within certain limits does it swell. After an hour, 1 c.c. of unswollen fibrin attains a volume of about 1.6 c.c., and after two hours, about 1.8 c.c. An apparent 50 % of undigested fibrin in a slowly digesting liquid will therefore represent a more advanced stage of digestion than 50 % of fibrin in a quickly digesting liquid. This is also true to some extent as regards 80 % of digested fibrin, but in this case the error is much smaller. The so-called time of digestion of 80 % really corresponds to a digestion of 88 to 90 % of the raw fibrin originally taken, as no allowance is made for the swelling it has undergone. When apparently all but about .35 to .4 c.c. of the fibrin in a centrifuge tube has dissolved, this tube is centrifugalised for 1 minute, and the volume—now reduced to about .2 c.c.—carefully read off. It is of course impossible to stop the digestion when exactly 80 % has been dissolved, but it is quite easy to hit it off to within 5 % of this amount, and to correct for the small error thereby introduced. It is unnecessary to centrifugalise for more than one minute, as in this time the swollen fibrin has attained to 1 % of the volume it would reach after 2 minutes' centrifugalisation, and 2 % of that after 4 minutes.

The digestions were invariably carried out at 38 ° C. It is necessary

to shake up the contents of the tubes from time to time, the frequency of shaking naturally depending upon the rapidity of digestion. One taking 30 minutes for 80 % solution would be shaken up four or five times in this period ; one an hour, seven or eight times, and so on.

*The relation of digestion to alkalinity.*

The evidence as to the concentration of sodium carbonate most favourable for tryptic digestion is somewhat contradictory. Heidenhain<sup>1</sup> found that in solutions containing moderate amounts of ferment, .9 to 1.2 %  $\text{Na}_2\text{CO}_3$  was the best concentration. Also he states—though he quotes only a single not very conclusive experiment in support of the statement—that weak ferment extracts require a more concentrated solution than active extracts for their maximum action. Kühne<sup>2</sup> found tryptic digestion to be most marked with .3 %  $\text{Na}_2\text{CO}_3$ , whilst Stutzer<sup>3</sup> obtained equally good results with .25, .5, or 1 %  $\text{Na}_2\text{CO}_3$ . These apparent contradictions will be partly accounted for by the results to be detailed, for these prove that it is impossible to fix the most favourable concentration in any general terms. A concentration favourable for one extract may be highly unfavourable for another. The reason of this depends chiefly on the fact, hitherto not properly realised, that trypsin is very rapidly destroyed by sodium carbonate, and also upon the fact that some extracts are very much more sensitive in this respect than others. With a sensitive extract, the most favourable concentration may be as low as .2 %, and with an insensitive one, as high as 1.5 %, or more. For instance, 1 c.c. of an alcohol extract of human pancreas took the following times to digest 80 % of fibrin. This fibrin had been previously swollen for an hour in 1 %  $\text{Na}_2\text{CO}_3$ , and then some of this  $\text{Na}_2\text{CO}_3$  replaced in each tube by the appropriate amounts of water or more concentrated  $\text{Na}_2\text{CO}_3$ .

.2 % $\text{Na}_2\text{CO}_3$	133	minutes
.3        "	82.5	"
.4        "	77.5	"
1.0       "	55	"
1.5       "	53	"

<sup>1</sup> *Arch. f. d. ges. Physiol.* x. p. 575. 1875.

<sup>2</sup> *Untersuch. a. d. physiol. Institut. d. Univ. Heidelberg*, i. p. 223. 1878.

<sup>3</sup> *Zeit. physiol. Chem.* xi. p. 207.

Here we see that, with 1·5%  $\text{Na}_2\text{CO}_3$ , the time of digestion was  $2\frac{1}{2}$  times more rapid than with ·2%  $\text{Na}_2\text{CO}_3$ , and possibly it may have been more rapid still with a higher concentration.

The following values, obtained with a sensitive glycerin extract of human pancreas, show that ·4%  $\text{Na}_2\text{CO}_3$  may act much more favourably than 1%  $\text{Na}_2\text{CO}_3$ , and they also show that the optimum percentage of  $\text{Na}_2\text{CO}_3$  varies according to the dilution. Thus in ·25%  $\text{Na}_2\text{CO}_3$ , the digestion time for ·125 c.c. of ferment was just the same as in ·4%  $\text{Na}_2\text{CO}_3$ , but with half this quantity of ferment it was only 65 minutes instead of 80 minutes. Again, with a large quantity of ferment, the difference in the digestion times with 1% and ·4%  $\text{Na}_2\text{CO}_3$  is scarcely appreciable, but with a small quantity, it varies as about 1 to 1·6.

Volume of extract		1% $\text{Na}_2\text{CO}_3$	·4% $\text{Na}_2\text{CO}_3$	·25% $\text{Na}_2\text{CO}_3$
1	c.c.	9·2 mins.	9 mins.	
·5	"	16·4 "	14·7 "	
·25	"	26·1 "	19·2 "	
·125	"	56·4 "	35 "	35 mins.
·0625	"	131 "	80 "	65 "
·0312	"	256 "	166 "	

A good many other observations were made upon this subject, but it is unnecessary to quote them here. At first ·3%  $\text{Na}_2\text{CO}_3$  was decided upon as the most generally favourable concentration, but as in the case of a few extracts ·4%  $\text{Na}_2\text{CO}_3$  was found to act much more advantageously, this concentration was eventually chosen, and was used in almost all the observations to be described. The tubes of fibrin, after being kept for an hour at 38° with 1%  $\text{Na}_2\text{CO}_3$ , were centrifugalised for a few seconds; 6 c.c. of the supernatant liquid were drawn off, and replaced by 6 c.c. of water, less the volume of extract to be added. Assuming that the swollen fibrin had contained 1%  $\text{Na}_2\text{CO}_3$ , the solution would now contain ·4%  $\text{Na}_2\text{CO}_3$ .

#### *The relative rate of digestion of fibrin.*

The relative rate at which the fibrin undergoes digestion was determined in a number of cases, so as to obtain data for "correcting" the digestion times to exactly 80% digestion. In addition to determining the time of digestion of approximately 80% of the fibrin, many of the tubes were centrifugalised when from 40 to 65% of the fibrin had been dissolved, and also when from 85 to 95%. The times of these various digestion percentages were now calculated proportionately

to that of the 80% digestion time taken as unity, and the values so obtained are given in the accompanying figure.

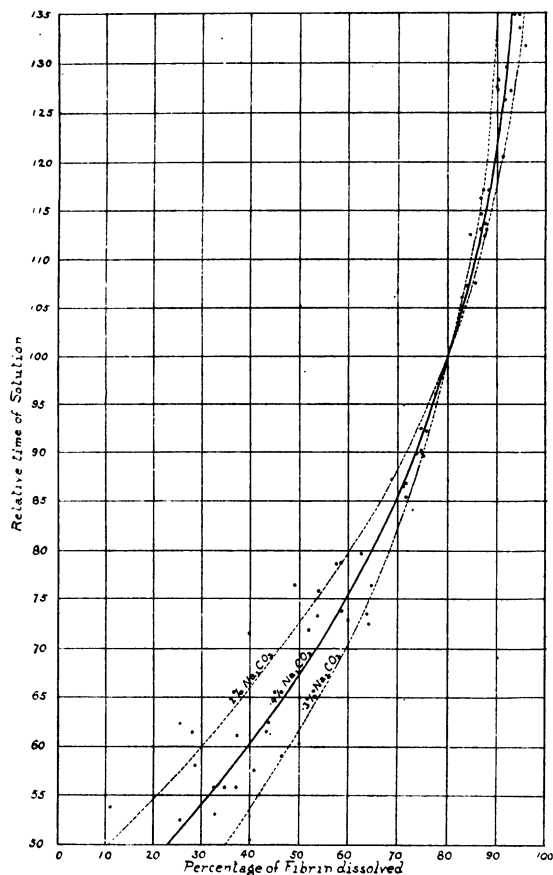


Fig. 1.

The thick line curve is a mean curve drawn as nearly as possible through the middle of these somewhat divergent values, and from it are taken the data used for correcting the various digestion times to exactly 80% digestion. These data are given in the accompanying table. Supposing, for instance, it had been found that 75% of fibrin had been digested in 37 minutes, then one can readily calculate that 80% would be digested in  $\frac{37}{.921} = 40.2$  minutes; or if 83% in 61 minutes, then 80% in  $\frac{61}{1.051} = 58.0$  minutes.

% dissolved	Time of solution	% dissolved	Time of solution	% dissolved	Time of solution	% dissolved	Time of solution
24	·505	62	·772	74	·907	82	1·033
27	·523	63	·781	74·5	·914	82·5	1·042
30	·541	64	·791	75	·921	83	1·051
33	·559	65	·801	75·5	·928	83·5	1·060
36	·578	66	·812	76	·935	84	1·070
39	·597	67	·823	76·5	·943	84·5	1·079
42	·616	68	·834	77	·951	85	1·089
45	·637	69	·845	77·5	·959	86	1·110
48	·658	70	·856	78	·967	87	1·133
50	·673	70·5	·862	78·5	·975	88	1·160
52	·689	71	·868	79	·983	89	1·188
54	·705	71·5	·874	79·5	·991	90	1·220
56	·721	72	·880	80	1·000	91	1·256
58	·737	72·5	·887	80·5	1·008	92	1·300
60	·754	73	·893	81	1·017	93	1·365
61	·763	73·5	·900	81·5	1·025	94	1·450

The divergences exhibited in the values given in the figure are for the most part real, and not due to experimental error. They depend largely on the facts already mentioned, viz., that different extracts vary in their degree of sensitiveness to the destructive action of sodium carbonate, and that the fibrin in slowly digesting solutions swells more than in rapidly digesting.

The thick line curve is for digestions in .4%  $\text{Na}_2\text{CO}_3$  solutions, but a considerable number of determinations of the rate of digestion were also made for 1% and for .3%  $\text{Na}_2\text{CO}_3$  solutions (respectively 49 and 73 values being obtained). In order to avoid confusion these values are not indicated in the figure, but their mean curves are given as dotted lines. From these we see that, taking the time of digestion of 80% of fibrin in each case as the standard, 1%  $\text{Na}_2\text{CO}_3$  digests fibrin relatively more slowly at first, and relatively more rapidly afterwards, whilst with .3%  $\text{Na}_2\text{CO}_3$  the reverse is the case. This is probably owing to the fibrin, on solution, combining with a good deal of the alkali present to form alkali albumin, and perhaps other weak combinations, and so being prevented from assisting the solution of the still undigested fibrin. Thus the less the amount of sodium carbonate present, the more and more do the latter stages of digestion become delayed. For instance, in one case it was found that with .1%  $\text{Na}_2\text{CO}_3$ , it took 36 minutes to dissolve 50% of the fibrin, but no less than 100 minutes to dissolve 80%. The same amount of ferment acting in .4%  $\text{Na}_2\text{CO}_3$ , took 57 minutes to dissolve 50% of the fibrin, but only 80 minutes to dissolve 80%.

The amount of fibrin subjected to digestion was always about 1 c.c. (after centrifugalisation), the actual volume never falling below .9 c.c., and scarcely ever rising above 1.15 c.c. No corrections for variations

in volume were applied, as it was found that the rate of digestion was but little influenced by moderate deviations from unit volume. The subjoined values show that even when the amount of fibrin is halved or doubled, the digestion time is altered by only about 20% in either direction. All these values were obtained with aqueous and normal saline extracts of dogs' pancreas and alcoholic extract of ox pancreas, the digestion time with 1.00 c.c. of fibrin being taken as 100.

Volume of fibrin	Digestion time
·53 c.c.	83.7 mins.
·56 „	90.9 „
·71 „	87.2 „
·89 „	97.7 „
1.00 „	100.0 „
1.82 „	113.6 „
1.93 „	126.4 „
2.00 „	125.6 „

*The instability of "trypsin."*

The extreme instability of trypsin, especially in the presence of sodium carbonate, does not seem to have been realised by most workers with the ferment. Heidenhain<sup>1</sup> quotes a single experiment, in which he exposed an active extract, diluted eight times with water, to a temperature of 35° for 24 hours. From the data given one can calculate that by this treatment 96% or more of the ferment was destroyed. He states that other observations, in which the ferment was kept with 1% Na<sub>2</sub>CO<sub>3</sub> instead of pure water, gave similar results. Kühne does not seem to have found the action of sodium carbonate very injurious, as in his method of obtaining pure trypsin<sup>2</sup>, pancreas powder is allowed to undergo auto-digestion for a week at 40° in .5% Na<sub>2</sub>CO<sub>3</sub>.

In my own observations, I have found that most extracts, when diluted with water and kept at 38° show a fairly rapid destruction of ferment, whilst the addition of sodium carbonate greatly accelerates the decomposition. For instance, volumes of .125 c.c. of a 20% glycerin extract of pig's pancreas (diluted in each case to 2 c.c.), were kept for an hour at 38° with various strengths of sodium carbonate, and were subsequently compared as to their digestive capacity with an equal quantity of the fresh extract. The digestion time of this latter was 30 minutes, and the other times are given as multiples of this

<sup>1</sup> *Arch. f. d. ges. Physiol.* x. p. 579.

<sup>2</sup> *Verhandl. d. naturh.—med. Ver. zu Heidelberg*, III. p. 463. 1886.



value: the manner in which the percentage of ferment destroyed was calculated will be indicated further on.

	Digestion time 30 × 1·00 mins.	Per cent. ferment destroyed
Fresh extract		
H <sub>2</sub> O for 1 hour	1·33 "	32·7
·4 % Na <sub>2</sub> CO <sub>3</sub> for 1 hour	2·04 "	60·5
1 " " "	4·18 "	82·6
1·4 % " "	3·93 "	81·5

Here we see that even in pure water no less than 32·7 % of the ferment was destroyed, or enough to increase the digestion time by 33 %. In ·4 % Na<sub>2</sub>CO<sub>3</sub> nearly twice as much underwent destruction, and the digestion time was double that of the fresh extract. With 1 and 1·4 % Na<sub>2</sub>CO<sub>3</sub>, however, we see that at the end of an hour less than a fifth of the ferment originally present was still remaining, so that the digestion time was now about four times its original value. Other observations made with active extracts gave somewhat similar results, but with comparatively inactive extracts, the reaction to sodium carbonate was strikingly different. For instance, an aqueous extract of human pancreas, having a digestion time of 132 minutes for 1 c.c. of extract, was found to have this time increased by only 5·5 % on keeping for an hour at 38° with ·3 % Na<sub>2</sub>CO<sub>3</sub>, and by 31·8 % on keeping with 1 % Na<sub>2</sub>CO<sub>3</sub> (*i.e.*, to have had respectively 4·9 % and 25·8 % of its ferment destroyed).

With a view to elucidating these remarkable differences of reaction, observations were carried out systematically upon all the pancreatic extracts available. These extracts were fairly comparable, as they were all of approximately 20 % strength, or made by adding 4 parts by volume of the extracting liquid to 1 part by weight of the finely chopped or minced gland substance. In the case of the dog, the pancreatic extracts had been made within half-an-hour of death; in that of the sheep and the ox, within two hours; of the pig, four hours; of one human pancreas (obtained from a woman who had died of diabetes), within eight hours; and of the other human pancreas (obtained from a young man, who died four days after an accident), within 36 hours. The extracting liquids consisted of chloroform water, ·9 % sodium chloride solution containing chloroform, saturated sodium chloride solution, dilute alcohol (3 parts of water + 1 part of methylated spirit), and glycerin, either pure, or containing 25 % or 50 % of water.

In the accompanying table are given the times of digestion of 80 % of fibrin (previously swollen 1 hour in 1 % Na<sub>2</sub>CO<sub>3</sub>) by 1 c.c. of the

extract, and in the next column, the tryptic values of the extracts deduced from these digestion times. Next is given the digestion time after exposure of a given volume of the extract, diluted 5 to 10 times, for 1 hour to the action of .4%  $\text{Na}_2\text{CO}_3$  at  $38^\circ$ . For convenience, these times are given as factors on the digestion time values obtained with an equal volume of the fresh extract. Such a volume of extract was generally taken as had a digestion time of about 30 to 50 minutes, but these factors hold fairly well for all proportions of extract. For 1 c.c. of extract, therefore, the increased time can be roughly calculated by multiplying the digestion times given in the third column of the table by the corresponding factor in the fifth column: *e.g.* for glycerin extract of human pancreas it would be  $9.0 \times 2.073 = 18.66$  minutes.

In those extracts marked with an asterisk, .3%  $\text{Na}_2\text{CO}_3$  was used instead of .4%, both during the hour's treatment of the extract at  $38^\circ$  and during the fibrin digestion. The differences of reaction and digestion produced thereby are but small, so it was deemed unnecessary to separate the observations.

Source of pancreas	Extracting liquid	Digestion time in mins.	Tryptic value	Digestion time after 1 hour at $38^\circ$	% ferment thereby destroyed	Age of extract in days
Dog	Glycerin	6.6	145.9	2.633	69.8	141
Pig	50 % glycerin	7.5	74.6	2.036	60.5	199
Man	Glycerin	9.0	64.9	2.073	59.6	92
Woman (diabetic)	75 % glycerin	14.7	26.6	2.625	72.7	101
*Dog	Alcohol	17.7	23.2	1.601	44.5	96
Pig	Alcohol	21.4	15.7	1.238	22.9	213
Man	50 % glycerin	27.2	11.3	1.343	28.5	148
*Sheep	50 % glycerin	30.2	10.2	1.986	55.0	174
Dog	Water	36.5	7.86	1.393	34.8	131
Dog (kept 70 hrs.)	.9 % NaCl	37.2	7.35	1.151	14.3	151
Sheep	Alcohol	42.5	6.19	1.195	17.7	213
Pig	Saturated NaCl	45	5.79	1.212	19.9	195
*Dog	.9 % NaCl	46	5.64	.987	—	124
Ox	Alcohol	50	5.10	1.055	6.7	181
Ox	Saturated NaCl	63	3.80	1.310	26.1	178
*Man	Alcohol	77.5	2.96	1.047	5.1	121
Man (kept 37 hrs.)	.9 % NaCl	81	2.86	.982	—	135
Woman	Alcohol	83	2.79	1.325	28.0	87
*Man	.9 % NaCl	85	2.72	1.079	8.1	118
Woman	.9 % NaCl	95	2.40	1.165	16.9	108
Sheep (kept 20 hrs.)	Saturated NaCl	97	2.34	1.190	19.1	213
Ox (kept 60 hrs.)	.9 % NaCl	98.5	2.31	1.176	17.8	187
*Man	Water	132	1.64	1.055	4.9	124
Ox	.9 % NaCl	260	.78	1.146	13.5	196
Sheep	Saturated NaCl	287	.70	1.064	5.7	198
Ox	Water	340	.59	1.021	1.9	198
Ox	Glycerin	380	.52	1.093	8.3	198

In the next column of the table is given the percentage of ferment calculated to have been destroyed in each extract by the hour's exposure at 38°, whilst in the last column is recorded the age of the extract in days. As a rule, the time of exposure of the ferment was nearly or exactly 60 minutes, but in some cases considerably more or less than this. The increase in the digestion time was always corrected to 60 minutes' exposure, however, in a manner to be subsequently indicated.

From this table one may see that altogether 27 different extracts were examined, their digestion times varying between the extremes of 6.6 minutes and 380 minutes. From various data, to be mentioned later on, it has been found possible to calculate the relative amounts of proteolytic ferment present in these extracts, and we see in the table that the tryptic value thus determined varies from 145.9 to .52, or as 281 to 1. However, the chief interest of the table lies in the next two columns. Here we see that whilst in the case of the four most active extracts, exposure for an hour to .4%  $\text{Na}_2\text{CO}_3$  at 38° more than doubled the digestion time, or brought about the destruction of 65.6% of the ferment present, in the case of the five least active it increased the digestion time by only 7.6%, or destroyed only 7% of the ferment. Extracts of intermediate activity showed an intermediate degree of reaction to the sodium carbonate. The five most active extracts (next to the initial four), with digestion times of 17.7 to 36.5 minutes, had on an average 37.1% of ferment destroyed per hour; the three next, with digestion times of 37.2 to 45 minutes, 17.3% per hour, and the ten next, with digestion times of 46 to 98.5 minutes, 12.8% per hour. One may conclude, therefore, that the destructive action of sodium carbonate on the proteolytic ferment diminishes concomitantly with the activity of the extract. Doubtless the individual values in this table are somewhat irregular, but taking them as a whole, I think there can be no doubt that they fully justify one in drawing this conclusion. If this is the case, then one is driven to still another conclusion, viz. that the ferment trypsin is not a single chemical substance. It seems rather as if there were a series of "trypsins" of gradually increasing degrees of stability. This stability does not concern only the reaction to sodium carbonate, but to all the conditions of environment, both chemical and physical, to which the ferment of an extract is subjected. As a kept extract in course of time gradually diminishes in activity, the most sensitive "trypsins" first undergo destruction, and then in turn the less and less sensitive ones, the least sensitive of all remaining till the last. That this is the case

is suggested also by a few observations made upon fresh gland extracts. Thus 20% normal saline and alcoholic extracts of sheep's pancreas, after 40 hours' extraction, were found to have tryptic values of respectively 23·7 and 12·25 (or digestion times of 16·4 and 25·6 minutes with 1 c.c. of extract). In their reaction to sodium carbonate, however, they were more sensitive than any of the extracts mentioned in the table, their digestion times being increased to respectively 2·832 and 2·761 their original amount, after keeping for an hour at 38°. These values correspond to a destruction of respectively 76·9 and 72·8% of the ferment.

As regards the individual values in the table, the digestion time given for the glycerin extract of dog's pancreas is a calculated one, a smaller quantity of ferment than 1 c.c. having been employed. Several of the extracts were of gland substance which had been kept for 20 to 70 hours in its chopped up condition, before the addition of the extracting liquid. In the case of two extracts, it will be seen that there was apparently no destruction at all of ferment on keeping at 38°. Such a result may perhaps be due to experimental error, as this is doubtless fairly considerable, depending as it does on the estimation and comparison of two ferment solutions.

The variations in the individual values, over and above those due to experimental error, may be attributable to two causes, viz., the nature of the extracting media, and the diverse origin of the glands themselves. The nature of the extracting liquid probably has very little to do with the differences of reaction, though the contents of the table lend some colour to this hypothesis. Thus it is noticeable that most of the glycerin extracts were very sensitive to  $\text{Na}_2\text{CO}_3$ , and the aqueous and saline extracts very insensitive, whilst the alcohol extracts occupied an intermediate position. As shown by the digestion time values, however, this must have been almost entirely due to the fact that glycerin is a very good preservative of the ferment, whilst water and normal salt solution are bad ones. Thus all the extracts of each individual animal were made at the same time with the same gland substance, and though they were afterwards tested at various times, yet the average age of the more active extracts is about the same as that of the less active. That the glycerin extracts were as a rule the more sensitive merely because they were also the more active, is also borne out by the fact that of the two glycerin extracts made of the same human pancreas, that in pure glycerin was exceedingly active, and also exceedingly sensitive, whereas that made in 50% glycerin, and examined nearly two months later,

contained only a sixth as much ferment, and also showed only 28.5 % destruction of ferment by the sodium carbonate, instead of 59.6 %. Still again, the glycerin extract of ox pancreas was the most inactive of the extracts examined, and it was also very insensitive to  $\text{Na}_2\text{CO}_3$ .

The other probable cause of the variations noticed in the table, viz., the diverse origin of the glands themselves, is best studied by classifying the extracts. This has been done in the accompanying table, which gives the tryptic values of the extracts.

Extracting medium	Dog	Pig	Man	Woman	Sheep	Ox
Glycerin	145.9	74.6	64.9	26.6	10.2	.52
Alcohol	23.2	15.7	2.96	2.79	6.19	5.10
Saline	5.64	5.79	2.72	2.40	.70	3.8 & .78
Water	7.86	—	1.64	—	—	.59
Average age } in days }	123	202	114	99	195	189

Here one sees that more or less the same order in the scale of ferment activity is kept, whatever the extracting liquid. Extracted with glycerin, dog's pancreas proved to be much the most powerful, pig's pancreas, and then the human pancreases, coming next. Extracted with alcohol, dog's pancreas is again the most powerful, and pig's pancreas again ranks second in activity, but ox and sheep's pancreases are more active than the human. In the case of salt solutions, however, the original order is more or less reverted to, though the activity of the pig's pancreas is slightly greater than that of the dog's. Probably this is because saturated sodium chloride was used instead of .9 % solution, for in the case of the ox pancreas the saturated salt extract was five times more active than the normal saline one. As regards the aqueous extracts, the observations are incomplete, but in their case also, as far as they go, the dog's pancreas provided the most active extract, and the ox pancreas the least.

One may conclude, therefore, that the dog's pancreas originally contained more ferment or zymogen than any of the other glands, whilst that of the pig came next. The extracts of this latter gland were about seven months old when examined, whereas those of the former were only four months, hence it is possible that they originally differed but little in ferment power. The human pancreases must have contained a good deal less ferment than either of them, but it should be remembered that neither of these glands was from an absolutely normal subject. It is, perhaps, a matter of surprise that extracts of a

gland obtained from a woman dying of diabetes (for post-mortem examination revealed no other cause of death) should be as active as the figures indicate.

The sheep and ox glands exhibited a moderate ferment activity, distinctly inferior to that of the dog and the pig, hence there is no doubt as to the inequality of the various glands. For equal degrees of ferment activity, therefore, one would expect extracts of dog's and pig's pancreas to be less affected by sodium carbonate than extracts of the other glands. The contents of the table given a page or two back seem, on the whole, to support this view. For instance, the glycerin extracts of the human pancreases had on an average 66.1% of ferment destroyed by the sodium carbonate, as against 65.1% in the case of the glycerin extracts of dog's and pig's pancreas, although they did not contain half their amount of ferment.

The existence of trypsins of various degrees of stability can be readily shown in any single extract, for it is found that the rate of decomposition of the ferment on exposure to sodium carbonate at 38° becomes gradually slower and slower, as less and less of the ferment is left. If the rate of increase of digestion time for various periods of  $\text{Na}_2\text{CO}_3$  treatment be calculated, however, the values obtained are more or less constant. For instance, .125 c.c. of a glycerin extract of human pancreas had a digestion time of 35 minutes, but on keeping 23 minutes with .4%  $\text{Na}_2\text{CO}_3$  at 38°, this time was increased to 51 minutes. Supposing the increase of digestion time be proportional to the time of exposure, then one can calculate that after one hour the digestion time would have risen to  $35 \times 2.192$  minutes. On keeping .125 c.c. of the extract for 70 minutes at 38°, the digestion time was found to have been increased to 73 minutes, or to  $35 \times 1.931$  minutes per 1 hour's exposure. Finally, extract kept for 172 minutes at 38°, had its digestion time raised to 155 minutes, or to  $35 \times 2.196$  minutes per 1 hour's exposure. Now these three factors, 2.192, 1.931 and 2.196 are sufficiently similar to warrant one in concluding that the rate of increase of digestion time is more or less directly proportional to the time of exposure of the ferment to  $\text{Na}_2\text{CO}_3$ . In the observations already recorded, as to the increase of digestion time of the extracts for 1 hour's exposure, it was mentioned that the actual times of exposure were frequently more, or less, than the standard 60 minutes. In such a case they were corrected to 60 minutes in accordance with this directly proportional relationship.

In the accompanying table are given the factors of the increased

digestion times (calculated per hour) obtained in the case of other extracts.

Extract	Time of exposure to $\text{Na}_2\text{CO}_3$	Relative increase of digestion time	Extract	Time of exposure to $\text{Na}_2\text{CO}_3$	Relative increase of digestion time
·5 c.c. glycerin (woman)	34 mins.	$20 \times 3.559$	·5 c.c. glycerin (sheep)	27 mins.	$55 \times 2.051$
	87 "	2.424		131 "	1.922
	147 "	2.296	·25 c.c. alcohol (dog)	28 "	$36.1 \times 1.611$
·0625 c.c. glycerin (dog)	20 "	$32 \times 2.407$		78 "	1.705
	64 "	2.860		133 "	1.486
·14 c.c. glycerin (pig)	37 "	$30 \times 1.945$	1 c.c. .9% NaCl (man)	69 "	$85 \times 1.071$
	71 "	2.127		140 "	1.086
	148 "	1.628			

It will be seen that they are somewhat variable, and also that they do not quite conform to the rule of direct proportionality. The relative increase of digestion time for long periods of exposure to  $\text{Na}_2\text{CO}_3$  is probably somewhat smaller than that for short periods (this being especially the case for glycerin extract of woman's pancreas), but the difference was not sufficiently constant or marked to permit one to allow for it in correcting the results above mentioned.

In spite of the digestion time increasing more or less proportionally to the time of exposure, it nevertheless follows that the rate of destruction of the ferment rapidly diminishes. Supposing, for instance, that such a quantity of the ferment is destroyed in the first hour as to double the digestion time, then if a similar proportion of what is still remaining be destroyed during the second hour, the digestion time would be quadrupled, and after a third hour, be increased to eight times its original amount. We have found, on the contrary, that it is only trebled after the second hour, and quadrupled after the third, or that the increase is in arithmetical proportion, not geometrical. From data recorded subsequently, it is possible to calculate the rate of destruction of the ferment, and in the accompanying table are given the results so obtained in the case of four of the extracts. Each of these extracts had its digestion time determined after about 30, 75 and 150 minutes' treatment with sodium carbonate at  $38^\circ$ , and so they are fairly comparable. In the case of glycerin extract of woman's pancreas, for instance, the increase of digestion time after 34 minutes was calculated to correspond to a destruction of ferment at the rate of 81.3% per hour. The actual digestion time after this 34 minutes' treatment was 49 minutes, whilst after 87 minutes' treatment it was found to be 61.3 minutes. The rate of destruction of ferment between the 35th

and 87th minutes could be calculated from these values to be 27·4 % per hour, or only about a third as much as in the first period. Finally the

Source of gland	Extracting liquid	Percentage rate of destruction of ferment per hour during		
		0—37 mins.	24—87 mins.	71—172 mins.
Woman	Glycerin	81·3	27·4	29·5
Man	Glycerin	62·0	35·5	43·8
Pig	Glycerin	58·2	51·4	7·4
Dog	Alcohol	45·0	43·2	9·1
Average		61·6	39·4	22·4

rate of destruction of ferment between the 88th and 147th minutes was calculated to be 29·5 % per hour, or about the same. However, not very much reliance can be placed on individual values, as the experimental error is necessarily large, and becomes much magnified by this method of calculation. The mean results of the four series of observations given in the table may probably be taken to afford one a fair idea of the average change of decomposition rate, and from these one gathers that for every three parts of ferment destroyed per hour during the first half-hour of sodium carbonate treatment, only about two parts are destroyed during the second half-hour, and only about one part during the  $1\frac{1}{2}$  hours after that. This diminishing rate of ferment destruction in individual extracts thus affords striking confirmation of the results obtained by a comparison of different extracts.

*The relation between quantity of ferment and rate of digestion.*

It was found by Schütz<sup>1</sup> that the rate of digestion of fibrin by pepsin varied as the square root of the quantity of ferment present, even when this quantity varied as 1 to 64. Subsequently Borissoff<sup>2</sup>, using Mette's method, showed that trypsin digested coagulated egg-albumin in accordance with the same rule, and Samojloff<sup>3</sup>, also using Mette's method, found that the amount of coagulated egg-albumin dissolved by pepsin varied as the square root of the quantity of ferment present only in dilute solutions. In concentrated solutions, it was less than that required by the law.

The digestion time values obtained when trypsin acts on swollen fibrin do not, as a rule, appear to conform very closely to the theoretical relationship. This is because of the rapid destruction of the ferment by

<sup>1</sup> *Zeit. f. physiol. Chem.* ix. p. 577. 1895.

<sup>2</sup> *Ibid.*

<sup>3</sup> *Arch. de Sci. Biol.* ii. p. 699. 1893.



the sodium carbonate, for the less the amount of ferment added, and the longer the digestion time, the greater is the relative amount of ferment destroyed. If this ferment destruction be allowed for, however, then the rate of digestion is found approximately to vary according to rule. For instance, in the case of alcohol extract of dog's pancreas, the following digestion time values were obtained:

Volume of extract	Digestion time	Corrected digestion time	$\times \frac{\text{Time}}{\sqrt{\text{ferment}}}$	Tryptic value
2 c.c.	11·8 mins.	11·14 mins.	16·2	22·0
1 "	17·7 "	16·25 "	16·3	21·4
·5 "	26·8 "	23·62 "	16·7	23·1
·25 "	36·1 "	30·57 "	15·3	30·5
·125 "	80·4 "	57·32 "	20·3	23·1
·0625 "	176 "	93·57 "	23·4	19·2

With large quantities of ferment, one finds that the digestion time values do not depart very greatly from the theoretical, for

$$11·8 \times \sqrt{2} = 16·7; \quad 17·7 \times \sqrt{1} = 17·7; \quad 26·8 \times \sqrt{5} = 18·9;$$

and  $36·1 \times \sqrt{25} = 18·05$ ; *i.e.*, the values are fairly concordant. With smaller quantities of ferment, however, they are very aberrant,  $176 \times \sqrt{0·0625}$  giving the value 44·0. Now it has been found that on an average, after 1 hour's treatment with  $\text{Na}_2\text{CO}_3$  at  $38^\circ$ , the digestion time is increased from 1 to 1·601, owing to the destruction of ferment. With a digestion time of, for instance, 11·8 minutes, the ferment—so far as regards its digestive action on the fibrin—would on an average be exposed to the destructive effect of the sodium carbonate for only  $\frac{11·8}{2} = 5·9$  minutes. The digestion time would thereby be increased

from 1 to 1·059. Supposing no ferment whatever had been destroyed during the digestion, therefore, the time taken would have been

$$\frac{11·8 \times 1}{1·059} = 11·14 \text{ minutes.}$$

Correcting in a similar manner for the ferment destroyed when the digestion time was 17·7 minutes, one finds it becomes reduced to 16·25 minutes; lastly, when it was 176 minutes, to 93·57 minutes, or not much more than half the amount. The products of these corrected values into the square root of the number of c.c. of extract used, are given in the fourth column of the table. Here one sees that with from ·25 to 2 c.c. of extract, the digestion time varied approximately in accordance with the rule, but that with smaller quantities of ferment it was considerably prolonged.

In the case of .0625 c.c. of extract, for instance, the time ought to have been about 65 minutes, instead of 93. Probably these apparent deviations are due to the correction applied for destruction of ferment being insufficient. A few observations were made which seemed to show that the ferment was destroyed somewhat more rapidly in presence of fibrin as well as sodium carbonate, than in sodium carbonate alone.

In the accompanying table are given the products of corrected digestion time into square root of volume of extract, obtained in the case of other extracts. The corrections applied were, of course, different in each case, the values used being those given in the full table some pages back.

Source of gland	Extracting liquid	Corrected digested time $\times \sqrt{\text{ferment}}$						
		2 c.c.	1 c.c.	.5 c.c.	.25 c.c.	.125 c.c.	.0625 c.c.	.03125 c.c.
Pig	50 % glycerin	—	7.0	8.1	9.7	10.7	14.5	—
Man	Glycerin	—	8.3	9.2	8.2	9.7	11.7	12.6
Woman	75 % glycerin	15.2	12.2	11.1	11.4	12.7	13.6	—
Sheep	50 % glycerin	21.1	24.2	26.8	25.7	24.4	—	—
Dog	Water	—	32.6	30.9	48.0	—	—	—
Ox	Alcohol	46.7	42.2	37.2	42.5	50.9	—	—

In the case of glycerin extract of pig's pancreas, the values increase steadily as the amount of ferment diminishes, so undoubtedly the correction applied must have been too small. With glycerin extract of man's pancreas, the values were fairly constant with from 1 to .125 c.c. of ferment, whilst with woman's pancreas extract the constancy extended more or less to the .0625 c.c. ferment value. With 2 c.c. of ferment, however, distinctly too high a value was obtained. This was probably due to the paralysing effect of the glycerin, for other observations showed that glycerin had such an effect. The glycerin extract of sheep's pancreas gave the most constant series of values obtained, they varying but little with from 2 to .125 c.c. of ferment. Alcohol extract of ox pancreas gave a moderately concordant set of values, but one of the numbers obtained with aqueous extract of dog's pancreas was very divergent.

It must be admitted, I think, that these values are sufficient to prove that the rate of digestion does in reality vary in proportion to the square root of the quantity of ferment present, though they are not so constant as could be desired, or as might be expected considering the care taken in determining them. The deviations are probably due to the extreme sensitiveness of the trypsin to very slight differences in the conditions of digestion.

*The determination of the tryptic value.*

From the results already discussed, it will be seen that in order to accurately compare the ferment strength of different extracts, it would be necessary to determine the rate of destruction of the ferment, as well as the actual digestion time, and then to calculate what the digestion time would be supposing no ferment at all had undergone destruction during the course of digestion. Such a procedure would be too laborious for ordinary comparisons of proteolytic power, which do not as a rule require great accuracy, so it was thought sufficient to

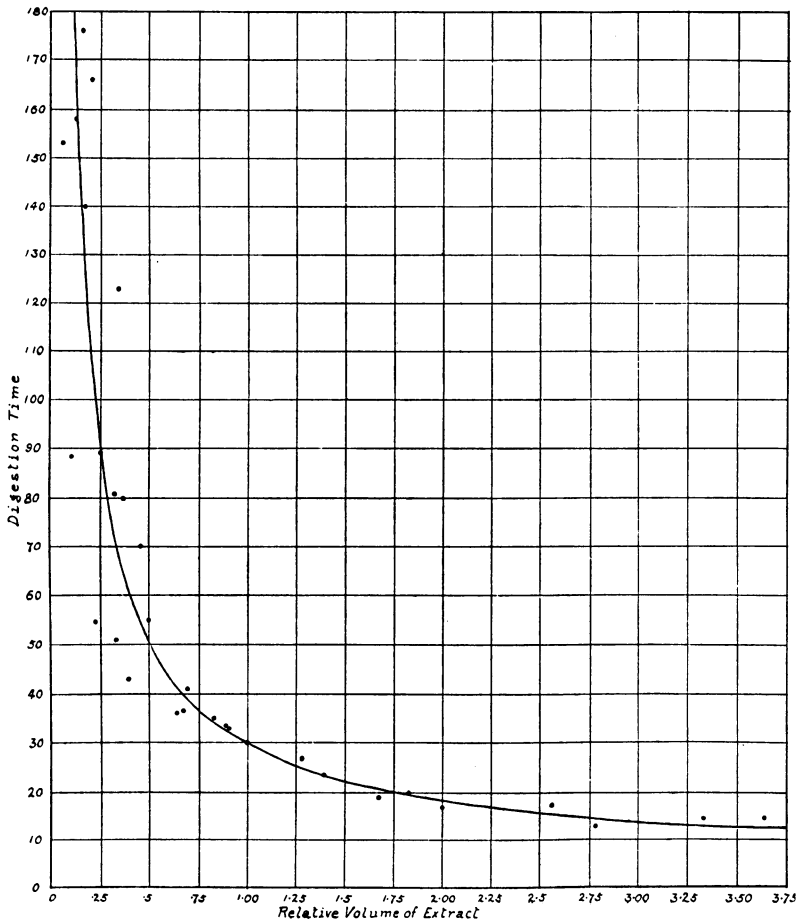


Fig. 2.

determine a series of values, representing the means of the relative digestion rates of the extracts mentioned above, and to use these as the basis of a table for calculating tryptic values. Accordingly the digestion time values for the alcohol extract of dog's pancreas and the six extracts given in the above table have been plotted out in the accompanying figure, and a mean smoothed curve drawn through them.

In each case, the volume of ferment required to give a digestion time of exactly 30 minutes was calculated, and this was divided into the other volumes. For instance, in the case of alcohol extract of dog's pancreas, it was calculated that .39 c.c. of extract would be required. Now it was found that for a digestion time of 17.7 min., 1 c.c. of extract was necessary, or 2.56 times that required for a 30 minutes' digestion time: for a digestion time of 80.4 minutes, .125 c.c. of extract, or .32 times that for a 30 minutes' digestion time, and so on. Digestion time values relative to that at 30 minutes taken as unity, have been calculated in a similar manner for the other six extracts, and the numbers so obtained plotted out in the figure. It will be seen that many of them are not in close agreement with the mean curve, but it is impossible that this should be so, considering the differences in reaction to sodium carbonate shown by the different extracts. In the accompanying table are given the volumes of ferment required to give digestion times of from 12.5 to 320 minutes, as determined from this mean curve. By means of it one can calculate the tryptic value of an extract without

Digestion time	Volume of extract	Digestion time	Volume of extract	Digestion time	Volume of extract	Digestion time	Volume of extract
12.5	3.81	24	1.337	46	.564	115	.190
13	3.47	25	1.265	48	.536	120	.182
13.5	3.20	26	1.200	50	.510	130	.167
14	3.00	27	1.145	52	.485	140	.153
14.5	2.83	28	1.095	54	.462	150	.143
15	2.69	29	1.047	56	.441	160	.133
15.5	2.56	30	1.000	58	.422	170	.125
16	2.45	31	.955	60	.404	180	.117
16.5	2.35	32	.912	64	.373	190	.110
17	2.26	33	.872	68	.348	200	.103
17.5	2.17	34	.835	72	.325	220	.093
18	2.08	35	.800	76	.307	240	.085
18.5	2.00	36	.767	80	.290	260	.078
19	1.916	37	.740	85	.272	280	.072
19.5	1.833	38	.714	90	.255	300	.067
20	1.755	39	.691	95	.240	320	.063
21	1.618	40	.669	100	.226		
22	1.510	42	.629	105	.213		
23	1.417	44	.595	110	.201		

any trouble, and with a moderate degree of accuracy. It was necessary to choose some arbitrary standards of time and amount of extract, so

30 minutes was chosen as the former, and 1 c.c. as the latter. Supposing, therefore, 1 c.c. of extract digested 80% of fibrin in 30 minutes, its tryptic value was said to be 10. Or if  $n$  c.c. of extract digested the fibrin in  $x$  minutes, then the tryptic value would be equal to  $\frac{10 \times (\text{equiv. of } x)}{n}$ . The equivalent of  $x$  (i.e. relative number of c.c.

of extract corresponding to the digestion time found) must of course be determined from the table. If 1 c.c. of extract be used, then it is necessary only to multiply the appropriate equivalent by 10: *e.g.* for a digestion time of 13.5 minutes, the tryptic value is 32.0; of 60 minutes, 4.04; and of 200 minutes, 1.03. For digestion times intermediate between those given in the table, intermediate values must be calculated.

In the table of digestion times for alcohol extract of dog's pancreas given a few pages back, the tryptic values are recorded in the last column. Though the digestion time varied from 11.8 to 176 minutes, the tryptic values, as calculated from the equivalents in the above table, kept more or less constant throughout. Hence a mean of the whole of them may be taken to represent the tryptic value with considerable accuracy. Unfortunately all the extracts do not give so even a set of values. With an extract very sensitive to sodium carbonate, they gradually get smaller and smaller the less the volume of extract added, and with one insensitive to sodium carbonate, gradually bigger and bigger. In such cases, the mean tryptic value is calculated only from the more or less constant values, those for small quantities of ferment, with digestion times of 80 to 100 minutes or more, being as a rule rejected.

In order to calculate the percentage of ferment destroyed on exposure of an extract to sodium carbonate at 38°, one obviously needs only to transmute the digestion times found before and after exposure into the corresponding tryptic values, and to determine the ferment destruction from them.

#### SUMMARY.

The method suggested for the estimation of the tryptic value of a pancreatic extract may be epitomised as follows: Introduce about 1.8 c.c. of finely chopped fibrin into a 10 c.c. graduated tube filled with water, and centrifugalise for two minutes. Carefully read off the volume of fibrin, now reduced to about 1 c.c. Replace 5 c.c. of the

water by 2% sodium carbonate, and allow the fibrin to swell for an hour at 38°. Then withdraw 6 c.c. of the 1%  $\text{Na}_2\text{CO}_3$ , and replace it with water + 1 c.c. or less of the extract to be tested. Shake up the tube from time to time, and when all but about .4 c.c. of the fibrin has undergone solution, centrifugalise again for one minute, and read off the volume of fibrin remaining. This is now reduced to about .2 c.c., so one has determined the time of digestion of about 80% of the fibrin originally taken. This time is corrected to exactly 80% digestion by means of a table, and then from another table the relative tryptic value can be ascertained.

This method is a fairly rapid one, the necessary manipulations for each estimation taking only about a quarter of an hour altogether.

It was found that trypsin is very rapidly destroyed by sodium carbonate, an active extract kept at 38° with .4%  $\text{Na}_2\text{CO}_3$  having about 65% of its ferment destroyed in an hour! 1%  $\text{Na}_2\text{CO}_3$  destroys over 80%, whilst pure water may destroy over 30% per hour. On investigating this phenomenon in a series of glycerin, alcoholic, saline and aqueous extracts of human, dog, pig, sheep and ox pancreases, which had been kept for some months, it was found that only the most active (*i.e.* least deteriorated) of these extracts showed this extreme sensitiveness to sodium carbonate, the least active, with about a hundredth the tryptic power, having only about 7% of their ferment destroyed per hour by .4%  $\text{Na}_2\text{CO}_3$ . Extracts of intermediate degrees of activity showed intermediate degrees of sensitiveness, or the destructive action of the sodium carbonate on the ferment diminished concomitantly with the activity of the extract. From this it seems to follow that the ferment trypsin is not a single chemical substance. It appears rather as if there were a series of "trypsins" of gradually increasing degrees of stability, so that when an extract is kept, and gradually deteriorates, the most sensitive trypsins are first destroyed, and the least sensitive ones last.

The law enunciated by Schütz, and by Borissoff, that the rate of digestion varies as the square root of the quantity of ferment present, was confirmed. It was necessary to allow for the ferment destroyed during the course of digestion.

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