LIV. THE ACTIVATION OF TRYPSINOGEN.

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In a previous paper I showed [1901, 2] that if minced pancreas were kept at room temperature with water or saline solution in presence of chloroform, or with dilute alcohol, the extracting liquid at first contained little or no free trypsin and rennin, but that after a variable time, it might be 5, 12, or 19 days, it suddenly began to develop activity, and in the course of the next few days reached its maximum tryptic and rennetic power. This clearly pointed to an auto-catalytic reaction, and I supposed trypsin itself to be the activator. Small quantities of it, liberated spontaneously from the trypsinogen, would in such a case activate the extract with rapidly accelerating velocity. In seeming proof of such a hypothesis I found that small quantities of active extracts containing trypsin had a very marked activating power upon inactive extracts, whilst a repetition of these experiments with pancreatic juice gave a similar result [1902, 1913]. Mellanby and Woolley [1912] showed that extracts of intestinal mucous membrane containing enterokinase, when added to pancreatic juice, liberated the trypsin at a rate which was slow at first, but which increased with rapidly accelerating velocity to a maximum as the end of the reaction was approached. This result appeared to me to confirm my own experiments, and to indicate that the later and rapid portion of the activation process is due chiefly to the catalytic action of the trypsin liberated in the earlier stages by the enterokinase. For reasons to be adduced later on, I find this hypothesis to be erroneous, and my fresh experimental data point to the following explanation of this vexed problem : *The trypsin liberated by the enterokinase gradually sets free an enzyme, termed deuterase (to indicate that it acts secondarily to enterokinase) from a precursor which is present in pancreatic juice and extracts, and this deuterase is mainly responsible for the later stages of the activation process.* Hence in all my previous

experiments in which I have attributed activating powers to free *trypsin*, I now conclude that they are really due to *deuterase liberated by the trypsin from a pro-deuterase*.

METHOD OF EXPERIMENT.

The method of estimation of *trypsin* adopted has already been explained in former papers [1901, 1; 1913]. It depends on Robert's metacasein reaction, suitably corrected, and as a rule takes 3 to 5 minutes to perform, or exceptionally from 2 to 60 minutes. As previously pointed out, this reaction is a test for the rennin rather than the *trypsin*, but under most conditions the two enzymes run parallel in activity, and so a measure of one is likewise a measure of the other. My earlier experiments were made with a fibrin digestion method which took 20 or 30 minutes for completion, and though it gave useful results it is clearly unsuitable for estimating rapid activation processes.

In all the experiments described below, unless special mention is made to the contrary, both the extracts and pancreatic juice were brought to a fivefold dilution before activation. To the extracts Na_2CO_3 was added up to 0.05% (*i.e.* a centinormal solution), whilst each sample of pancreatic juice used was titrated against N/10 HCl, using methyl orange as indicator, and its alkalinity after dilution reduced to one of 0.05% Na_2CO_3 by the addition of suitable amounts of N/10 HCl.

The enterokinase was prepared by scraping off the mucous membrane from the upper half of the small intestine of the cat or monkey, grinding it with a little sand, and mixing thoroughly with two to six times its weight of chloroform water. The extract was filtered off 20 to 40 hours later, and kept with chloroform. All the extracts and pancreatic juice were kept in a refrigerator at a temperature of 1° to 5° when not in use.

THE ACTIVATION OF PANCREATIC JUICE BY ENTEROKINASE.

In the few experiments recorded previously on the time relations of the activation of pancreatic juice by enterokinase, I pointed out that the latter part of the activation showed approximately the same velocity whatever the quantity of enterokinase employed. This velocity is so great that its determination is necessarily somewhat inexact, but the plan adopted in the present experiments was to estimate the time taken over the *final half* of the activation process. It was possible to make two or three *trypsin* estima-

tions during the most rapid stage, as well as other estimations before and after it. These results were plotted out, and the straight line joining the estimations made during the most rapid stage continued till it reached the point of maximum tryptic activity. That is to say, for the purpose of calculating the velocity of the final half of activation it was assumed that there was no slowing down of the activation process as it tended towards completion. Doubtless some slowing does exist, but it is so slight that no good experimental evidence of it could be obtained.

In Fig. 1 is shown the effect of activating pancreatic juice with cat's enterokinase. The 10 % of extract used took 39 minutes to activate at 37°,

Fig. 1.

but 172 minutes at 17°. These values give a temperature coefficient of 2.10 for each rise of 10° between the limits mentioned, or a similar value to those obtained in most other enzyme actions. Nevertheless temperature does not by any means affect every portion of the curves in the same manner. The last half of the activation process apparently took 12 min. at 37°, but only 8 min. at 17°. The larger value obtained at the higher temperature is doubtless an experimental error due to the difficulty of drawing the slope of the curves correctly when only a small number of experimental results are available, but all the results agree in showing that the latter half of the

activation process is comparatively little affected by temperature. In the other two curves shown in the figure, which indicate the activation produced by 5 % and 2.5 % of enterokinase at 17°, the times taken for the last half of the process were 11 and 8 minutes respectively, or the average time taken at 17° is 9 minutes. In an experiment at 27° the time taken was 13 minutes. Experiments made at 37° with other samples of juice gave the following times for the last half of activation : (a) with 10 % of E., 7 min. ; with 5 %, 7 min. ; (b) with 6 % of E., 5 min. ; with 3 %, 6 min. ; (c) with 10 % of E., 9 min. ; (d) with 10 % of E., 5 min. (average of five experiments). Hence the average value obtained at 37° works out at 7 min., whilst the average at 17° and 27° is 10 min. In my previous paper I stated that the time taken for the most rapid doubling of tryptic activity was about 4 min., but if the results there quoted are re-calculated in accordance with the method now used, the time taken over the final half of activation works out at about 5 min.

As will be pointed out again later on, the small influence of temperature upon the final stages of activation of pancreatic juice by enterokinase is due to their being induced for the most part by deuterase, the activating power of which is almost unaffected by temperature.

It will be noted that the time taken for the last half of activation is independent of the quantity of enterokinase used. On the other hand the time taken over the whole process is greatly influenced by it, though not quite in inverse proportion. Thus in the experimental results above recorded activation by 10 % of E. took 172 min., by 5 % took 296 min., and by 2.5 % took 512 min., or in the ratio of 1 : 1.7 : 3.0, instead of 1 : 2 : 4. Another sample of juice was activated at 37° by 10 % of E. in 30 min., and by 5 % of E. in 50 min. Another sample was activated by 10 % of E. in 69 min., and by 4 % of E. in 137 min. On the other hand a fourth sample was activated by 6 % of E. in 32 min., and by 3 % of E. in 83 min., but this exception cannot be held to counterbalance the other three results. Mellanby and Woolley [1913, p. 356] quote one experiment in which the activation time was almost exactly in inverse proportion to the quantity of enterokinase, but in another experiment [1912, p. 375] it was less affected than in any of the results above quoted.

It is of interest to compare the minimum and maximum velocities of activation effected by enterokinase at the beginning and end of its action. For instance, in the above recorded experiment when 2.5 % of E. acted on pancreatic juice at 17°, the tryptic value increased from 7 units to 20 units

in the first 337 minutes, or at the rate of 1 unit in 26 minutes, and as already mentioned it finally doubled itself in 8 min. This increase works out at the rate of 1 unit in 0.057 min., or is 456 times more rapid than the initial rate. Probably it is in reality quite 1000 times more rapid than the real initial rate, as there can be no doubt that the velocity of activation towards the end of the 337 minutes interval was considerably greater than at the beginning. In the experiment with 5 % of E. the velocity observed during the 0 to 99 min. interval was 210 times slower than the final rate observed. With smaller quantities of E. than 2.5 % the difference between the initial and final rates would be greater still, and differences of such an order are seen with most samples of pancreatic juice. Even when kept in a refrigerator they activate themselves spontaneously in about five days. This is presumably due to the presence of minute quantities of enterokinase, which for the first two or three days liberate scarcely appreciable quantities of trypsin, but which finally lead to the familiar rapid activation. Mellanby and Woolley find that extracts of every tissue in the body accelerate the activation of pancreatic juice, and so presumably contain small quantities of enterokinase. The activation of pancreatic extracts observed by me in my original experiments must likewise have been due to the presence of variable traces of enterokinase.

The extreme slowness of the initial rate of activation induced by small quantities of enterokinase, coupled with the fact that the final rate is practically independent of the amount present, clearly points to the existence of a dual reaction in the process. It is not possible to explain such a result by assuming that a co-enzyme is liberated during the course of activation which augments the action of the enterokinase, for in such a case the final velocity would be influenced by the amount of enterokinase present.

THE ACTIVATION OF PANCREATIC EXTRACTS BY ENTEROKINASE.

For most purposes it is much more convenient to use pancreatic extracts than pancreatic juice, as they can be more readily obtained, and are much more constant in their properties [Vernon, 1901, 2, p. 316]. If fresh sheep's or pig's pancreas be minced up and mixed with four times its weight of glycerin, the extract filtered off one to three weeks later contains large quantities of trypsinogen and only traces of trypsin, and does not activate itself for months if kept in a refrigerator. If, on the other hand, the minced gland be mixed with three parts of glycerin and one of water, the extract begins

to activate itself in about three weeks, and attains its maximum tryptic power in about 5 or 6 weeks. This tryptic power it retains with very slight diminution for many months. If the minced gland be extracted with a mixture of equal parts of glycerin and water it affords an active extract in a fortnight or less, for the larger the proportion of water present in the extracting liquid the quicker the traces of enterokinase in the gland substance exert their action.

The pancreas of animals other than the pig and sheep may yield different results on extraction, partly because the amount of enterokinase contained

Fig. 2.

in them is variable, and because they may contain an anti-body. For instance, a concentrated glycerin extract of monkey's pancreas was found on two occasions to activate spontaneously in a fortnight. On the other hand a 75 % glycerin extract of ox-pancreas did not activate at all in three weeks.

Inactive glycerin extracts of sheep's pancreas are activated by enterokinase in a very similar manner to pancreatic juice, as can be seen from the curves given in Fig. 2. The times required for complete activation at 37° by 5, 2.5, 1.25 and 0.625 % of E. were 26, 36, 49 and 70 minutes respectively, or were very far from being in inverse proportion to the quantity of enterokinase. The times taken for the last half of activation were 8, 8, 8, and 10 minutes respectively, or were almost independent of the quantity of enterokinase. The results obtained at 17° are represented as dotted line

curves. The time scale on which the values are plotted is four times smaller than that for the values at 37° , and so it can be seen at a glance that the rate of activation at 17° by 5 % and 1.25 % of E. was about four times slower than in the corresponding experiments at 37° . The actual temperature coefficients work out at 1.93 and 2.07 respectively. It will be noted that the slope of the final portion of the dotted line curves is considerably steeper than that of the other curves, and in fact the times taken for the last half of activation by 5 and 1.25 % of E. were 24 and 17 minutes respectively, or two to three times longer than in the corresponding experiments at 37° instead of four times longer. The more marked influence of temperature

Fig. 3.

on the final stages of activation than in the experiments with pancreatic juice probably indicates that the deuterase factor is relatively less responsible for them.

Extracts of pig's pancreas are not activated by enterokinase in nearly such a typical manner as those of sheep's pancreas. As can be seen from Fig. 3, they much more nearly approach straight lines, or show comparatively little acceleration of velocity in the latter part of the activation. That is to say, the activation is throughout induced very largely by enterokinase, and deuterase is much less important than in the activation of sheep's pancreas extracts. Still it probably accounts for over half of the total activation. This is shown by determining the time taken over the last half of activation, and calculating it as a percentage on the total activation time. The per-

centage times observed are given in the table. They were obtained with various enterokinase preparations, two of which were much more active than the others, and so the percentage amounts of enterokinase given in the first column only hold for about two-thirds of the data. For the remaining third they represent a fivefold diluted solution.

Enterokinase added	Percentage time taken over last half of activation at			Mean
	37°	17°	1°	
10 %	37, 31	50, 37, 28	22, 20	32
5	—	43, 28	21	31
2.5	42, 32, 28	30, 30	25	31
1.25	34, 22, 22	44	—	30
0.625	21	—	—	21
Mean	30 % at 37°	36 % at 17°	22 % at 1°	

It will be seen that the relative time taken for the last half of activation was about 31 % of the whole time, or was about two-fifths of that required for the first half of activation. Hence even if deuterase were not responsible for any of this first half, it must have induced the larger part of the second half of activation. It will be noted that with a single exception the percentage time is almost constant whatever the amount of enterokinase used for activation. That is to say, with diminishing quantities of enterokinase the slope of the last half of the activation curves changed in almost the same proportion as the time taken for the whole of activation. This points to the final stages of activation being chiefly induced by enterokinase, but evidence indicating that it is largely due to deuterase is afforded by classifying the results according to temperature. In addition to experiments at 37° and 17°, a number were made in which the activating mixture was kept in a vessel surrounded by pounded ice and water, or at a temperature of 0–1°. From the bottom line of the table it will be seen that on an average in these experiments the last half of activation took only 22 % of the total time, as against 33 % taken in the experiments at 17° and 37°. From these data it can be calculated that deuterase must have been responsible for nearly twice as much of the last half of activation at 1° as at 17° and 37°. This result is in accordance with the conclusion that the action of deuterase is almost unaffected by temperature. The considerable difference in the form of the activation curves at 1° and 17° is well shown by comparing Figs. 4 and 3. These results were obtained with extracts of the same pig's pancreas, but the sample used for the experiments shown in Fig. 4 had remained longer in contact with the gland substance before filtration, and so contained much more trypsinogen.

It will be seen from the curves in Figs. 3 and 4 that the time of total activation of pig's pancreas extracts does not vary in inverse proportion to the quantity of enterokinase. Thus the activation times at 17°, with 10, 5, 2.5 and 1.25 % of E. were 52, 109, 148 and 248 min. respectively, whilst at 1°, with 10, 5, and 2.5 % of E. they were 143, 200 and 282 min. respectively. In some observations at 37°, with 10, 2.5 and 1.25 % of E. they were 14, 45 and 65 min. respectively. Experiments with other extracts of pig's pancreas gave similar results. Six sets of experiments made at 37° and 17° with 10 to 1.25 % of E. gave temperature coefficients of 2.07, 1.97, 1.93, 1.93, 1.86 and 1.81, or 1.93 on an average, whilst two sets of experiments at 17° and 1° gave temperature coefficients (likewise for a 10° interval) of 2.24 and 1.94.

Fig. 4.

Extracts of ox-pancreas gave activation curves similar to those obtained with pig's pancreas. 5 % of E. took 32 minutes to activate at 37° and 108 min. at 17° (temp. coefficient 1.84), whilst the last half of activation took 28 and 31 % respectively on the total activation time.

The difference in the character of the activation curves obtained with extracts of sheep's and of pig's pancreas probably depends on a number of factors. As will be shown later on, various extracts not only contain different proportions of pro-deuterase and of an anti-body, but the rate of liberation of deuterase by trypsin and the action of deuterase on trypsinogen is influenced by various conditions. For instance, products of proteolytic activity retard the formation, or the action, of deuterase much more than the action of enterokinase. In Fig. 5 is shown the effect of adding 0.2 to 3.5 % of Witte

peptone to sheep's pancreas extract which was then activated by 5 % of E. at 37°. It will be seen that with increasing quantities of peptone the rate of the last part of activation, as compared with that of the first part, was more and more diminished. The actual percentage time of the last half of activation on the total time when 0, 0.2, 0.6, 2.0 and 3.5 % of Witte peptone was present was 16, 29, 27, 31 and 46 % respectively. Probably the peptone acts chiefly by retarding the action of the trypsin on pro-deuterase, for it is well known to retard its action on proteins. For instance, I found [1904, 2, p. 350] that 2 % of Witte peptone reduced the digestive action of trypsin on fibrin by 12 %.

Fig. 5.

Under other conditions the action of enterokinase is much more retarded than that of deuterase. For instance, 5 % of it took 157 minutes to activate a glycerin extract of sheep's pancreas which was diluted with half its volume of water but without the addition of Na_2CO_3 , as against 44 minutes when it was diluted to 5 volumes of 0.05 % Na_2CO_3 . The last half of activation, however, took only 35 min. as against 15 min., or 22 % of the total activation time as against 34 %. Again, a pig's pancreas extract diluted 50 % with water only, took 203 min. as against the normal 56 min., but took only 12 % of the total time over the last half of activation instead of 28 %. The same extract, diluted 50 % with Na_2CO_3 up to a concentration of 0.08 %, took 139 min. for activation, but only 12 min., or 9 % on the total activation time, over the last half of activation. Presumably this great retardation of the enterokinase is due chiefly to the glycerin itself. Arguing on the

supposition that the gland substance which was extracted by four times its weight of glycerin contained 75 % of water, these activating solutions must have contained about 56 % of glycerin.

THE ACTIVATION OF TRYPSINOGEN BY DEUTERASE.

In previous experiments made with pancreatic juice and extracts I found [1902] that the addition of active pancreatic extracts caused a more rapid activation than the addition of intestinal extracts containing entero-kinase. This result I have fully confirmed in my recent experiments.

Fig. 6.

In Fig. 6 is shown the effect of adding 0.25 to 8 % of an active glycerin extract of pig's pancreas to an inactive extract at 17°. It will be seen that 8 or 4 % of extract activated almost completely in about 16 minutes, whilst 2 % activated in about 40 minutes. The form of the activation curve is entirely different from that obtained with enterokinase. Activation begins very rapidly and gradually slows down to zero as the available mass of trypsinogen diminishes, or deuterase appears to act logarithmically like most other enzymes. However, its action does not as a rule obey the logarithmic law, for it will be noted that the activation slows down and nearly ceases before the whole of the trypsinogen is converted. This is especially well seen when 1 % or less of deuterase extract was employed. In these experiments the activation almost stopped in the first 10 or 20 minutes, and then continued

with such extreme slowness that even 22 hours later, as can be seen from the data on the extreme right of the figure, a large portion of the trypsinogen was still unconverted. As will be shown later, this very slow and continuous conversion must be due to the action of fresh deuterase gradually liberated by the trypsin from the pro-deuterase contained in the inactive extract.

It should be stated that in these experiments the tryptic value of the active extract added was always deducted from the tryptic value observed. Also it was found that the deuterase continues to activate the trypsinogen of the extract to some extent after the sample has been added to the diluted milk for estimation, though not so rapidly as in the original solution. Hence in plotting out the curves *half* of the time taken for each estimation—which was generally 3 or 4 minutes—was added to the actual time from start of experiment to beginning of the estimation. In the enterokinase experiments no such correction was made, as the enterokinase appeared to cease activating on mixing with the milk.

A large number of activation experiments have been made with different substrates and different deuterase extracts, and they show that no single law of action will suffice to explain them. From evidence which will be adduced later it seems probable that both the trypsinogen and the deuterase in pancreatic extracts exist in various colloidal states, the condition of which can be readily changed, and which greatly influence their interaction. For instance, in Fig. 10 (see below) is recorded another set of experiments made with the same pancreatic extracts as were used in the experiments just described, but three months previously. It will be seen that the activation curves obtained with 0.7 and 1.2 % of deuterase approach the logarithmic type much more closely than those in Fig. 6. In still another experiment with the same inactive glycerin extract of pig's pancreas, but with a different deuterase extract (obtained by extracting pig's pancreas with two parts of N/10 HCl; slightly over-neutralising 17 hours later with solid Na_2CO_3 , and filtering off), [*cf.*, Mellanby and Woolley, 1914, 1], the activation curve was definitely logarithmic in type. It is impossible to determine the total amount of trypsinogen present in an extract with great accuracy, for in the first place the method of trypsin estimation employed is only moderately accurate, and it does not give quite the same results from day to day as the samples of milk vary somewhat in quality. This glycerin extract had on previous occasions developed a maximum tryptic value of 236, 229, 219, 212 and 211, when kept at 17° with deuterase, but in the present experiments developed a value of only 200. Taking this to represent the effective maxi-

num, and deducting from it the 7 units of free trypsin present in the extract before activation, it will be seen from the table that the results obtained at 17° and 37° roughly follow the logarithmic law for uni-molecular reactions, viz. :

$$K = \frac{1}{t} \log \frac{1}{1-x}.$$

The most exceptional values were those obtained in the two initial estimations. They are probably due to the correction made for the activation which continues during the trypsin estimation being too great.

Fig. 7.

At 17° C.			At 37° C.		
Trypsin liberated (x)	Time (t)	K	Trypsin liberated (x)	Time (t)	K
46	3.5 min.	0.034	48	3.25 min.	0.038
69	8.4	0.023	69	8.0	0.024
95	13.0	0.023	101	15.5	0.021
109	18.0	0.020	114	23.75	0.016
127	23.5	0.020	155	35.5	0.020
170	69.0	0.014	182	42.75	0.029

In another series of experiments, made with extracts of sheep's pancreas, the rate of activation as a rule followed the linear law. In Fig. 7 is shown the effect of adding active glycerin extract of sheep's pancreas to inactive extract, and it will be seen that the activation curves are almost straight lines,

and continue so nearly to their completion. Yet these very same extracts, when tested some four months later, gave activation curves more or less midway between the linear and logarithmic types. Also, the inactive extract, at the same time that it was giving linear curves when activated by the active extract of sheep's pancreas, under some circumstances gave nearly logarithmic curves when activated by the HCl extract of pig's pancreas above mentioned. It should be stated that this extract had begun to activate spontaneously immediately it was prepared, but since at the time it was required for this experiment it had not quite completed its activation, a trace of enterokinase (0.1 %) was added to it and it was kept 24 hours before use. The action of 10 % of this mixture is shown in the dotted line curve of Fig. 7. Similar curves of a logarithmic type were obtained on activating with 6 and 4 % of the mixture, but 3, 2, 1.5 and 1 % of it gave absolutely linear activation curves. In their case the available mass of substrate must have been in excess of the maximum upon which the enzyme could act at any moment.

Five days after the HCl extract had activated itself completely its action on inactive sheep's extract was again tested, and 10 % of it, acting at 37° and at 17°, yielded curves almost identical in form and position with those shown on the extreme left of Fig. 7. The changed action seemed to depend partly on the lack of a trace of enterokinase, for after keeping some of the completely activated HCl extract for 24 hours with 0.1 % of E., it again yielded a curve approaching the logarithmic type. On the other hand some of the HCl extract likewise gave logarithmic instead of linear curves when diluted and kept five days with 0.05 % Na_2CO_3 . Also, the inactive extract of sheep's pancreas under certain conditions, to be mentioned later, yielded curves very similar to those shown in Fig. 6, whilst it yielded curves about midway between the logarithmic and linear types when activated by active extract of pig's pancreas.

A glycerin extract of ox-pancreas, acted on by an active glycerin extract of monkey's pancreas, gave nearly linear curves (*cf.* Fig. 16).

These very conflicting results suggest that a change in the colloidal condition of enzyme and substrate, and in their manner of interaction, is effected very easily. More striking proof of the existence of different kinds of trypsinogen in pancreatic extracts came to light in quite another way. It was found that the maximum tryptic value induced on addition of enterokinase to inactive extract was always less than when it was kept with active extract (*i.e.* deuterase). The difference in the two maxima

attained varied considerably even in the same extract at different times. For instance, a glycerin extract of pig's pancreas was filtered off from the gland substance after 23 to 26 days' extraction, and was then found to develop a maximum tryptic value of 132 on addition of enterokinase. Four days later the maximum was 160; twelve days later it was 187, but three months later it averaged 215 in various experiments. On keeping this same extract with various deuterase extracts it developed an average tryptic value of 221, hence after the three months' interval practically all of its trypsinogen was in the same form. On the other hand a glycerin extract of sheep's pancreas, even after it had been kept three months, developed a maximum tryptic value of only 252, 236, and 244 in various experiments on addition of

Fig. 8.

enterokinase, but one of 334, 313 and 312 when deuterase was added. That is to say, on an average only 76 % of the trypsinogen was converted into trypsin on the addition of enterokinase. Of course the extract did attain the same maximum after addition of enterokinase as after the addition of deuterase if it were given sufficient time, and if the destruction of the liberated trypsin were avoided by carrying out the experiment at 17° or 1°, but this only occurred slowly and was quite distinct from the rapid activation to a nearly constant level which is so typical of the action of deuterase.

After the glycerin extract just mentioned had been kept another four months in cold storage, it was found that nearly *half* of its trypsinogen had passed into the relatively stable form. This is strikingly proved by the series of experiments recorded in Fig. 8, in which it was acted on by 10 to

1.25 % of enterokinase at 17°. With 10 % of enterokinase the typical activation was completed in 41 minutes, the last half of activation taking only 15 % of the total time. Then the activation of the second half of the trypsinogen proceeded at a rate about seven times slower than the previous maximum rate, and it took about 97 minutes for its completion. The action of 5 % of E. was equally striking, but with 2.25 % and 1.25 % of E. the change in the slope of the curve after activation of the first half of the trypsinogen was not so marked. It will be noted that within the limits of experimental error the slope of the last half of the activation curves is almost the same whatever the amount of enterokinase present, *i.e.*, it is a deuterase effect.

The dotted line curve shows the action of 5 % of E. (another preparation) on this same extract of sheep's pancreas four months previously. It is one of the results already recorded in Fig. 2. Unfortunately the rate of activation in these earlier experiments at 17° was not investigated after the supposed maximum had been reached. It had been investigated in the corresponding experiments at 37°, and was then found to fall off rapidly. The reason is that the trypsin is so unstable at 37° that its destruction rate is greater than its activation rate, when such activation occurs at the slow speed shown by the latter half of the curves in Fig. 8.

THE ACTIVATION OF PANCREATIC JUICE BY DEUTERASE.

Pancreatic juice is activated by deuterase in the same way as pancreatic extracts. In Fig. 9 (p. 510) is shown the effect of 1 to 3 % of a glycerin extract of pig's pancreas at 37°, and of 3 % at 17°. The curves are rather irregular in form, owing largely to the fact that the final stages are complicated by the activating effect of fresh deuterase liberated from the pro-deuterase present in the juice. The difference in the rate of activation induced by 3 % and by 2 % of deuterase at 37° is so remarkable that it might be thought to be due to some experimental error: but a repetition of the experiment a day later gave almost the same result. The two sets of values obtained with 3 % of deuterase are shown in the figure.

Experiments on the activation of pancreatic juice by the deuterase of activated juice will be described in a subsequent section.

THE TEMPERATURE COEFFICIENT OF DEUTERASE.

As already mentioned, the activation of trypsinogen by deuterase is only slightly affected by temperature. A striking proof of this statement

Fig. 9.

is afforded by the table showing the relationship between time and trypsin liberated which was given a few pages back. The average velocity coefficient, K , was 0.0223 at 17°, and 0.0247 at 37°, or nearly the same. A number of

Fig. 10

other experiments in which an active glycerin extract of pig's pancreas was allowed to activate an inactive glycerin extract of pig's pancreas gave almost the same result. One series is shown in Fig. 10, and here we see that though 3 % and 0.7 % of deuterase activated rather more quickly at 37° than at 17°, 1.2 % activated more quickly at 17° than at 37°, or the average of the three sets of experiments indicates that the temperature change was practically without influence. At 1°, however, there is a distinct retardation in the rate of activation, especially in the initial stages. Later on there is an acceleration, and in the experiments with 1.2 and 0.7 % of deuterase we see that 30 minutes after the start the activation was proceeding at about the same rate at 1°, 17°, and 37°. This acceleration is due in part to the greater stability of deuterase at the low temperature, but there must be some other factor concerned, as deuterase is only very slowly destroyed at 17°.

As can be seen from the curves given in Fig. 7, the action of deuterase on extract of sheep's pancreas is considerably affected by temperature; but the actual temperature coefficient is not nearly so high as might be imagined at first sight. It is true that the rate of activation by 3 % of deuterase at 37° was nearly three times greater than at 17°, and by 1.5 % of deuterase, three and a half times greater, but it can be calculated from the curves that 3 % of deuterase at 37° activated the trypsinogen nine times more rapidly than did 1.5 %, and at 17°, fourteen times more rapidly. Hence in order to arrive at the real temperature coefficient, the relative amounts of deuterase required to induce the same rate of activation at 37° and at 17° must be calculated. It is found that 2.5 % of deuterase at 37° would activate at the same rate as 3.5 % at 17°, or that the temperature coefficient for this extract is 1.18.

It is a more accurate method to determine by direct experiment the relative amounts of deuterase required to activate at the same rate at 37° and 17°, and this has been done in two instances. It was found that 2 % of an active glycerin extract of pig's pancreas at 37° activated glycerin extract of ox pancreas at almost exactly the same rate as 3 % at 17°, or that the temperature coefficient was 1.22. Again, 2.5 % of active glycerin extract of sheep's pancreas at 37° activated pancreatic juice at the same rate as 3.0 % at 17°, or gave a temperature coefficient of 1.09.

The temperature coefficients for the activation of trypsinogen by enterokinase and deuterase are collected in the table for the sake of contrast. Only the data obtained between the temperature limits of 17° and 37° are included.

		Temp. coefficient (10°)
Enterokinase activating	Pancreatic juice	2.10
	Extract of sheep's pancreas (2.07, 1.93)	2.00
	„ „ pig's pancreas (2.07, 1.97, 1.93, 1.93, 1.86, 1.81)	1.93
	„ „ ox pancreas	1.96
		1.09
Deuterase activating	Pancreatic juice	1.08
	Extract of sheep's pancreas	1.18
	„ „ pig's „	1.00
	„ „ ox „	1.22

A large number of experiments were made in which the data were insufficient to give an accurate temperature coefficient, so in order to compare

Fig. 11.

the temperature effects the method adopted was to read off from the plotted curves the times taken for liberation of 50 % of the total amount of trypsin set free in 30 or 40 minutes at 37°, and then find the time taken for the liberation of the same amount of trypsin at 17°. The time of liberation of 50 % of trypsin is taken for comparison rather than that of the completion of activation, as it can be estimated more accurately.

The differences of temperature effect observed with extracts of pig's pancreas and of sheep's pancreas appear to depend on the quality of both enzyme and substrate. Taking first the experiments made with glycerin extracts, it was found that when pig's pancreas deuterase activated sheep's pancreas trypsinogen, it took 1.4 times longer at 17° than at 37°, instead of 1.5 to 3.1 times longer as in the corresponding experiments when sheep's

deuterase activated sheep's trypsinogen. In the converse experiment when sheep's deuterase activated pig's trypsinogen it took, in various experiments, 1.2, 1.3 and 2.3 times longer at 17° than at 37°, whereas we have seen that pig's deuterase activated pig's trypsinogen as quickly at the lower temperature as at the higher. Hence the temperature effect depends partly on enzyme, but probably the physical condition of the substrate is the most important factor, and it can readily be modified so as to influence the temperature effect enormously. This is well shown by the two series of curves reproduced in Fig. 11. In one experiment, the results of which are represented by continuous line curves, some of the same glycerin extract of pig's pancreas, which on fivefold dilution with 0.05 % Na_2CO_3 was activated as quickly at 17° as at 37°, was diluted fivefold with water only. It was activated by 5 % of active pig's pancreas extract, and we see that its activation was distinctly slower at 17° than at 37°, and very much slower at 1°. In the other experiment the same extract was diluted with an equal volume of water only, and then it took four times longer to activate at 17° than at 37°, and much longer still at 1°. The curve obtained at 17° happens to be identical in position with the 1° curve of the more diluted extract.

Experiments with the active HCl extract of pig's pancreas previously mentioned gave similar results. When allowed to activate glycerin extract of pig's pancreas it took 1.6 times longer at the lower temperature, and when activating sheep's extract it took, in various experiments made at different times with different amounts of enzyme, 2.2, 2.7, 2.8, 3.8 and 6.4 times longer at the lower temperature.

THE RATE OF LIBERATION OF DEUTERASE.

We have seen that the addition of small quantities of active pancreatic extracts to inactive extracts rapidly activates the trypsinogen, but if a sample of this freshly activated extract be added to more inactive extract it is found to possess little or no activating power. For instance, in Fig. 12 we see the effect of adding 3 % of active extract of pig's pancreas to inactive extract at 17°. Activation was almost complete in 30 minutes, and a minute after this time (marked by an arrow in the figure) a sample of the mixture containing 3 % of the extract was added to more inactive extract at 17°, and we see from the lowest curve in the figure that it had extremely little activating power. Another sample of the mixture taken 48 minutes after had distinctly more activating power: one taken 115 minutes after much more activating

power still, whilst a sample taken 175 minutes after had almost as much activating power as the original pancreatic extract. It is concluded, therefore, that a freshly activated extract contains scarcely any deuterase, but that this enzyme is gradually liberated from the pro-deuterase present in the extract by the action of the free trypsin. In proof of this hypothesis, it is found that the rate of development of activating power is dependent on temperature. On repeating the experiment at 1° , it was found that 3 % of the active extract activated the inactive extract to about the same point as that indicated in Fig. 11 in 90 minutes. At times ranging from 145 to 696 minutes later, 3 % samples of it were added to inactive extract at 17° ,

Fig. 12.

and it was found that the activation curves were of the same character as those shown in Fig. 11, except that the rate of generation of activating power in the mixture—which was kept throughout at 1° —was about four times slower than before. This is shown by the curves given in Fig. 13, which indicate the percentage of trypsin liberated in 40 minutes on addition of 3 % samples of the regenerating mixtures to inactive extract. When the results recorded in Fig. 12 are plotted out in this manner, it is seen that the rate of development of activating power gets more and more rapid from start to finish. The results obtained at 1° are plotted out on a time scale reduced fourfold, and we see that the curve obtained is of similar form and position to that obtained at 17° ; *i.e.* that the rate of development of activating

power in freshly activated extracts is four times slower at 1° than at 17° . At 37° it is probably about four times quicker than at 17° , but the results are unsatisfactory owing to the extreme instability of the liberated deuterase at this temperature. Thus an experiment was made in which 3 % of active extract was added to inactive extract at 37° , and it activated it almost completely in 24 minutes. At times ranging from 5 to 139 minutes later 3 % samples of this mixture—kept throughout at 37° —were added to inactive extract at 17° , and the percentages of trypsin liberated in 40 minutes are shown in Fig. 13. We see that though the activating power of the mixture

Fig. 13.

at first developed much more rapidly than that kept at 17° , it quickly slowed down, and after 105 minutes began to deteriorate.

The rapidly accelerating rate of formation of deuterase in freshly activated extracts is a mysterious and unexpected phenomenon. It is true that within certain limits the amount of trypsin liberated in an inactive extract on the addition of active extracts increases in greater proportion than corresponds to the extract added. For instance, the curves in Fig. 6 indicate that the addition of 0.25, 0.5, 1 and 2 % of active extract to inactive extract caused the liberation, in 40 minutes, of 12, 28, 79 and 193 units of trypsin respectively, or in the proportion of 1, 2.3, 6.6 and 16.1, instead of 1, 2, 4

and 8: but the rate of acceleration of deuterase formation is much more rapid than this. It occurs in geometrical progression, as is shown by comparing the curves of deuterase formation in Fig. 13 with the dotted line curve. This curve indicates the percentage of trypsin liberated by 0.25 to 4 % of active extract (*cf.* Fig. 6), but plotted out on the logarithmic scale given at the top of the figure. The curve is very similar in form to those indicating the rate of formation of deuterase at 17° and 37°, and hence it follows that this likewise occurs in geometrical progression.

In contradistinction to this geometrical increase in the rate of deuterase liberation is the fact that on addition of small quantities of deuterase to inactive extracts a certain amount of trypsinogen is activated almost at once, but then the activation slows down and continues so slowly as to be incomplete even 24 hours later (*cf.* Fig. 6). One would have thought that the trypsin liberated by the deuterase added would at once begin to liberate more deuterase from the pro-deuterase present, and that in consequence the activation would soon begin to accelerate again as it does in the presence of enterokinase. So far from that, the results recorded in Fig. 6 indicate that 0.5 % of deuterase, though it liberated 26 units of trypsin in 40 minutes, liberated only an extra 42 units in 22 hours. Again, 1 % of deuterase liberated 78 units in 40 minutes and an extra 90 units in 22 hours, though at the end of this time 52 units of trypsin still remained in the zymogen form. Experiments with sheep's pancreas extracts showed that the activation induced by deuterase continued much more steadily than that in pig's pancreas extracts, but even in them no acceleration was observed. The explanation of this result is unknown, but it seems to be dependent on conditions, such as the presence of anti-bodies, which do not obtain in pancreatic juice. As already pointed out, the results recorded in Fig. 9 clearly indicate that the addition of deuterase to pancreatic juice causes not only a rapid initial activation of a portion of the trypsinogen, but after an interval of slow activation this is succeeded by a second acceleration which must be due to the liberation of fresh deuterase. The results obtained in another experiment at 17° are recorded in Fig. 14 (see below), and two of the curves there reproduced show this secondary acceleration. In a similar experiment carried out at 37° the secondary acceleration appeared in every case.

It is evident that if time be given for the deuterase in freshly activated extracts to be liberated, the capacity for activation can be transmitted from extract to extract indefinitely. For instance, 5 % of an active extract of pig's pancreas was used to activate inactive extract of pig's pancreas,

and 4.5 hours later 5 % of this mixture was added to more inactive extract. 15 hours later 5 % of *this* mixture was used to activate still more inactive extract, and 5.5 hours later 5 % of this last mixture was added to still another sample of inactive extract. All the activation curves in the series were practically the same in form.

Experiments with glycerin extracts of sheep's pancreas gave results similar to those obtained with extracts of pig's pancreas. For instance, 5 % of active sheep's extract was added to inactive extract at 17°, and 36 minutes later the addition of 5 % of this mixture to more inactive sheep's

Fig. 14.

extract at 17° liberated 8 units of trypsin; 51 minutes later it liberated 13 units; 150 minutes later 24 units, but 343 minutes later, 165 units. Hence there was a rapid acceleration in the rate of deuterase formation in the latter part of the experiment, as in the previous series of experiments. The activation curves were somewhat similar in form to those shown in Fig. 12, and were not linear like the sheep's pancreas curves shown in Fig. 7.

Pancreatic juice yielded similar results to pancreatic extracts. In an experiment at 17°, 3 % of active extract of pig's pancreas activated the juice completely in 47 minutes, and 6 minutes later 5 % of the active juice was added to inactive juice at 17°, but as can be seen in Fig. 14 its activating power was but small. When it was tested again 98 minutes after it had probably reached its maximum activating power, for when tested 142 minutes

after it was distinctly less active. When tested 5 hours and again 21 hours after it had less activating power still. Hence the curves shown in the figure seem to indicate that the activating power of freshly activated pancreatic juice reaches its maximum two to four times more quickly than in pancreatic extracts, and that the deuterase formed is so unstable as to undergo rapid destruction even at 17°. In a similar experiment at 37° 3 % of active extract activated the juice completely in 13 minutes, and the immediate addition of 5 % of this active juice to inactive juice at 37° had as much activating effect as that shown in the lowest curve in Fig. 14. A sample taken 25 minutes later had nearly as much effect as that shown in the "142 minutes after" curve of Fig. 14, whilst a sample taken 104 minutes after had slightly less effect than that shown in the lowest curve of Fig. 14. Another experiment at 37° showed that the freshly activated juice had more activating power 14 minutes after it had completed activation than either immediately after or 40 minutes after. Hence it is evident that at 37° the deuterase of freshly activated juice is extraordinarily unstable. It is quickly liberated, but within a very few minutes most of it is destroyed.

A COMPARISON BETWEEN THE ACTIVATING AND TRYPTIC POWERS OF EXTRACTS.

The experimental results thus far described are explicable on the hypothesis which I put forward in previous papers to the effect that trypsin itself is a powerful activator of trypsinogen. I found that fresh pancreatic extracts, rich in trypsin, had relatively much greater activating power than extracts which had deteriorated to some extent in tryptic power as the result of being kept. Also I found that the trypsin (or rennin) in freshly prepared extracts is so unstable that even when kept with water only (and no Na_2CO_3) at 38° about 50 % of it is destroyed in an hour. That in kept extracts is much more stable, and may be destroyed at the rate of only 12 % per hour [Vernon, 1901, 1, p. 196]. Hence I concluded that trypsin exists in various states of stability, and that only unstable trypsin has much activating power. It was suggested to me by Prof. W. M. Bayliss that I had no good evidence that the activating substance was this unstable trypsin, and further experiments supported his contention, for I found that in various freshly prepared active pancreatic extracts there was no relationship between activating power, trypsin content and stability of trypsin. It is quite true that if any extract deteriorates as the result of being kept, its activating power diminishes

much more rapidly than its tryptic power, but this is because the deuterase it contains is much more unstable than the trypsin.

Anything more than a rough comparison between the activating powers of extracts is useless, in that such comparisons do not hold at all closely at different concentrations. A comparison of the activation curves obtained with pig's pancreas extracts and sheep's pancreas extracts which are shown in Figs. 6 and 7 proves this. In experiments made at 37° it was found that 3 % of active sheep's extract activated inactive sheep's extract at the same rate as 1.25 % of active pig's extract, or that its activating power was 42 % as great. A similar experiment at 17° showed it to be 40 % as great. In other experiments made at different times upon inactive pig's extract it was found that 6, 4.5 and 3 % of active sheep's extract activated at the same rate as 2.0, 1.5 and 0.65 % of active pig's extract, or was 33, 33 and 22 % as active in the respective experiments. On an average, therefore, the sheep's extract had only 34 % of the activating power of the pig's extract, in spite of the fact that its tryptic power was almost exactly the same. The HCl extract of pig's pancreas, though it had three-fourths the tryptic power of the glycerin extract, had only a sixth its activating power, whilst a watery extract of pig's pancreas (made by extracting for 42 hours with 2 parts of chloroform water, filtering off and keeping with chloroform) had about half the tryptic power of the glycerin extract, but only a thirtieth the activating power. These experiments with pig's pancreas extracts confirm those formerly made by me [1901, 2, p. 295], in which I found that 0.9 % NaCl extracts of the pancreas of various animals had much less activating power, relative to their trypsin content, than their glycerin extracts.

The most striking evidence of the lack of correspondence between activating power and tryptic power was obtained with the other two extracts recorded

Pancreatic Extract	Relative activating power Mean	Trypsin	% Trypsin destroyed in 1 hour at 37°
Glycerin extract of pig	100	294	12
„ „ „ monkey	83, 62, 52	66	83
„ „ „ sheep	42, 40, 33, 33, 22	34	293
HCl „ „ pig	17	218	17
H ₂ O „ „ pig	3.5	144	—
Glycerin „ „ ox	1	183	78

in the table. A glycerin extract of monkey's pancreas was found to have only about a fourth as much tryptic power as the glycerin extract of pig's pancreas, but two-thirds its activating power. This relative richness of

the monkey's gland in pro-deuterase may help to explain the fact that both the concentrated glycerin extracts made with the pancreas of healthy Macaque monkeys, which had been killed instantaneously, activated themselves spontaneously within a fortnight of their preparation.

In contradistinction to monkey's pancreas extract, a 75 % glycerin extract of ox-pancreas, though it had nearly two-thirds the tryptic power of the pig's extract, had no activating power at all in most experiments. As there was a slight indication of it in one or two instances, its actual power is recorded in the table as 1 % on that of pig's extract. As will be shown in the next section, the extract did contain some deuterase, but its activating powers were masked by the presence of an anti-body.

The stability of the trypsin in pancreatic extracts was tested by diluting them fivefold with water and Na_2CO_3 to 0.05 %, and keeping for an hour (or two hours) at 37°. Under such conditions no less than 78 % of the trypsin of the ox-pancreas extract was destroyed, and 65 % of that in the sheep's extract. The other extracts were much more stable, and the glycerin extract

Extract		% trypsin destroyed in 1 hr. at 37°	Mean
Glycerin extract of ox pancreas		82, 76, 76	78 %
" " " sheep's "		71, 70, 70, 69, 64, 59, 52	65
HCl " " pig's "		21, 19, 17, 12	17
Glycerin " " pig's "		27, 26, 23, 23, 22, 21, 21, 14 (two hours)	(22)
" " " monkey's pancreas		17, 16	16

of pig's pancreas had only 22 % of its trypsin destroyed in *two* hours. The stability of the trypsin probably depends on its colloidal state, and so varies according to the manner in which an extract of a gland is prepared, for my earlier experiments with pig's pancreas extracts gave a very different result from the present ones. When tested by a fibrin digestion method it was found that the trypsin of a glycerin extract was destroyed at the average rate of 69 % per hour by 0.4 % Na_2CO_3 at 38°, and at the rate of 39, 32, 24 and 23 % per hour by water only (in this case tested by the metacasein method). That is to say, it was destroyed about three times more rapidly than in the present experiments, if we assume that 0.05 % Na_2CO_3 is rather more injurious than water only. A glycerin extract of sheep's pancreas was destroyed at the average rate of 80 % per hour by 0.4 % Na_2CO_3 , and at the rate of 66, 60, 53 and 39 % per hour by water only, or was about as unstable as in the present experiments.

The great instability of deuterase as compared with trypsin is proved by several experiments described in the previous section, in which the activating power of juice kept at 17° and 37° was found to deteriorate rapidly,

though the tryptic power was almost unaffected at 17° , and only moderately affected at 37° . Also I showed previously [1913, p. 335] that pancreatic juice activated at 37° has much less activating power on inactive juice than if it is activated at 17° . Deuterase retains its activity almost undiminished for months in glycerin extracts, but is very unstable in aqueous extracts. This is indicated by the small activating power of the HCl and aqueous extracts in the present experiments, and of the 0.9 % NaCl extracts described in my original experiments. Possibly the trypsin itself attacks it. Arguing from the great retardation to the action of deuterase exerted by Witte peptone, it seems probable that pro-deuterase is a protein substance which is first converted into deuterase, and then destroyed, by the digestive action of trypsin.

Fig. 15.

THE AUGMENTOR ACTION OF INTESTINAL EXTRACT ON DEUTERASE.

It was stated in an earlier section that when ox-pancreas extract is acted on by enterokinase, it shows the typical acceleration in the velocity of the last half of activation. On an average this latter half took 29 % of the total activation time, so the acceleration was not marked, but the mere fact that it exists at all points to the liberation of deuterase in the extract, or is in apparent contradiction to the result described above. Further experiment showed clearly that there is a formation of deuterase in the course of activation. The addition of 10 % of E. to the extract at 17° gave the curve shown in Fig. 15. Forty-one minutes after the point in this curve marked with an arrow had been reached, 20 % of the activated mixture (containing 2 % of E.) was added to more inactive ox-extract together with 8 % of fresh E. That is to say, the inactive extract was now being activated by 10 % of E., together

with any deuterase which may have been present in 20 % of the previously activated mixture. The curve obtained shows the rapid initial velocity of activation followed by a gradual slowing which is the typical deuterase effect. This is probably followed by a second acceleration of activation, though by an oversight sufficient experimental data were not obtained to prove it, but in another experiment made with 10 % of E. + 10 % of the activated extract 138 minutes after activation, this second quickening of acceleration is shown. Presumably it is dependent on the enterokinase present. In another experiment made 76 minutes after activation, with 10 % of E. + 5 % of the activated mixture, the activation is considerably accelerated, but at a steady rate throughout.

This experiment leaves no doubt that a moderate amount of deuterase must have been liberated from pro-deuterase in the extract of ox-pancreas during the course of its activation by enterokinase, but in such a case how is one to explain the apparent absence of deuterase as shown in the experiments of the type described in the last section? The subject is a difficult and complex one, and I did not attempt to solve it in detail, but the experiment to be described points to the existence of an anti-body which neutralises the deuterase unless its action is augmented by the presence of enterokinase. Some of the extract of ox-pancreas was activated at 17° by the addition of 5 % of active extract of pig's pancreas, and 8, 56, 128 and 174 minutes after activation was practically complete 5 % samples of this activated extract were added to more inactive extract, together with 5 % of E. In every case activation occurred more rapidly than in the control experiment in which E. alone was added. With the 128 minutes sample it was completed in 81 minutes instead of the 109 minutes taken in the control experiment, and with the other samples in 84 to 90 minutes. In the absence of E. the samples had no activating effect at all.

The augmentor action of intestinal extract upon deuterase was proved by a number of experiments. For instance, Fig. 16 shows the effect upon inactive extract of ox-pancreas at 37° of (a) 1.25 % of deuterase (active glycerin extract of monkey's pancreas); (b) 1.25 % of deuterase which had been diluted tenfold and kept with 10 % of E. at 17° for 2 minutes before mixing with the inactive ox extract; (c) 1.25 % of deuterase kept with the E. for 62 minutes before mixing with the inactive extract. We see that in this last experiment activation occurred considerably quicker than in the first or even in the second experiment. In fact it was almost doubled in rate, as can be seen on comparing the curve with the control

curve obtained with 2.5 % of active extract. Hence it is evident that the trace of E. in some way augments the action of the deuterase, and that to get the best result it is necessary for the deuterase and E. to be mixed for some little time before they are added to the inactive extract. In another experiment, with different extracts, it was found that when the deuterase and E. were mixed for 32 minutes before, they had just the same activating effect as when they were mixed 69 minutes before, activation being in each case completed in 18 minutes instead of 24 minutes, the time taken when they were mixed only 1 minute before. If the time for which they are mixed be too prolonged the activating effect is diminished rather than increased,

Fig. 16.

as the enzymes appear to destroy one another. Thus in one experiment it was found that if they were mixed 148 minutes before adding to the inactive extract there was no augmentation of activation at all, whilst in experiments in which they had been mixed for 24 hours there was a considerable diminution of activation.

It might be thought that the increased rate of activation was due directly to the E. but this is not so, for the actual amount added in the experiment recorded in Fig. 16, viz., 0.125 %, had by itself no appreciable activating effect in the time for which the experiment lasted. In another experiment the proportion of E. added was large enough to produce a considerable amount of activation. An extract of sheep's pancreas was activated at 37° by (a) 2 % of deuterase (active extract of pig's pancreas); (b) 0.25 % of E.

(a very active preparation); (c) 2 % of deuterase + 0.25 % of E., mixed together in presence of 0.05 % Na_2CO_3 and kept at 37° for 1.5 minutes before adding to the inactive extract. This mixture liberated 43, 104, 164 and 191 units of trypsin after 10, 15, 20 and 25 minutes respectively, whilst the sum of the amounts liberated by the deuterase and the E. after the same time intervals amounted only to 22, 43, 77 and 145 units.

An experiment in which enough E. was taken to activate a glycerin extract of pig's pancreas completely in 60 minutes at 17° is recorded in Fig. 17. This activation is compared with that produced by (a) 1 % of deuterase (active extract of pig's pancreas); (b) samples of 1 % of deuterase + 0.5 %

Fig. 17.

of E. mixed together at 17° in presence of 5 volumes of 0.05 % Na_2CO_3 , and added to the inactive extract, 2, 67 and 107 minutes later. The curve obtained with the 2 minutes mixture shows a less rapid initial rate of activation than that produced by 1 % of deuterase alone, and much less than the sum of the effects produced by 1 % of deuterase + 0.5 % of E. The 107 minutes mixture, however, yielded an activation curve the earlier part of which would about correspond to the sum of the deuterase and E. curves, and the later part of which represents a distinct augmentation on such effects. The retardation produced when deuterase and E. solutions are mixed in certain proportions and added immediately to inactive extract appears to be greater the larger the proportion of E. used, for in another experiment at 17° , upon inactive sheep's extract, the addition of a mixture of 5 % of deuterase (active sheep's extract) + 10 % of E. (an old and rather weak preparation) caused

activation in 95 minutes when added immediately after mixing, though the 10 % of E., when acting alone, caused activation in 58 minutes. The mixed deuterase + E., when tested 62 minutes after mixing, took 77 minutes to activate; 116 minutes after mixing, 62 minutes to activate; 187 minutes after, 36 minutes, and 229 minutes after, 32 minutes. The 5 % of deuterase, when acting alone, activated three-fourths of the trypsinogen in the first 33 minutes, and then almost stopped, but even the activation induced by the 229 minutes' mixture was only equal to the sum of the activation rates induced by the deuterase alone and the E. alone. Hence in this experiment the large quantity of E. added, or some anti-body contained in the preparation, exerted a retarding action throughout.

GENERAL CONCLUSIONS.

It is desirable to discuss briefly some of the objections which may be levelled against my hypothesis, and to show in what respects it can be reconciled with previous observations. In the first place it may be said that it is inherently improbable that two different enzymes should be required to bring about one and the same effect. In reply to this I would point out that several different agents are capable of liberating secretin from pro-secretin. In addition to acids the liberation is effected by soaps, alcohol, 0.6 % NaCl and other substances [Matsuo, 1913]. These liberating substances cannot all act in the same way; neither can pepsin and trypsin attack proteins in the same way, and so there is no real difficulty in the fact that enterokinase and deuterase attack trypsinogen in different ways. The extremely low temperature coefficient observed for deuterase, viz., 1.12, is unique, but comparatively low coefficients have been observed with other enzymes. Kastle and Loevenhart [1900] obtained one of 1.3 for the action of esterase on ethyl butyrate. Tammann [1892], Kjeldahl [1881], and O'Sullivan and Thompson [1890] independently obtained the same coefficient, viz., 1.4, for the action of invertase on cane sugar, whilst Senter [1903] obtained a coefficient of 1.5 for the action of catalase on hydrogen peroxide. Most other enzymes investigated, including enterokinase, have been found to give coefficients of 2.0 to 2.6 [for literature see Euler, 1910], but Bayliss [1904] observed one of 5.3 for the action of trypsin on caseinogen, and as I [1904, 1, p. 364] observed one of 2.2 to 1.8 for the action of trypsin on Witte peptone one must conclude firstly that different enzymes have very different coefficients from one another, and secondly that even with the same

enzyme the coefficient varies with the nature of the substrate. In this connection it will be remembered that deuterase gave coefficients varying from 1.0 to 1.2 with different substrates.

Bayliss [1911] points out that the temperature coefficient of adsorption is, so far as investigated up to the present, a low one. He himself found that the rate at which equilibrium is attained in the adsorption of Congo red by paper is accelerated 1.36 times for each rise of 10° between 10° and 50° . Presumably the still lower coefficient obtained with deuterase is likewise compatible with the view that its action is an adsorption process, and involves no chemical change such as is associated with the action of enterokinase. The low coefficient of deuterase cannot be taken to indicate that it is not a true enzyme, for we have seen that it acts catalytically according to the logarithmic or the linear law like other enzymes. Moreover it is an extremely unstable body.

As another objection to my hypothesis it may be said: What is the need of a dual mechanism of activation? A convincing answer is supplied by the experiments of Terroine [1910] and of Mellanby and Woolley [1914, 2], who have shown that pancreatic steapsin is extraordinarily unstable in the presence of free trypsin. If enterokinase is added to pancreatic juice, the steapsin persists only so long as the quantities of trypsin present are minute. Hence, as Mellanby and Woolley point out, it is essential that the digestion of fat in the small intestine should take place in the absence of much free trypsin. This condition is observed in the upper part of the small intestine provided that there is not much enterokinase present, for in such a case the rapid liberation of trypsin from the trypsinogen of the juice may be delayed for many minutes.

An objection may be raised to the fact that enterokinase apparently has two distinct actions. We have seen that it not only liberates trypsin from trypsinogen, but it heightens the activity of deuterase on trypsinogen. This fact naturally suggests that deuterase is merely a co-enzyme of enterokinase, but arguments precluding this view have been adduced in a previous section. It may be found that these two properties of enterokinase are in some way related to one another, though at present we cannot say how. Again, it is possible that enterokinase acts by neutralising an anti-body to deuterase which is present to a greater or less extent in all extracts. Moreover, the augmentor effect on deuterase may not be due to enterokinase at all, but to some other unknown substance present in intestinal extracts.

The nature of the change involving the conversion of trypsinogen into trypsin is unknown. Mellanby and Woolley suggest that enterokinase is a proteolytic ferment which activates trypsinogen by digesting it, and splitting off free trypsin from a protein moiety with which it is firmly combined. Some of my earlier experiments [Vernon, 1903] show this hypothesis to be very improbable. I determined the proportions of trypsinogen precipitated from a glycerin extract by the addition of various strengths of alcohol, and on repeating the experiment a few days later when the trypsinogen had been converted into trypsinogen I found the precipitability of this trypsin to be almost exactly the same. It might be argued that the alcohol really precipitated protein from the extract, which absorbed and carried down trypsinogen or trypsin with it, but in reply I would point out that I found the precipitability of another enzyme in the extract, viz. amylopsin, to be quite different from that of the trypsin. Hence it is highly improbable that a protein + trypsin body would be adsorbed exactly to the same extent as trypsin alone. In all probability the conversion of trypsinogen into trypsin is a transformation involving no change in the size of the molecule, and this can be induced either by a mechanism which involves a chemical process at some stage, or by a purely adsorption process, *i.e.*, by enterokinase or by deuterase.

If the results described in this paper be compared with those given previously [Vernon, 1913], it might be thought that they are to some extent mutually contradictory. Thus I showed that inactive juice could readily be activated by the addition of activated juice, whether this contained enterokinase or not, but the activation curves which I gave do not, except in one experiment, show the initial rapid activation which is typical of the action of deuterase. The explanation is that I did not as a rule happen to catch my samples of activated juice at a time when they still retained much of their unstable deuterase, and that in consequence the curves given in Figs. 2 and 4, and the curve on the right of Fig. 3 of my previous paper, are comparable to the lowest curve in Fig. 9 of the present paper, whilst the curve on the left of Fig. 3 of my previous paper is comparable to that on the extreme left of Fig. 9 of the present paper. Again, Mellanby and Woolley [1913, p. 355] quote an experiment in which they activated juice by enterokinase, and every few minutes during the course of activation they took 10 % samples of it and added them to inactive juice. On plotting out their results it can be calculated that the sample taken when the primary activation was just completed had nearly twice the activating power of the sample

taken when it was just begun, and yet they do not admit that this result affords proof of the existence of any activating agent other than enterokinase. As is indicated by the results described in this paper, they would have found that their activated juice had a much greater activating power if they had tried it a few minutes after it had completed its activation, and still better, if they had carried out the experiment at 17° instead of 37°.

SUMMARY.

1. The experiments described in this paper lead to the conclusion that in the activation of trypsinogen by enterokinase, the trypsin liberated in the earlier stages by the direct action of the enterokinase gradually sets free an enzyme (termed *deuterase*, to indicate that it acts secondarily to enterokinase) from a precursor, and that this deuterase is mainly responsible for the later stages of the activation process.

2. In support of this hypothesis it is found that in the activation of pancreatic juice by enterokinase, the rate of activation during the last half of the process may be 1000 times more rapid than the initial rate, and it is independent of the quantity of enterokinase used. The total activation time varies with the temperature (temp. coefficient for 10° = 2.00), but the time taken over the last half of activation is only slightly influenced by temperature. This is because it is chiefly dependent on deuterase, the temperature coefficient of which ranges from 1.00 to 1.22. Enterokinase and deuterase are also distinguished by the fact that the former is relatively much more retarded in its action by glycerin, and the latter, by Witte peptone.

3. The addition of active pancreatic extract or juice to inactive extract or juice causes activation which is rapid at first, but which quickly slows down almost to zero. This is due to its containing deuterase, which acts according to a logarithmic or linear law like other enzymes. The freshly activated extract or juice contains very little deuterase, but it is gradually liberated from pro-deuterase in the course of the next few minutes (at 37°), or next hour or two (at 17°). The rate of its liberation increases in geometrical progression.

4. There is no relationship between the amounts of deuterase and trypsin present in pancreatic extracts prepared from various animals. Glycerin extract of monkey's pancreas contained only a fourth as much trypsin as glycerin extract of pig's pancreas, but two-thirds as much deuterase. Some experiments seemed to show that extract of ox pancreas contained no deuterase

at all, but this was because its presence was masked by an anti-body. Deuterase is extremely unstable, except in presence of glycerin, and therefore aqueous pancreatic extracts have relatively little activating power, whilst freshly activated pancreatic juice was observed to lose most of its activating power in two hours at 37°. Probably the deuterase is destroyed by the trypsin.

5. The stability of the trypsin in different extracts is extremely variable, and bears no relationship to activating power. That in ox pancreas extract was destroyed at the rate of 78 % per hour by 0.05 % Na_2CO_3 at 37°, whilst that in pig's pancreas extract was destroyed at 12 % per hour. Trypsinogen also exists in different degrees of stability, and in one extract half of it was found to be activated by enterokinase seven times more slowly than the other half.

6. The activating power of deuterase may be nearly doubled by the addition of small quantities of intestinal extract which themselves have no appreciable activating power. Nevertheless deuterase is not a co-enzyme of enterokinase.

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