RESEARCHES ON THE FORMATION OF DIASTASE BY ASPERGILLUS NIGER.

II.

By

G. L. FUNKE.

Contents.

I. Introduction.

The researches which I will describe here, are a continuation of those which have been done in 1921—1922 (9); I then devoted my attention chiefly to the question how the fungus Aspergillus niger forms amylase from day to day during the first six or eight weeks of its life time, when cultivated on substances such as glucose and amylum, which lend themselves well to the development of this enzyme. This time my chief purpose was to see how amylase is formed on other kinds of sugars and also to investigate how far the influence of acid and neutral phos-

phates goes, as many authors attach great importance to them.

The researches have been done in a room in which the temperature was somewhat higher than ordinary room temperature, with oscillations between 18°—20°. I do not think that this circumstance could present any difficulties, as oscillations of physiological processes are of no great importance at these temperatures.

The method of doing this type of research was essentially the same as in my former investigations; a very brief description therefore should suffice this time. The fungus was cultivated in globe shaped flasks with straight necks of 100 c. c. M. capacity; 50 c. c. M. of culture solution was used which had a surface area of ± 32 c.M.^{2}. This culture solution contained 1 % or 0.5 % of the sugar and the following salts:

NH_4NO_3	0.5 %
K_2HPO_4 or KH_2PO_4	0 1 „
$MgSO_4$	0.05 „

The concentration of the sugars was never higher than 1 % because it was formerly shown that the amylase will be formed most easily at low concentrations. The flasks with the culture solutions were sterilised during 25 à 30 min. at 120°.

Inoculating was very simple by bringing a rather large quantity of conidia with a platinum wire into the solution. There was always a nearly equal development in all the flasks inoculated at the same time.

The first observations mostly took place 7 days after inoculating and further on from week to week. The cultures were filtered off through a dried and weighed filterpaper which was dried again in a dessicator afterwards until the weight was constant; in this way the dry weight of the mycelium was accurately ascertained. Of the cul-

ture solution 10 or 20 c. c. M. were mixed with an equal volume of a 0.08 % solution of soluble potato starch; from this mixture from time to time a portion was tested for the presence of starch by adding some drops of a dilute solution of J in JK. When the liquid remained yellow without the slightest touch of red, I took it for granted that all the starch and erythrodextrine were hydrolysed. The concentration of amylase was estimated by taking the amount to be = 100 if the time, taken for hydrolysis was 150 min.

I hardly ever investigated the amount of amylase in the mycelium, as it had appeared that when a certain quantity of amylase is formed, it is secreted almost directly and fully out into the culture solution; only when no or hardly any amylase was found, I sometimes controlled the mycelium, but in those cases I never found any quantity of amylase worth mentioning.

In most cases, however, I did investigate whether the mycelium contained glycogen by putting a small bit of it into a not too diluted solution of J in JK; on a white underground (e.g. in a porcelain dish) the typical brown colouring, when glycogen is present, is very easily seen. As seems to be the fact with most fungi, Aspergillus appears to produce glycogen on every sort of food and mostly in the first days of development; the reaction was strongest after 7 days, after 14 days it was still clear, after 3 weeks it became doubtful or disappeared altogether; it never reappeared later on. I have not the impression that there could exist any relation between glycogen and the formation of amylase but I will return to this subject later on. When in the tables the amount of glycogen is indicated, + + means very distinct, + distinct, (+) indistinct, + ? dubious and — none.

The pH of the culture solution of all cultures was determined in the colorimetrical way; it is mostly not in-

dicated in the tables as it appeared to remain nearly always within the limits for optimal amylase action (between 3.0 and 4.5), it is only then indicated when it was not favourable for the hydrolysis; in these cases however the solution was always brought to optimal hydrogen ion concentration by adding acids or bases and only the corresponding amounts of amylase are communicated.

I also considered the question whether the age of the conidia, from which the cultures arise, has any influence on the growth and the formation of amylase; therefore an equal number of flasks with glucose solution were inoculated with conidia which were 7, 14, 35 and 56 days old. It appeared however that the cultures showed no differences worth mentioning; one might object that in a culture of e. g. 35 days old, the conidia may have been newly formed. I do not believe that this is often the case; it may be taken for granted that in a culture of 35 days, the conidia are about 4 weeks older than in one of 7 days, as they are formed very soon on the new mycelium, esp. when low concentrations of food material are used. Moreover, once the conidia are formed one gets the impression that they remain the same during the whole life time of the fungus; neither is new mycelium formed after the maximum of dry weight has been reached, on which new conidia could possibly grow.

The cultures were kept in a dark cupboard; I found this condition to be absolutely necessary. The idea that light should not be injurious to enzyme action (4) is not true, at least not for the amylase of Aspergillus niger; this may appear from the next table: it relates to quantities of enzyme from one culture, one half of which was kept in the light, the other one in the dark:

Table 1

Original time of hydrolysis.		Light.	Dark.
12 min.	after 1 day	42 min.	12 min.
	„ 8 „	180 „	12 „
20 min.	„ 1 „	80 „	20 „
	„ 8 „	500 „	22 „
17 min.	„ 1 „	65 „	17 „
	„ 8 „	1080 „	17 „

Cultures which remained for some days in the light, lost their amylase altogether and required at least a week to restore part of it.

Formerly I had already been struck by the fact that solutions of reducing sugars, in being sterilised, became light brown or yellow; I saw the cause of this phenomenon in the presence of alkali which is splitted off from the glass: this alkali probably causes an enolisation, also giving rise to ketones. This is the more likely as this yellow colour does not appear in the flasks with non-reducing sugars or where KH_2PO_4 is added in stead of K_2HPO_4; the acid phosphate apparently binds the alkali directly after it is splitted off. To avoid the colouring in using neutral phosphate, I sterilised 25 c.c.M. both of the sugar- and of the salt solutions apart in 2 flasks; in both the liquids then remained white, from which one may conclude that the K_2HPO_4 seems to act as an intermediary between the alkali and the sugar; after sterilising, the solutions were put together with the necessary care. In sterilising with neutral phosphate, substances most probably are formed which can not be assimilised (ketones and other sorts of sugars, saccharine, saccharinic acid etc.); this may be inferred from several data: firstly the brown or yellow colouring never disappears, no matter how long

the culture is continued; further the dry weight always remains below that of cultures on "purely" (if I may say so) sterilised liquids, as part of the food material has been lost; some figures may illustrate this:

Table 2.

		Dry weight in m.Gr. of parallel series on liquids sterilised	
Food material.	Days.	"Impurely".	"Purely".
Glucose . . .	13	141	225
	27	116	200
Fructose . . .	28	172	183
	28	160	172
	28	197	217
	18	189	200
	32	182	188
	46	176	180
Mannose . . .	7	49	121
	14	117	207
	26	125	197
	35	111	185

Besides a mycelium, cultivated with acid phosphate, has mostly got, in the first weeks of its development, a habitus, quite different from one which is grown on impurely sterilised neutral phosphate; this can be seen in the formation of the conidia: these are formed at least ten times more abundantly on acid than on neutral phosphate and they are formed much earlier; when a culture on KH_2PO_4 looks quite black already, the one on K_2HPO_4 is at the utmost light grey; further the conidiophores and their stems too differ rather wideley; all this is to be seen immediately with the naked eye, so that one glance

will suffice to decide whether we have to deal with a KH_2PO_4 — or a K_2HPO_4 culture.

The next figures will make this still clearer:

Table 3.

The figures are averages of 10 measurings.

Parallel series of 7 days old on.	Diameter of conidiophore + sterigma in μ.	length of stems in μ.
Fructose + K_2HPO_4	145.8	3540
„ + KH_2PO_4	169.2	4635
Mannose + K_2HPO_4	72.-	1740
„ + KH_2PO_4	226.8	4075
Galactose + K_2HPO_4	151.2	2340
„ + KH_2PO_4	216.-	3765
Lactose $^3/_4$% + starch $^1/_4$% + K_2HPO_4	114.5	3750
„ „ „ + KH_2PO_4	190.8	4770
Glucose + K_2HPO_4	118.8	2040
„ + KH_2PO_4	172.8	4295

The measurements of the conidia themselves were the same on both sides.

I had ascribed all those differences to the influence of the phosphates, but later on the idea struck me that those "injurious substances," originated by sterilising with neutral phosphate, might be the real cause. This becomes very probable when we see that the greater part of these differences is absent between cultures on KH_2PO_4 and "pure" K_2HPO_4. This is not only true for the dry weight, but also for the growth of the conidiophores; vid. table 4.

Table 4.

	Diameter of conidiophores.	Length of stems.	Dry weight.
Glucose + K_2HPO_4. . . .	118.4	2040	141
			116
Glucose + K_2HPO_4, "pure"	163.2	3960	225
			200
Glucose + KH_2PO_4. . . .	172.8	4295	203
			188

A certain influence of these substances on the formation of amylase could also be observed, but I prefer to deal with this point and some others in describing the cultures concerned.

Formerly I worked with one tribe of Aspergillus, this time with three. I began with a tribe which I found in the laboratory for technical botany in Delft; as it made the impression of being rather „worked out", I soon proceeded with one which was sent to me from the botanical laboratory in Utrecht; this one was more active through it very seldom produced such quantities of amylase as 4 years ago; unfortunately absolute certainty was not to be got whether it was really the same tribe; only I was informed that there had never been more than one tribe in Utrecht. It is possible that it has gradually changed its physiological character, as has been observed in more cases, but this can not be proved now.

In addition to this, I cultivated a "wild" tribe from oak galls; these were put into Erlemeyer flasks, half way in aqua dest.; these flasks were kept at a temperature of 35° and after some weeks Aspergillus niger had developed so prodigally that, though some other species of fungi appeared, I had obtained a pure culture after the first oculating off.

Externally there were no differences to be seen between these tribes, physiologically there were, but more quantitatively than qualitatively. I mostly worked with the tribes from Utrecht (called U) and the one from the galls (called G) and so most of the researches were done twice. How far they differed precisely in formation of amylase will be seen in the tables and their descriptions.

With an eye to the strong aftereffect of the former culture medium on the conidia, which are used for the oculation, I generally stuck to the principle of only then considering an experiment as final when the conidia were taken from cultures on solutions of the same chemical composition as those on which I intended to grow them; even this is not always sufficient: very often the aftereffect of a medium is perceptible after 3 or 4 times passing into another; a few examples may once more illustrate how carefully one should proceed in this matter:

Table 5.

Starch 0.5 %.

Foregoing culture medium.	Days.	pH.	Glycogen.	Amylase.	Dry weight in m.gr.
Starch	21	3.2	+ +	210	114
	42	3.9	—	3000	87
Fructose	21	3.2	+	60	121
	42	3.6	—	60	114
Glucose 1 %.					
Glucose.	14	3.3	+	160	248
	21	3.6	+ ?	175	215
Lactose	14	3.4	+	0	208
	21	3.6	—	2	195

Sometimes I deviated from this course, in order to force some result, but I will mention these cases later on.

II A. Results obtained with the tribe from Utrecht.

Only few cultures were necessary to make out that the tribe U was capable of producing considerable quantities of amylase, when grown on glucose 1 % + K_2HPO_4, as appears from table 6: (in the first column are indicated the consecutive culture solutions from which came the conidia which were used for the experiment).

Table 6.

Glucose 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Glucose-agar	5	+ +	100	215
	14	+	170	198
	21	—	165	181

Also on amylum + K_2HPO_4 a very abundant quantity of enzyme was formed:

Table 7.

Starch 0.5 % + K_2HPO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Starch-glucose-agar . .	14	+	3000	118
	28	—	2200	98

On fructose + K_2HPO_4 only little amylase was developed and besides very irregularly; the liquid was dark brown after sterilising; I therefore also investigated the behaviour on pure fructose + K_2HPO_4 as well as on fructose + KH_2PO_4.

Table 8 A.

Impure fructose 1 % + K_2HPO_4.

Foregoing culture medium.	Days,	Glycogen.	Amylase.	Dry weight.
Fructose-agar	6	(+)	0	205
	14	—	18	212
	21	—	80	171
	42	—	0	143
	49	—	15	140
	61	—	2	117
	126	—	30	93
	140	—	35	98
8 B. Pure fructose 0.5 % + K_2HPO_4.				
Pure fruct. - pure fruct. - fruct.	7	(+)	0	149
	14	—	0	160
	21	—	0	147
	35	—	17	141
	54	—	0	123
8 C. Pure fructose 0.5 % + KH_2PO_4.				
Pure fruct. - pure fruct. - fruct.	7	+?	0	165
	14	—	0	150
	21	—	2	155
	35	—	4	126
	63	—	0	112

Attention is called to the very slight formation of glycogen.

It appears that next to no amylase is formed, as well on neutral as on acid phosphate; it does not seem too bold to suppose that the quantities of amylase that came out on impure fructose are the result of, or have at least some connection with the substances formed by sterilising; in any case the acid phosphate has been here of no influence on the production of the enzyme.

The question arises whether fructose itself prevents the formation of amylase or whether metabolic substances, formed out of it by the fungus, are the cause of it. In

order to settle this, I made the following investigation: when a culture solution appeared not to contain any amylase, it was divided into two halves, to each of which 25 c.c.M. of 1 % glucose solution was added. As after filtering off the mycelium and taking away the 10 c.c.M. for the analysis of the enzyme, there remained about 30 à 35 c.c.M. of the liquid, the mixture contained somewhat more than 0.5 % glucose, besides the metabolic substances; the mixture was boiled during a few seconds and then inoculated with conidia of the very mycelium that had been grown on the solution. If it should appear that in this mixture there was formed amylase, this would favour the theory that fructose itself prevents the producing of amylase and not its metabolic substances. The results can be found in tables 9 and 10.

Table 9 vid 8 B.

Culture solution + 25 c.c.M. 1 % glucose

Liquid of the	Days.	Glycogen.	Amylase.	Dry weight.
14th day . .	7	+	0	112
	21	—	40	97
21 day . .	7	(+)	0	152
	21	—	60	103
35 day . .	7	(+)	5	142
	21	—	60	114
54 day . .	9	—	75	111
	31	—	25	104

Table 10 vid 8 C.

14 day . .	7	+	8	90
	21	—	40	81
21 day . .	7	(+)	60	154
	21	—	60	114
35 day . .	7	+ ?	140	142
	21	—	60	136
63 day . .	7	+	20	136
	22	—	83	130

One should not expect big quantities of amylase taking into consideration the aftereffect of the former circumstances. Therefore I think we may conclude from these data that it is really the fructose itself that prevents amylase from coming out (or from being formed at all) and not its metabolic substances; this becomes still more probable by the next investigations: these concern the series cultivated on mixtures of fructose and glucose and of fructose and starch.

Table 11.

Fructose 0.5 % + glucose 0.5 % + K_2HPO_4.

Foregoing culture medium.	Days.	pH.	Amylase.	Dry weight.
Fruct. + gluc. - fruct. - fruct. - agar.	7	3.-	0	217
	14	2.8	35	254
	21	3.-	70	229
	28	± 5	55	170
	35	± 6	170	203
	42	± 6	175	167
	49	± 6	230	144

Table 12.

Fructose 0,5 % + starch 0.5 % + K_2HPO_4.

Foregoing culture medium.	Days.	pH.	Amylase.	Dry weight.
Fruct. + starch - fruct. - fruct. - agar.	7	3.-	20	258
	14	3.2	85	218
	21	3.6	500	215
	28	4.-	250	189
	35	± 5.-	190	185
	42	± 5.5	30	147
	49	± 6.-	85	128
	56	± 6.-	115	—
	63	± 6.-	100	—

In table 11 we see the amylase only then developing well when the fructose must have been consumed and then it increases bye and bye, as it were "coming free from the fructose." In table 12 we see at first a slow development of the enzyme which stays considerably behind the production on starch only, then a descend and then another rise, which, however, remains rather low too.

The various liquids as a rule keep their enzymatic power for an indefinite time, at least during 6 à 8 weeks; now it is worth while noting that in this case the liquid of the 28th day shows a gradual decrease of amylase concentration, that of the 34th and 42d day a very rapid one, where as the amounts of the 49th, 56th and 63d day appeared to be once more constant; a phenomenon therefore, parallel to what goes on apparently in the mycelium.

A result, similar to that obtained with the mixtures of fructose and glucose may be expected when Aspergillus is grown on saccharose. The investigations which I made with this sugar are only few, but they seem to answer the expectations.

Table 13.

2 Par. cultures on saccharose 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Glucose - glucose - glucose-agar.	7	++	0	246
	19	(+)	80	186
Fructose - fructose - fructose-agar.	7	++	0	217
	19	(+)	60	188

Where there is no amylase formed on fructose, one might expect the same negative result with inuline as source of carbon, since inuline seems to be hydrolysed into fructose only; it appeared however that this was not exactly the case, c.p. table 14.

Table 14.

Inuline 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	pH.	Glycogen.	Amylase:	Dry weight.
Inuline - fructose - fructose - agar.	7	3.2	+	0	174
	14	3.2	(+)	0	193
	21	5.5	+?	50	237
	28	± 6.-	—	25	207
	35	± 6.-	—	12	197
	42	± 6.-	—	30	151
	49	± 6.-	—	30	143
Inuline - inuline - fruct. - fructose - agar.	7	3.2	+	0	123
	21	± 6.-	—	60	136
	35	± 6.-	—	25	179

The quantities of amylase formed here can not be caused by substances from sterilising as the liquids remained wholly uncoloured; in my opinion the production of amylase may be due to metabolic substances of the fungus itself; the fact that in the first 14 days no production of enzyme takes place, would plead for it; so it might be possible that inuline is not all hydrolysed into fructose, but that also e.g. some γ-glucose is formed (18), which, whether or not condensed into higher compositions, could further the amylase production.

The results with galactose appear from table 15 A and B.

Table 15 A.

Galactose 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Galact. - galact. - galact. - amylum.	14	+?	45	88
	21	—	40	66
	35	—	50	86
	49	—	0	111
	63	—	33	120

Table 15 B,

Galactose 1 % + KH_2PO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weigt.
Galact. - galact. - galact. - amylum.	14	+ +	18	86
	21	+ ?	22	129
	35	—	8	115
	49	—	0	192

The fungus developes very poorly on this sugar; the mycelium remains immersed in the liquid; here and there it rises to the surface where spots, black with conidia, appear. Yet these 2 series have been grown from conidia which had passed 3 times already through a galactose culture; so they had had ample opportunity to get "used" to it; moreover, those initiating cultures gave similar results. The amylase production is rather slight and once more the phosphate, whether acid or neutral, appears to be of no decisive importance. The amylase which was formed, lost its power without exception after a very short time; in my opinion this could prove that galactose itself does not prevent the formation of amylase, but that its metabolic substances do. In this way might also be explained that after the 5th week the enzyme production stops altogether and that in the culture solutions with 25 c.c.M. 1 % glucose added to it, no amylase is formed at all, without exception. It is remarkable, however, that in both series the dry weight of the mycelium shows a rise at the end, in one case even combined with an increase of (new) amylase.

When grown on mannose, Aspergillus gave the following results:

Table 16 A.

Mannose 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Mannose - amylum.	7	+ ?	0	49
	14	—	0	117
	26	(+)	0	125
	35	(+)	0	111
	42	—	0	79
	57	—	0	103

Table 16 B.

Mannose 1 % + KH_2PO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Mannose - amylum.	7	(+)	0	129
	14	(+)	6	238
	26	—	2	219
	42	—	3	211
	57	—	0	193

The initiating cultures gave similar data. It is very striking here that on acid phosphate the mycelium develops so much stronger; yet I did not get the impression that during sterilising an extra quantity of substances was formed that opposed development; the colouring of the liquid was very light yellowish. Probably the K_2HPO_4 combines with the sugar in another way than KH_2PO_4, so that in the last case assimilation becomes easier (compare 7, 13 and the results with milk sugar).

Formation of amylase is not found at all, at least on mannose + K_2HPO_4; the liquids of this series with glucose added to it, did not give any development of the enzyme either.

Table 17 vid. 16 A.

Culture solution + 25 c.c.M. 1 % glucose.

Liquid of the	Days.	Glycogen.	Amylase.	Dry weight.
7th day . .	7	+ +	0	129
	21	+ ?	0	121
14 day . .	12	+ +	0	105
	28	—	0	87
26 day . .	7	+	0	107
	21	—	0	110
35 day . .	7	+	0	70
	21	—	0	69
42 day . .	7	+ +	0	67
	21	—	0	113
58 day . .	13	+ +	0	130
	27	—	0	100

So the mannose itself appears to prevent the production of amylase, but also its metabolic substances. In order to decide whether perhaps we have to deal here with a very strong aftereffect of the former culture, I sometimes added, for the second time, 25 c.c.M. 1 % glucose to the liquids which I then oculated in the same way as described above; in the greater part of these cases, there was no formation of amylase either.

By way of exception, I added for a single occasion starch i • st. of glucose; I otherwise never did this, because I think that by giving starch the amylase is rather forced and so we do not get a pure idea of the conditions under which it can be formed; but it here appeared that the mannose and its metabolic substances so strongly oppose the formation of the enzyme, that even on this food it was almost entirely prevented from coming out;

the development of the dry weight too was very slight. Even after this had grown for some time, the liquid reacted blue with J in JK, so that we may ascribe at least part of the dry weight to remnants of other feeding substances that had stayed behind in the culture solution.

Table 18 vid. 16 A.

Cult. sol. of the 42 day + 25 c.c.M. starch 0.5 %.

Days.	Glycogen.	Amylase.	Dry weight.
7	++	trace	47
21	+?	trace	101

On acid phosphate the development of amylase is so slight that also here I added 25 c.c.M. glucose 1 % to the liquids and grew new cultures on them, the results of which are to be seen in table 19.

Table 19 vid. 16 B.

Culture solution + 25 c.c.M. 1 % glucose.

Liquid of the	Days.	Glycogen.	Amylase.	Dry weight.
7th day . .	7	+?	3	195
	21	—	0	149
14 day . .	12	—	45	139
	21	—	0	134
26 day . .	16	—	5	100
	31	—	1	149
42 day . .	7	+?	35	138
	21	—	30	101
57 day . .	13	—	115	142
	27	—	8	121

In the beginning little or no amylase; in the later liquids which were most "worked out", bye and bye small quan-

tities appear, later on still more; none of these quantities of enzyme remained constant in their hydrolysing power. When we compare these results with the corresponding ones of the fructose series, it is striking that as contrasted with the latter, there here appears a quantity of enzyme rather early which disappears later on. This clearly shows that also in these cases the metabolic substances of the mannose make their influence felt.

When starch in stead of glucose was added to the culture solution, a similar phenomenon appeared as with the K_2HPO_4 series; the solution reacted violet with J in JK after 7, red after 21 days.

Table 20 vid 16 B.

Cult. sol. of the 42d day + 25 c.c.m. starch 0.5 %.

Days.	Glycogen.	Amylase.	Dry weight.
7	+	trace	87
21	—	15	141

On lactose with K_2HPO_4 Aspergillus niger can not grow as I have stated already formerly. Just as Euler and Johannsen (6) made a yeast get used to the forming of lactase, I tried the same by repeatedly transferring the conidia of a culture on a new solution of milk sugar. There were always formed such scanty bits of pellicle that they only became visible when coloured here and there by conidia; after some weeks the whole sank to the bottom, apparently dead. I also tried to force growth by using conidia from cultures which had grown on mixtures of lactose 0.9 % + starch 0.1 % or lactose 0.75 % + starch 0.25 % but these also yielded no result. Some mycelia which seemed to have developed somewhat better, have been examined but gave only negative results: hydrogen ion concentration very low (pH ± 6.-), no amylase, dry weight 50 m. Gr. at the highest. On culture solutions

to which 25 c.M. % glucose was added (but in which the milk sugar had remained almost unaltered!), development was not prevented, but amylase did not appear.

On a mixture of lactose 0.75 % + amylum 0.25 % or of lact. 0.9 % + am. 0.1 % the fungus developed well but hardly any amylase was secreted into the solution, although the starch had been hydrolysed; nor was there any in the culture solutions with glucose added to it; these facts remained the same, even when the fungus had been grown several times in succession on the same nutrient base. It may be safely concluded that milk sugar prevents formation of amylase.

Table 21 A.

Lactose 0.75 % + amylum 0.25 % + K_2HPO_4

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Amylum	14	+ +	0	78
Lact. 0.75 % + am. 0.25 % — amylum	14	+	40	129
Lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	14	+	0	116
Lact. 0.75 % + am. 0.25 % 3 × — amylum. . . .	14	(+)	trace	132
Lact. 0.75 % + am. 0.25 % 4 × — amylum. . . .	16	—	8	96

Table 21 B.

Lactose 0.9 % + amylum 0.1 % + K_2HPO_4

Lact. 0.75 % + am. 0.25 % — amylum	21	—	0	140
Lact. 0.9 % + am. 0.1 % — lact. 0.75 % + am. 0.25% — amylum	21	—	trace?	104

It is remarkable that the presence of starch makes the fungus assimilise at least part of the lactose; indeed, the dry weight seems too high for the 0.25 or 0.1 % of starch.

It may, however, not be said that Aspergillus has not got lactase as I thought formerly. This becomes quite clear when it is cultivated on lactose + KH_2PO_4. Then growth takes place, though not abundantly; the fungus forms a closed, thin pellicle over the surface of the liquid, which soon looks dark grey with conidia; formation of amylase however is absent or very scanty, though I tried to increase it by using conidia from cultures on mixtures of starch and milk sugar.

Table 22.

Lactose 1 % + KH_2PO_4 (the figures between ( ) bear upon cultures, grown on the liquids + glucose of the cultures concerned).

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry Weight.
Amylum.	14	++	0	170
Lact. 0.9 % + am. 0.1 % — Lact. 1 % — amylum.	26(16)	— (—)	0 (85)	117 (198)
Lact. 0.9 % + am. 0.1 % — Lact. 0.75 % + am. 0.25 % 2 × — amylum.	19(16)	(+)(—)	5 (17)	176 (135)
Lact. 0.75 % + am. 0.25 % 3 × — amylum	21(21)	— (+?)	25 (50)	117 (217)
Lact. 0.9 % + am. 0.1 % — Lact. 0.75 % + am. 0.25 % 2 × — amylum.	28(14)	++(+)	0 (83)	129 (189)
Lact. 1 % — lact. 0.9 % + am. 0.1 % — lact. 0.75 % + am. 0.25 % 2 × — amylum	21(14)	—(++)	8 (28)	137 (176)

In the liquids with 25 c.c.M. glucose amylase appears in rather considerable quantities, so that it is apparently the lactose itself and not (in the same degree) its metabolic substances that prevents formation of amylase. We are led to a similar conclusion by tables 23 A and B which represent the results with cultures on mixtures of milk sugar and starch. Notwithstanding the presence of starch, the quantities of amylase are strongly lowered by the lactose.

Table 23 A.

Lactose 0.75 % + amylum 0.25 % + KH_2PO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry Weight.
Amylum	14	++	85	190
Lact. 0.75 % + am. 0.25 % — amylum	7	++	55	144
Lact. 0.75 % + am. 0.25 % 2 × — amylum	21	—	40	223
Lact. 0.75 % + am. 0.25 % 3 × — amylum	21	+?	30	177

Table 23 B.

Lactose 0.9 % + amylum 0.1 % + KH_2PO_4.

Lactose 1 % — amylum .	14	+	165	150
Lact. 0.75 % + am. 0.25 % 2 × — amylum	21	—	10	175

Contrary to galactose, lactose itself prohibits the coming free of the enzyme; in their effect on growth however, they behave some what similarly: no or very little development on the sugar when K_2HPO_4 is used but much more in presence KH_2PO_4. There may exist a certain connection too between the acid phosphate and the lactose molecule which facilates its assimilation by the fungus (7,13).

I made a few more investigations to get certainty whether it is really the K_2HPO_4 itself that prevents development in this case and not the alien substances formed by sterilising.

To this purpose I made the following culture solutions: lactose 1 % + K_2HPO_4 0.1 % + KH^2PO_4 0.1 % and lactose 1 % + K_2HPO_4 0.1 % + KH_2PO_4 0.1 % +, after sterilising, once more the same quantity of KH_2PO_4. In this way I thought that the formation of alien substances could be prevented and that possible inhibitory influence of K_2HPO_4 on growth could be counterbalanced and even surpassed by the KH_2PO_4; to avoid a possible unfavourable action of too much of phosphates, I made a parallel investigation with only $^1/_5$ of their quantities. The results were negative, all liquids were coloured equally intensely yellow and development of mycelium was as scanty as in other cultures.

On purely sterilised lactose solutions + K_2HPO_4, development was very little too, though somewhat stronger; 87 m. Gr. of dry weight were found at the utmost and only after 5 weeks; pH came to ± 3.- but amylase was not formed. We may conclude that in this case it is principally the neutral phosphate that prevents growth and not the inhibitory substances formed during sterilising.

When the same mixtures of phosphates, ascribed above, were added to glucose, brown colouring was also as intense as ever. Here, however, all cultures developed very equally, with the habitus of the ordinary ones with 0.1 % K_2HPO_4, so that it appears once more that in the case of glucose, the alien substances are the principal cause of the unsatisfactory growth.

It seems worth while to note here that on Ca-lactate 1 % + K_2HPO_4 no development at all takes place.

II. B. Results obtained with the tribe from Delft.

I can be very brief about the results obtained with the Delft tribe; I did not make very many investigations with it because it gave me the impression of being rather worked out. On glucose it formed large quantities of amylase, only irregularly, on fructose none at all and on inuline it gave similar results as tribe U.

table 24.

Inuline 1 % + K_2HPO_4

Foregoing culture medium.	Days.	Amylase.	Dry weight.
Glucose - agar	7	0	175
	14	6	247
	21	6	250
	28	10	250
	42	6	224
	60	1	226
Inuline - glucose - agar	7	0	167
	14	0	258
	21	4	249
	35	25	235
	46	0	226

On starch amylase was very abundant; after 8 à 14 days there was always a quantity between 2000 and 4000.

On glycerine no amylase was formed at al.

Till now we have always seen that when Aspergillus is grown on a mixture of two substances, one of which prevents formation of the enzyme, the production is influenced by both (fructose + glucose; fructose + starch; lactose + starch); therefore it seems peculiar that on a mixture of + 0.25 % starch + 0.25 % glycerine quite another result appears, viz. the amylase production takes place without

showing the least influence of the glycerine, whereas the previous culture conditions of the conidia seem to be quite indifferent.

Table 25.

Glycerine 0.25 % + starch 0.25 % + K_2HPO_4.

Foregoing culture medium.	Days.	Amylase.	Dry weight.
Glucose	7	500	109
Gluc. 0.25 % + am. 0.25 %	7	2300	108
— glucose	20	1850	102
Glyc. 0.25 % + am. 0.25 % 2 × — glucose.	13	1250	106
Glycerine.	7	1800	107
	14	3300	107
Glyc. 0.25 % + am. 0.25 %	7	300	101
— glycerine	20	1850	99
Glyc. 0.25 % + am. 0.25 % 2 × — glycerine.	13	2000	111

The dry weight of 7 cultures on starch 0.5 % between the 7th and 42d day was resp. 118—106—96—109—98—93—111 m.Gr. In table 25 we see that the dry weight is, as an average, somewhat higher which proves that not only the 0.25 % starch, but the glycerine too has been consumed; the course of the development makes this still clearer: after 7 days the cultures on the mixture had always sufficiently grown to be examined whereas on starch only, I mostly had to wait a week longer; so the development in the first week on the mixture may be ascribed for the greater part to the glycerine. Unlike sugars as mannose, lactose etc., the molecule of glycerine seems to be indifferent to the formation of the enzyme, viz. it neither stimulates, nor prevents it.

II C. Results obtained with the tribe, cultivated from galls.

My idea in cultivating this "race" from galls was to get a tribe which would not be influenced by a long stay in the laboratory and so would be more vigorous in its various manifestations of life, namely in its production of amylase. This expectation has not been realised as can be jugded from table 26.

Table 26.

Glucose 1 % + K_2HPO_4.

Foregoing culture medium.	Days.	Amylase.	Dray Weight.
Galls.	14	0	239
Glucose - galls.	14	0	231
	21	2	192
	28	50	190
	42	70	176
Glucose - glucose - galls.	7	0	91
	14	0	202
	21	25	207
	28	35	163
Glucose - glucose - glucose - galls. Cultivated at 35°.	7	32	247
	14	2	207
	18	3	205
	36	0	222
	49	2	163
	63	2	161

Development was vigorous with abundance of conidia, but the formation of amylase remained scanty, even after having been oculated subsequently, 3 à 4 times on glucose no increase could be observed. In the end I cultivated the

fungus at 35°, the amount of amylase being determined at room temperature. The maximum of dry weight was reached much sooner as appears from the figures; in the first week there was a small quantity of amylase which was not constant in vitro and later on less of it than ever.

When cultivated on starch, it takes very long before the fungus begins its development; in the end rather large quantities of amylase are produced which keep their concentration after some lapse of time, but which remain far under those which are found in other tribes.

It is worth while noting that the first time a considerable quantity of amylase appeared, the course of hydrolysis was somewhat different from the norm; mostly the hydrolysis goes on gradually but in these cases the liquid kept for a long time reacting blue with J in JK, until rather suddenly the colour changed; so e. g. it was observed in the solution of the 21st day of a culture on starch:

after	40	min.	blue
„	90	„	blue
„	180	„	violet
„	240	„	orange
„	270	„	light orange
„	290	„	nearly yellow
„	310	„	yellow

or in a culture on glucose on the 28th day:

after	40	min.	blue
„	110	„	blue
„	200	„	reddish violet
„	260	„	orange
„	290	„	nearly yellow
„	300	„	yellow

while the normal case would be:

after	60	min.	blue-violet
„	100	„	violet
„	160	„	reddish violet
„	200	„	light red

after 270 min. orange
„ 290 „ light orange
„ 300 „ nearly yellow
„ 310 „ yellow

(from a culture of tribe U, 7th day, on glucose)

The enzyme is, as it were, seen struggling in its attack on the molecule of starch. (Or it is the amylase sensu stricto only which is not easily formed, whereas the erythrodextrinase is ready to do its part). A similar phenomenon was once observed in a culture of tribe U on a mixture of lactose 0.75 % and starch 0.25 % + KH_2PO_4 (foregoing culture medium: amylum 0.5 %; so it was the first time the fungus was grown on lactose):

after 60 min. blue
„ 100 „ blue
„ 120 „ blue-violet
„ 150 „ light red
„ 160 „ orange
„ 170 „ light orange
„ 180 „ yellow

In the succesive series on starch, the amylase does not increase at all; cultivating at 35° did not avail any more than with the glucose series.

Table 27.

Amylum 0.5 % + K_2HPO_4.

Foregoing culture medium.	Days.	Amylase.	Dry weight.
Glucose - glucose - galls.	21	25	103
	28	85	115
	35	200	119
	39	250	90
	57	300	86
Amylum - galls.	21	50	133
	35	250	116

Foregoing culture medium.	Days.	Amylase.	Dry weight.
Glucose 3 × - galls cultivated at 35°.	7	75	94
	14	710	108
	21	250	110
	28	180	110
	32	350	87
Amylum (35°) - glucose 3 × - galls	18	35	68
	35	450	156
	42	450	103
	49	420	128
	84	300	100
Amylum 2 × - galls.	21	25	115
	28	67	130
	35	350	111
	39	300	100
	57	750	92

Some series on glucose, inoculated with conidia from cultures on starch did not show anything of the expected aftereffect:

Table 28.

Glucose 1 % + K_2HPO_4.

Foregoing culture medium	Days.	Amylase.	Dry weight.
Amylum 2 × - galls.	7	8	192
	14	0	213
	21	1	179
Amylum 2 × - galls.	7	0	133
	14	0	207
	21	25	221
	28	30	218
	39	50	195

On saccharose no development of the enzyme took

place, not even after the 45th day, neither in cultures grown at room temperature, nor at 35° and the same can be said of fructose; it is remarkable however that on inuline a similar phenomenon presents itself as with tribe U:

Table 29.

Inuline 1 % + K_2HPO_4.

Foregoing culture medium.	Days	Amylase.	Dry weight.
Glucose 2 × - galls.	7	0	183
	14	0	245
	21	10	186
	28	10	219
	35	20	223
Inuline - glucose 2 × - galls	7	0	195
	14	10	269
	28	50	192
	46	35	198

On maltose the formation of amylase was very scanty.

Table 30.

Maltose 1 % + K_2HPO_4

Foregoing culture medium.	Days.	Amylase	Dry weight.
Amylum - glucose 3 × - galls.	21	30	211
	35	2	—
Maltose - amylum -	7	2	256
glucose 3 × - galls.	14	6	239
	28	15	210
	42	10	207

When I took to cultivating this tribe on milk sugar, I hardly expected to find any amylase; yet the amounts did not remain under those obtained with U on that sugar; so I can omit a detailed description. Only one

remarkable fact should be pointed out, viz. that in the culture solutions with the addition of 25 c.c.m. glucose 1 % there appears generally more amylase than in the cultures on pure glucose.

Table 31 A.

Lactose 0.75 % + amylum 0.25 % + K_2HPO_4

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Amylum	14	+ +	10	120
Lact. 0.75 % + am. 0.25 % — amylum	14	+	5	144
Lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	14	—	10	127
Lact. 0.75 % + am. 0.25 % 3 × — amylum. . . .	21	+?	30	99

Table 31 B.

Lactose 0.9 % + amylum 0.1 % + K_2HPO_4.

Lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	21	—	2	119
Lact. 0.9 % + am. 0.1 % — lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	14	+	68	125

Table 32.

Lactose 1 % + KH_2PO_4 (the figures between ( ) bear upon cultures, grown on the liquids + glucose of the cultures concerned).

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Amylum	14	+	0	134
Lact. 0.09 % + am. 0.1 % — lact. — amylum . .	26 (16)	— (+?)	20 (72)	117 (195)

Foregoing culture medium.	Days.	Glucogen.	Amylase.	Dry weight.
Lact. 0.75 % + am. 0.25 % 3 × — amylum. . . .	21 (21)	— (—)	25 (68)	116 (215)
Lact. 0.9 % + am 0.1 % — lact. 0.75 % + am. 0.25% 2 × — amylum. . . .	28 (14)	+?(+)	0 (75)	144 (193)
Lact. 0.9 % + am. 0.1 % — 2 × lact. 0.75 % + am. 0.25 % — amylum. . .	21 (22)	+ (—)	2 (20)	105 (104)
Lact. — 3 × lact. 0.75 % + am. 0.25 % — amylum.	29 (13)	— (+)	0 (105)	128 (168)

Table 33 A.

Lactose 0,75 % + amylum 0.25 % + KH_2PO_4.

Foregoing culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
Amylum	14	+ +	48	194
Lact. 0.75 % + am. 0.25 % — amylum	7	+ +	45	136
Lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	21	—	30	200
Lact. 0.75 % + am. 0.25 % 3 × — amylum. . . .	21	—	30	175
Lact. 0.75 % + am. 0.25 % 4 × — amylum. . . .	21	—	50	191

Table 33 B.

Lactose 0.9 % + amylum 0.1 % + KH_2PO_4.

Lactose—amylum	14	+	115	177
Lact. 0.75 % + am. 0.25 % 2 × — amylum. . . .	21	—	38	173

III. Discussion of the results.

Briefly recapitulating the results, I will chiefly consider those which have been obtained with tribe U; those of the other tribes, as far as they can be compared, generally agree with them.

The cultures with KH_2PO_4 differ from those with K_2HPO_4 principally by better development of the mycelium, by forming more conidia, bigger conidiophores with longer stems; an important difference in the production of amylase could on no occasion be found, in any case never so striking as with the other phenomena; now it is a little bigger on one side, now on the other. And when we see that just those other differences disappear as soon as we take care to sterilise purely, so that no alien substances can be formed in the liquids, we may feel justified in taking it for granted that those differences are due to those alien substances and not to the kind of phosphate (exc. for the growth on mannose and milk sugar). I never should have continued my researches so far in this line, were not the opinion about the decisive role of the acid phosphate, though for other species of fungi, so widely spread (e.g. Euler). I found however that authors are not always justified in ascribing such an influence to the phosphates. When e.g. Haehn and Berentzen (11) examine the influence of the hydrogen ion concentration in combination with several kinds of phosphates on the hydrolysing power of their "Normal System", it is to the pH that they should ascribe the action on it and not to the phosphates.

Phosphates certainly play an important part in the dissimilation of the sugars as may be seen from the series on mannose and lactose (compare 7 and 13) but once more, the amylase seems to have nothing to do with it.

Now we have seen:

Production of amylase:

on glucose....	good,
fructose ...	none, prevented by fructose itself,
galactose ..	little, probably prevented by metabolic substances of galactose,
mannose...	none, prevented by mannose itself as well as by its metabolic substances,
lactose	very little, mainly prevented by lactose itself,
inuline.....	little, only later on, probably due to metabolic substances such as γ-gluc.,
amylum....	strong.

By cultivating on mixtures, results were obtained that agree with these above mentioned.

It would be certainly too bold to form an hypothesis which could account for these facts. I am fully aware that every word of it will perhaps have to be repealed later on and that till now the given data are not sufficient. Yet one could imagine;

— amylase to be a simple substance, perhaps anorganic (comp. 1 and 11), which in its construction is somewhat kindred to, or has some connection with, or shows some similarty to the glucose molecule;

— this substance to be easily formed, (with or without the aid of phosphates), in the presence of, if not out of glucose;

— that as a matter of fact the fungus always tends to form it;

— but that in presence of exclusively otherwise built sugar molecules it can not be formed;

— that those other molecules, as well as their metabolic substances, can even prevent its formation (or its appearance), although glucose is present;

— or that on the other side just the metabolic substances

are of such construction (e. g. γ-glucose) that they can do what their original substances can not.

This hypothesis would not only explain to a certain extent the above facts, but also;

— that in some culture solutions the amylase keeps its concentration for an indefinite time, in others not;

— that when not purely sterilised, amylase appears in solutions, which otherwise are not apt to form it;

— that amylase can be developed on inuline;

— that in the mixture starch + glycerine, the glycerine molecule appears to be indifferent to the formation of amylase.

I know very well that this hypothesis is not at all new in its foundations; Fischer, Pekelharing and others have expounded similar views and therefore it may seem unneccessary to explain them once more; but as the ideas, here written down, came to me bye and bye while investigating and seemed to go rather well with the facts, I thought that I might be justified in communicating them in my way.

To throw more light on these questions, it seems reasonable for the time being to make experiments mainly with monoses; in fact, what can we know of what goes on when organisms are cultivated on bioses, to say nothing of higher compositions.

Pringsheims views (18) have made everything uncertain here; though we knew that out of monoses higher compositions are formed which make their influence felt, especially the γ-glucose has suddenly changed our ideas and I will not yet venture to take a fixed point of view in these matters. Only about the glycogen I want to observe that it is formed to almost the same extent on every food material; so for the time being, I can not accept that it should have any influence on the formation of amylase. (Very unfortunately I was not able to cultivate

Aspergillus on a pure glycogen solution). The glycogen very probably disappears, having served neither for the formation of the enzyme, nor for the development of new dry weight, as the figures seem to point out.

I never observed at all that after the first maximum of dry weight a second one is formed (the only exception could be the series on galactose, but it is doubtful whether in the beginning a real maximum had been reached here); indeed, either there would be no room for it in the flasks or the necessary food supply has been since long consumed. Even in those cultures where the mycelium leaves large areas of the surface free, there never originates any new growth from the surrounding numerous conidia. Schenker (19) believes a second maximum to be formed, but he only observed the dry weight during the first 10 days of the development, which certainly is not long enough. For the rest, though the investigated on lipase, there seem to be rather striking parallellities in our results.

There is something left to say about the tribes; I made use of this word on purpose and avoided the word "race" because I strongly doubt if we may speak of races here, My 3 tribes were not to be distinguished as far as the habitus is concerned; physiological distinct differences are not to be observed either. I can only state that during the time of investigation (about a year) the tribe G produced much less amylase than U, especially when cultivated on starch and glucose. But one should not forget 2 things here:

Firstly, for the time being, we can not say exactly what a race is; I used to take a large quantity of conidia (some hundreds) for oculating; it may be that in this way I got often, if not always, a mixture of several races. Perhaps it might be better in future to cultivate mycelia from one single conidium. I had not time enough to make any investigations in this direction, but I also doubt very

much if the labour would be worth while. There will certainly appear physiological differences between the mycelia, which can be due to several causes, whether or not to be traced; but it will be hardly possible to state really genotypically distinguished races.

Then there is the embarassing aftereffect of the foregoing medium; I noticed already that unfortunately it could not be made out with certainty where the tribe of Utrecht came from. But do we know anything more of the tribe G? There were conidia on the galls which probably originated from a mycelium that had grown itself on galls; but where did that come from; how long had that tribe been accustomed to that sort of medium?; and how are we to calculate its aftereffect? I believe that a long stay in a certain medium where there is no special reason for the formation of amylase, such as agar, galls etc. and where amylase (therefore) is not produced, makes its influence felt during a corresponding length of time, so that even after several cultures it manifests itself so strongly that one can get the impression to have to deal with a constant variation. In this connection I once more refer to the series of cultures of G on glucose - amylum - glucose. Another investigation may throw some more light on this question: I wanted to know how long the aftereffect of lactose lasts on the enzyme production. G as well as U were oculated on glucose and starch, after having been cultivated 7 times in succession on milk sugar + K_2HPO_4 (where there is, as may be remembered, hardly any growth). The results are rather surprising.

Table 34 concerning U.

(The figures between ( ) bear upon cultures, grown on the liquids + glucose of the cultures concerned).

Foregoing culture medium.	Culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
7 × lactose + K_2HPO_4	Glucose 1 % impure + K_2HPO_4	15 (13)	— (+ +)	0 (125)	122 (144)
The former	"	14 (14)	+ + (—)	0 (0)	131 (124)
The former	"	13 (14) 27	+ ((+)) —	0 (0) 0	97 (98) 130
7 × lactose + K_2HPO_4	Glucose 1 % pure + K_2HPO_4	15 (13)	+ ? (+ +)	' (0)	214 (95)
The former	"	14 (14)	+ + (—)	0 (65)	219 (116)
The former	"	13 27	+ —	8 2	280 180
7 × lactose + K_2HPO_4	Glucose 1 % + KH_2PO_4	15	—	8	205
The former	"	14	—	30	239
The former	"	13 27	— —	32 2	245 207
The former	Starch 0.5 % + KH_2PO_4	14 21 35	+ + ? —	60 125 3	120 118 106
The former	Glucose 1 % + KH_2PO_4	7 14	(+) (+)	4 20	144 116
Starch 0.5 % + KH_2PO_4	Glucose 0.6 % + K_2HPO_4	7 21 27	+ — —	0 2 2	155 117 110

Table 35. concerning G.

(The figures between ( ) bear upon cultures grown on the liquids + glucose of the cultures concerned).

Foregoing culture medium.	Culture medium.	Days.	Glycogen.	Amylase.	Dry weight.
7 × lactose + K_2HPO_4	Glucose 1% impure + K_2HPO_4	15 (13)	— (+ +)	0 (0)	152 (117)
The former	"	14 (14)	(+) (+ +)	0 (0)	141 (137)
The former	"	13 (14)	++ ((+))	0 (0)	141 (114)
		27	—	0	116
7 × lactose + K_2HPO_4	Glucose 1% pure + K_2HPO_4	15	(+)	2	230
The former	"	14 (14)	(+) (—)	0 (42)	251 (146)
The former	"	13 (14)	++ (—)	0 (5)	225 (135)
		27	—	2	200
7 × lactose + K_2HPO_4	Glucose 1% + KH_2PO_4	15	—	8	224
The former	"	14	—	28	221
The former	"	13	++	25	203
		27	—	3	188
The former	Starch 0.5% + KH_2PO_4	14	+	100	133
		21	—	135	121
		35	—	6	108
The former	Glucose 0.5% + KH_2PO_4	7	+	75	164
		14	—	110	160
		28	—	25	148
		34	—	500	136

Formation of amylase is not or hardly found, nor in the liquids to which a new quantity of glucose was added (in 6 six cases the addition of glucose was done twice successively but they yielded the same negative result), both in presence of K_2HPO_4 as of KH_2PO_4 and as well on purely as on impurely sterilised liquids. Where enzyme was formed, it appeared to loose its concentration very soon, without exception. Even on amylum the quantities are small and inconstant, whereas the culture on glucose which follows this last one is "paralysed" in its amylase production again. (N.B. that there is not the least difference between U and G in this case excepted the sudden rise of G in the last culture).

I think this result will suffice to point out how cautious we should be in judging if we are dealing with a race when only the formation of amylase is concerned. So I should not wonder if the tribe U were really the same as the one with which I worked in 1921 - '22 and which, after a stay of 3 years on agar, had not yet regained its former power of enzyme formation in the course of the year during which I experimented with it.

On the whole it may be said that changes in physiological behaviour want a long time to get into a state of apparent equilibrium. An analogical fact is stated by Euler and Laurin (8): Saccharomyces Thermantitonum, a tropical yeast, whose optimum of fermentation was at 42°, was kept for some years in Europe; it then appeared that the optimum was constantly at 33°. Euler elsewhere (4) makes similar observations which, though mainly referring to yeasts, still have their interest in this question too.

These results should be also a warning for those who too soon admit the existence of so called biological races; here in most cases the organisms can not be cultivated on artificial mediums, but when we see how great the inconstancy is of Aspergillus, due to foregoing

mediums and when we consider for how many years a biological race may have been accustomed to its mother plant, we cannot be too careful with our judgement.

The authors who worked with different tribes of Aspergillus niger, all agree in this respect. Hanna Lappalainen (15) had got 10 races from different parts of Europe which differed more or less from each other in physiological conduct, but her opinion is that they should be looked upon as food modifications only; Brenner (2) obtained 3 races by cultivating the fungi at different temperatures but succeeded in getting one race out of another by varying the resp. temperatures. And we need not wonder any more that Grèzes (10) got different results with 2 tribes which had been cultivated on different culture solutions during 60 generations.

The question whether new enzymes can be induced in an organism could not be answered any further by my researches; but as we see that, due to external influences the faculty of amylase production seems sometimes to disappear, caution is needed here too; so Euler and Johannsen (6) do not say anything definitive on this subject when dealing with the question, viz. if a yeast yielded a really new enzyme in fermenting galactose, which before could not be assimilised, (on the other hand Kluyver (13) affirms two years later that a new enzyme, the galactozymase, was formed in one of his yeast cultures which formerly was not able toferment galactose and that there is no question of stimulating here). Pringsheim (17), though having the strong belief that it is possible, admits that still then (1910) there was nothing that had proved it. The behaviour of Aspergillus niger supports their opinion.

A similar view may be noticed here from a quite other range of biology; d'Hérelle (12) states that virulence of of a bacteriophage for some race of bacteria can be weakened or intensified, but that a not existing virulence was never induced in his experiments.

IIII. Summary.

1) A culture solution which contains a reducing sugar together with a neutral phosphate is coloured yellow or light brown during sterilising; this must be caused by substances which are formed by the alkali out of the glas and the sugar; acid phosphate binds the alkali, so that the sugar remains unaltered.

2) These substances retard growth; they promote the formation of amylase when fructose is used, they prevent it in other sugars.

3) It is indifferent for the formation of amylase whether K_2HPO_4 or KH_2PO_4 is used.

4) Glucose and amylum further the formation of amylase; fructose, mannose, lactose and inuline prevent it; galactose does not prevent the enzyme production, but its metabolic substances do; this holds true also for mannose.

5) The results, mentioned under 4) were completed by investigating cultures on worked out culture solutions to which glucose was added and by growing the fungus on mixtures of substances which were favourable and unfavourable for the formation of amylase.

6) Glycerine does not favour the formation of amylase but neither prevents it when mixed with another substance which is conducive to it.

7) Not all sorts of sugar are assimilised to the same degree; galactose and lactose appear to be bad sources of nutrition.

8) An hypothesis is formed that there must be a certain narrow relation of structure between the amylase molecule and that of the substance which is given as food supply or which is formed during the life time of the fungus, if amylase is to be formed.

9) The researches were made with 3 tribes which probably were not distinct races.
10) Apparently in many cases where races of fungi are accepted, we have to deal with modifications only.

Before I close I have great pleasure in thanking professor Dr. G. van Iterson for his kindly allowing me to work in his laboratory and for putting at my disposal all its facilities. I also wish to express my heartfelt thanks for his very valuable suggestions and personal interest which encouraged me to make this investigation.

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