

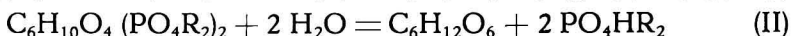
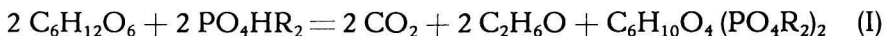
Biochemistry.— *The first phases of the chemistry of the dissimilation of the hexoses.* By A. J. KLUYVER and A. P. STRUYK.

(Communicated at the meeting of June 25, 1927).

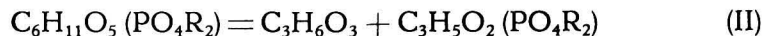
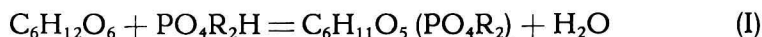
§ 1. *Introduction.*

At the meeting of January 30th 1926 a paper by the authors ¹⁾ was presented in which a new scheme — based on the extensive experimental material available — was developed regarding the first phases of the chemistry of the dissimilation of the hexoses.

As the result of the observations regarding the behaviour of the inorganic phosphates by the so-called cell-free fermentation, it was up to that time generally accepted ²⁾ that the reactions involved in alcoholic fermentation could be represented by the following equations, which were first given by the English investigators HARDEN and YOUNG in 1908 :



It was pointed out by us at that time, how a number of arguments plead in favour of replacing this representation, less satisfactory from a logical point of view, by the following scheme of the process of the splitting up of the hexose :



where the $C_3H_6O_3$ produced in equation IIIb, is split up afterwards, also according to IIIa, into carbonic acid and alcohol.

The by-product of the cell-free fermentation of the hexoses, which had been isolated by ROBISON ³⁾ already four years before, was in this scheme for the first time (however cf. note 2) proclaimed to be the first normal reaction product of the hexoses in fermentative dissimilation.

A further consequence of our view was that the hexose biphosphoric acid, which only appears in case of cell-free fermentation originated from

¹⁾ These Proceedings 29, 322, (1926) and also: Die Naturwissenschaften, Vol. 14, p. 862 (1926).

²⁾ An exception must be made in favour of the American investigator RAYMOND whose publication relating to the matter in question (Proc. Nat. Acad. of Sciences Vol. 11, p. 622 (1925)) was unknown to us at that time. This very preliminary paper contains, however, practically no documentation, whereas RAYMOND's scheme also deviates from ours in this respect that hexose biphosphate is assumed to be a normally occurring intermediary product.

³⁾ R. ROBISON, Biochem. Journ., Vol. 16, p. 809 (1922).

a carbolygatic synthesis of the intermediately formed triose-monophosphoric ester. Whereas this view as to the origin of the hexose-biphosphoric ester had been given before, a.o. by H. VON EULER¹⁾, yet our assumption differs fundamentally from the Swedish investigator's view, owing to the fact that the latter only aims at giving a further interpretation of the mutual connection of the equations of HARDEN and YOUNG. In doing so the hexose-biphosphoric ester is accepted as an intermediary product of the normal alcoholic fermentation of the hexoses ; whereas in our fermentation scheme there is no room for this ester and its appearance in case of cell-free fermentations is looked upon by us as an incidental product, in consequence of the breaking-up of the co-ordination of the successive reactions.

We put in view a further experimental test of our assumption, and it was our intention to return to this more in detail in the thesis of one of us (Str.) Various experimental difficulties which presented themselves together with the desirability to pay attention first of all things, in this connection, to the problem (which was in an extremely confused state) of the so-called "co-enzyme" of the alcoholic fermentation²⁾, were the cause that the anent investigation was not yet finished.

That we already proceed to publish some of the results obtained by us in this investigation at the present moment, finds its cause in the fact, that from 1926 onward the various hexose-monophosphoric esters, especially the one isolated by ROBISON in 1922, have come to be regarded with the utmost interest by a number of the leading investigators who are occupied with the study of the hexose-dissimilation. We only mention such names as : EMBDEN, MEYERHOF, NEUBERG and VON EULER. We are far from venting the suggestion that this altered situation might be due to the publication of our view, yet it seems desirable to ascertain here, how the exactness of our scheme has derived considerable support from later investigations. As the problem under discussion is now actively and intensively investigated in various laboratories, we thought it advisable not longer to postpone the provisional publication of some of the results obtained by us in this line.

§ 2. *Survey of the more recent investigations which support the hypothesis of the formation of a hexose-monophosphoric ester as the first intermediary product in the dissimilation of the hexoses.*

The first investigator, who, in the course of 1926, declared himself in favour of the supposed formation of a hexose-monophosphoric ester as an intermediary product in the dissimilation of the hexoses, may have been G. EMBDEN. He did so at an oral discussion at the Physiological congress³⁾

1) H. VON EULER, *Chemie der Enzyme*, I Teil, 3e Aufl., p. 336, 1925; Cf. also: *Die Naturwissenschaften*, Vol. 13, p. 938 (1925).

2) *These Proceedings*, 30, 569 (1927).

3) O. MEYERHOF, *Die Naturwissenschaften*, Bd. 14, p. 1179 (1926).

held at Stockholm in August 1926. In this connection it must be borne in mind, that EMBDEN, in 1917¹⁾, announced to have proved that the hexose-phosphoric ester, which occurs as the normal precursor of the lactic acid which is produced in the muscular tissue, and to which he had given the name of "Lactacidogen", produces a phenylosazone which is identical with the well-known LEBEDEFF osazone, which is obtained from the hexose-biphosphoric ester formed in the case of cell-free alcoholic fermentation. In how far the lactacidogen itself is identical with the above-mentioned ester, was then left undecided. In 1924²⁾, however, EMBDEN arrives at the conclusion: "Hiermit ist der Nachweis erbracht, dass dem Lactacidogen die Struktur einer Hexosediphosphorsäure zukommt, welche mit der bei der Hefegärung gebildeten Hexosediphosphorsäure vollkommen identisch ist". However, he adds to this that he does not exclude the possibility, that at the same time a hexose-monophosphoric ester also occurs in the muscular tissue. In 1927 there appears the fifth paper on the chemistry of the lactacidogen, in which EMBDEN revokes his former statements to a certain extent³⁾. It now has appeared to him that the ester, obtained until that time from the muscular tissue by adding fluoride of sodium, does not occur in the undamaged muscular tissue, but that, on the other hand, it contains a hexose-monophosphoric ester which in fact must be looked upon as the lactacidogen proper. This ester is isolated by him and studied, and he arrives at the conclusion that it is neither identical with NEUBERG's ester, nor with ROBISON's. As regards the latter part of this statement, it seems to us that there is still room left for doubt.

Whereas MEYERHOF until shortly still adhered to the hypothesis on the function of the phosphoric acids in the dissimilation of the hexoses given by HARDEN and YOUNG, as appears from his book "Chemical Dynamics of Life Phaenomena" (finished in April 1924), and even gives new evidence in support of it, he, for the first time, pronounces the opinion of the necessity "einer Umdeutung und Neuformulierung der HARDEN—YOUNG'schen Gärungsgleichungen" in a "Zuschrift" sent to "Die Naturwissenschaften"⁴⁾ on June 30th 1926. As a result of new investigations he has arrived at the following conception of the splitting-up of the hexoses in the muscular-extract: "In der I Periode werden beide Zuckermoleküle phosphoryliert. Das eine Estermolekül zerfällt rasch in statu nascendi was so lange geht, als noch neuer Ester entstehen kann. Das andere Estermolekül stabilisiert sich als Hexosediphosphorsäure". In this publication MEYERHOF leaves as yet undecided the nature of the primarily formed

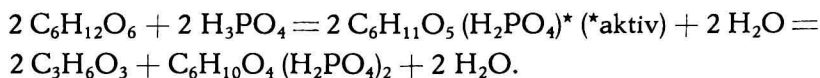
1) G. EMBDEN und F. LAQUIER, Zeitschr. f. physiol. Chemie, Vol. 98, p. 181 (1917).

2) G. EMBDEN und M. ZIMMERMANN, Zeitschr. f. physiol. Chemie, Vol. 141, p. 225 (1924).

3) G. EMBDEN und M. ZIMMERMANN, Zeitschr. f. physiol. Chemie, Vol. 167, p. 114 (1927).

4) O. MEYERHOF, Die Naturwissenschaften, Vol. 14, p. 756 (1926). Cf. also: Idem, Biochem. Zeitschr., Vol. 178, p. 462 (1926).

ester. In a paper ¹⁾, published towards the end of 1926, he defines this ester in agreement with EMBDEN's above-mentioned statement, as a labile *monophosphoric ester*, and gives the following scheme :



Consequently, MEYERHOF too, here comes to the conclusion that a hexose-monophosphoric ester is the first reaction-product in the dissimilation of the hexoses. Starting from this hypothesis MEYERHOF and LOHMANN ²⁾ have, quite recently, made a detailed investigation to ascertain the behaviour of the various hexose-monophosphoric esters towards muscular-extract and yeast maceration-juice.

In his last summary of the chemistry of the hexosedissimilation (which treatise bears the date of December 1, 1924), NEUBERG ³⁾ mentions but incidentally the hexose-monophosphoric ester isolated two years before that time by ROBISON, whereas he makes the remark : "seine Bedeutung für den Gärungsvorgang ist noch ungeklärt".

In the same treatise, however, the possibility is left open that, in contradistinction to HARDEN and YOUNG's theory, the formation of hexose-diphosphoric ester "ein unphysiologischer Prozess ist". In a publication ⁴⁾ which appeared in 1925, this statement, however, is further developed as follows : "somit ist die *extrazelluläre Anhäufung* ⁵⁾ von Zucker-phosphorsäure-ester von C. NEUBERG und seinen Mitarbeitern nicht ohne Recht als ein unphysiologischer Prozess bezeichnet". On the other hand, the fact, that the phosphoric ester does not accumulate in the sugar-fermentation by living yeast-cells, is ascribed to the perfect co-ordination of the binding and splitting-off of the phosphate present in these cells. From this it must be inferred that NEUBERG at that time looked upon the hexose-biphosphoric ester as a normal intermediary product of the alcoholic fermentation of the hexoses. In agreement with this the hexose-biphosphoric ester continues to remain the focus of interest in some later publications of NEUBERG and KOBEL ⁶⁾.

In a paper ⁷⁾ published towards the end of 1926 the following statement strikes us : "In den Vordergrund des Interesses ist nun neuerdings die Hexose-*monophosphorsäure* gerückt". In connection herewith observations are given on the fermentability of the three different hexose-*monophos-*

¹⁾ O. MEYERHOF, Die Naturwissenschaften, Vol. 14, p. 1175 (1926).

²⁾ O. MEYERHOF und K. LOHMANN, Biochem. Zeitschr., Vol. 185, p. 113 (1927).

³⁾ C. NEUBERG in OPPENHEIMER, Handbuch der Biochemie des Menschen und der Tiere, 2te Aufl., II Vol., p. 453 (1925).

⁴⁾ C. NEUBERG und A. GOTTSCHALK, Biochem. Zeitschr., Vol. 161, p. 254 (1925).

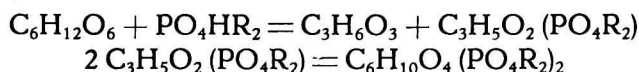
⁵⁾ NEUBERG himself prints in italics.

⁶⁾ C. NEUBERG und M. KOBEL, Biochem. Zeitschr., Vol. 166, p. 488 (1925). Ibid. Vol. 174, p. 480 (1926).

⁷⁾ C. NEUBERG und M. KOBEL, Biochem. Zeitschr., Vol. 179, p. 451 (1926).

phoric esters. In a very recent publication¹⁾ the question is moved how far "im Mono-phosphorsäure-ester wirklich das lang gesuchte obligatorische Zwischenprodukt der Gärung vorliegt", and NEUBERG gives here as his opinion that the high yields of ROBISON's ester obtained by him are "vielleicht ein Argument zugunsten der Ansicht, dass dem Gärungsmono-phosphat der Rang eines wichtigen Durchgangsgebildes zukommt".

In 1924 VON EULER and MYRBÄCK²⁾ still write: „Durch die hier mitgeteilten Versuche gewinnt die HARDENSche Theorie über die Beteiligung des Hexose-diphosphates als Zwischenprodukt der Gärung eine neue Stütze". Shortly afterwards VON EULER and BRUNIUS³⁾ express themselves even more positively, as may appear from the following quotation: "dass also die Bildung des Hexose Diphosphates $C_6H_{10}O_4(PO_4R_2)_2$ eine notwendige einleitende Teilreaktion der alkoholischen Gärung ist, kann nunmehr als feststehend angesehen werden". In a "Zuschrift" to "Die Naturwissenschaften"⁴⁾ which bears the date of the 24th of September 1925, VON EULER expresses himself as follows: "Für die normale alkoholische Gärung können die HARDENSchen Gärungsgleichungen als *bewiesen*⁵⁾ gelten". It is true that VON EULER, in writing this, feels the want of giving a more detailed representation of the connection, which, on the strength of these equations, must exist between the fermentation of the first and the esterification of the second hexose-molecule. He therefore develops a conception according to which the chemistry of the hexoses might be represented as follows:



VON EULER imagines that the reacting glucose molecule is first split up into two molecules, each containing 3 C atoms, the former of which is afterwards esterified with phosphate, whereas the latter is fermented into alcohol and carbonic acid. Further, the synthesis of the hexose-biphosphate it said to take place "auf Kosten der energieärmeren, vergärenden Glukosehälfte". Without entering into a more direct consideration of this assumption, we only want to point out here, that it only tallies in one respect with our scheme, viz. as regards the formation of the hexose-biphosphate from two molecules of triosephosphate. For the rest it is altogether different from it. For, according to VON EULER the fermentation is made subservient to the synthesis of the hexose-bi-phosphate, whereas the main point of our conception lies in the fact that the phosphorylation of a hexose molecule is made subservient to the splitting up of the same molecule. Moreover according to VON EULER it is the triosemolecule which is

¹⁾ C. NEUBERG und J. LEIBOWITZ, Biochem. Zeitschr., Vol. 184, p. 489 (1927).

²⁾ H. VON EULER und K. MYRBÄCK, Zeitschr. f. physiol. Chemie, Vol. 139, p. 15. (1924).

³⁾ H. VON EULER und E. BRUNIUS, Svensk Kemisk Tidskrift, Vol. 37, p. 301 (1925).

⁴⁾ H. VON EULER, Die Naturwissenschaften, Vol. 13, p. 938 (1925).

⁵⁾ We print in italics.

esterified, whereas, in our conception, it is the esterification of the glucose-molecule, i.e. the formation of the hexose-mono-phosphate that comes to the fore. In a recent paper, however, VON EULER¹⁾ also hints at the possibility of the hexose-monophosphate playing a part in the first splitting-up to which the hexosemolecule is subjected in the dissimilation.

§ 3. *Personal observations on the appearance of hexose-monophosphoric ester in the cell-free fermentation.*

As it has already been stated, the consideration which induced us to publish our experiments, though incomplete, was the fact that some weeks ago an extensive communication was published by NEUBERG and LEIBOWITZ (l.c.), which contains numerous data regarding the coming to the front of hexose-monophosphoric ester when maceration-extract reacts on hexoses in presence of inorganic phosphates.

In connection with what follows it may be pointed out, that these investigators give no explanation whatever of this remarkable fact, which differs from the results obtained by former investigators, and derive no conclusion whatever from their experiments, with the exception of the above-quoted guarded statement given in a footnote.

As regards our own observations, which partly date from the latter half of 1926, the following introductory remarks may be made.

The scheme made up by us, naturally includes that the hexose-biphosphoric ester can only be formed, if, by the side of the phosphatase, the oxydoreducing agent of the yeast, the zymase in the narrower sense, can also come into action. This agent is both indispensable for the splitting-up of the hexose-monophosphoric ester, and the carbolytic synthesis of the triose-monophosphoric ester formed. The most beautiful demonstration of the exactness of our view would have been a separation of the phosphorylating from the oxydoreducing agent of the yeast, for, in that case, the esterified phosphate ought to have been found back exclusively as hexose-monophosphoric ester. A similar isolation of the phosphorylating agent has been tried more than once, and as far as we know a positive result has been reported but only once.

In 1912 EULER and OHLSEN²⁾ state that an extract of a special kind of yeast brings about esterification under very special circumstances, without producing carbonic acid. A further investigation of the ester formed has, however, not taken place. A repetition of the said experiment, with the yeast-species that were at our disposal, yielded, however, a negative result, in accordance with the experience other investigators had obtained on this point. Starting from the fact that fluoride of sodium in the muscular tissue, does not interfere with the phosphorylation, but stops the formation of

¹⁾ H. VON EULER und K. MYRBACK, Zeitschr. f. physiol. Chemie, Vol. 165, p. 44, (1927).

²⁾ H. VON EULER und HJ. OLSÉN, Zeitschr. f. physiol. Chemie. Vol. 76, p. 468 (1912).

lactic acid, it was now tried, by adding this salt to produce a similar effect in case of the reaction of maceration extract on hexoses.

Experiments made with various concentrations of Na F showed that neither in this way a separation of the two functions could be brought about. Neither esterification nor fermentation took place with the higher concentrations, and as soon as the esterification did set in when the concentration of the poison was reduced, carbonic acid was always formed at the same time. Incidentally it may be stated that after that time MEYERHOF¹⁾ has also come to the same result.

Attempts to come to a phosphorylation with a total absence of fermentation in the reaction of zymase-preparations and hexoses, had to be given up for the present. Meanwhile, attempts aiming at isolating the triosephosphate which IWANOFF found at one time in the fermentation medium, had drawn our attention to the great differences which manifested themselves in the nature of the rate of fermentation in presence of inorganic phosphates, when different maceration juices were used. It was tempting to trace to what extent this divergent behaviour manifested itself also in the nature of the hexosephosphoric esters appearing in the fermentation media.

It may be stated at once that this appeared to be the case.

We subjoin the result of two typical experiments which, however, are only instances of a series of observations which yielded similar results.

In order to prevent misunderstanding, it may be stated that the yeast used for the preparation of the maceration juice was the same yeast in all these experiments, viz. bottom-yeast of the d'Oranjeboom Brewery at Rotterdam²⁾. Meanwhile it has appeared to us from other experiments, that the properties of the maceration juices prepared from this yeast, vary a good deal, according to the previous history of the yeast which is worked up, and according to the duration of the operations to which the dry yeast was subjected before the maceration.

Two typical instances are given below of the progress of the production of carbonic acid in the fermentation of glucose, in the presence of inorganic phosphates, with the aid of maceration-juices prepared from two different portions of dried yeast.

With these experiments 5 grams of glucose were brought to fermentation with 25 cc. of the maceration-juice which were kept shaking in a thermostat at 30° C.

When the rate of fermentation had become constant, 25 cc. of a 6% solution of one part of NaH_2PO_4 and five parts of Na_2HPO_4 were added.

The two curves *A* and *B* of Figure 1 mark the progress observed of the rate of fermentation, i.e. the quantity of carbonic acid liberated every 5 minutes. Whereas with the aid of the maceration-juice obtained from

¹⁾ O. MEYERHOF, Biochem. Zeitschr., Vol. 183, p. 176 (1927).

²⁾ We herewith express our heartfelt thanks towards the directors and the bacteriologist of this brewery for the readiness with which they have always put yeast at our disposal.

yeast-sample *A*, a progress of carbonic-acid development is obtained which is characterised by the rapid reaching of the maximum and a subsequent,

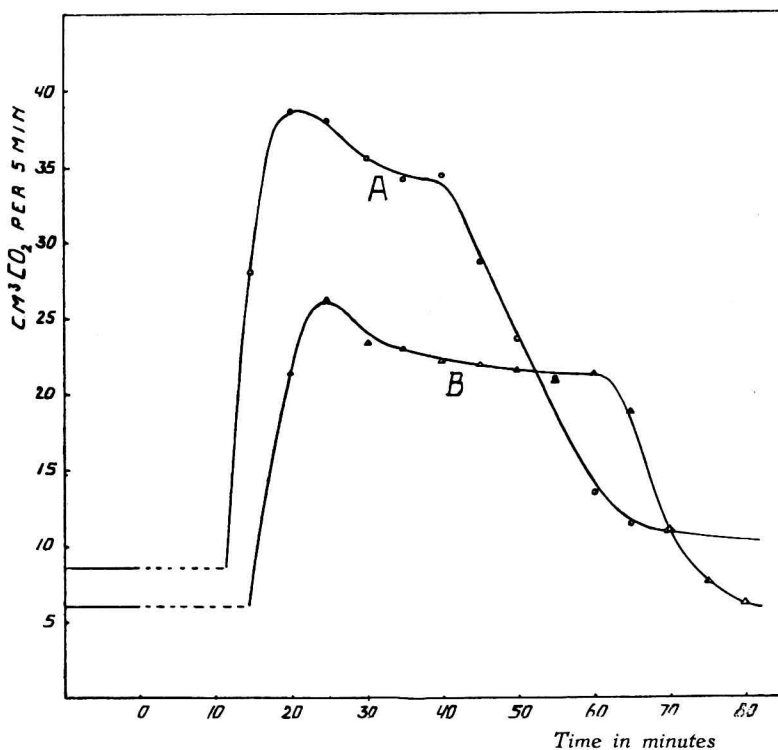


Fig. 1.

constant decrease in the rate of fermentation, it is seen that the addition of phosphate to the maceration-juice prepared from yeast-sample *B* has a different influence. The increase in rate of fermentation is considerably less in this case, but on the other hand, the increase in velocity continues to exist for a longer time. After complete esterification had set in, which, at the same time, revealed itself by the fact that the rate of fermentation fell back to its original value, we proceeded, in both cases, to a further investigation of the phosphoric esters present at that point of time. In doing so, the method of separation (elaborated by ROBISON¹) of the hexose-mono-phosphoric and hexose-biphosphoric esters with the aid of the Ba-salts, was applied. For this purpose the yeast-mixture was first slightly acidified towards litmus, and afterwards poured out into half a volume of boiling water, to stop the enzyme-action spontaneously. After having boiled this for a short moment, the liquid was sucked off from the protein precipitated while still hot, and the precipitate was washed with hot water. The required quantity of solid acetate of barium was added to the combined filtrates and the liquid was neutralised with baryta towards phenolphtha-

¹) l. c.

leine. Independent of the appearance or non-appearance of a precipitate with these operations, one or two volumes of 96 % alcohol were added to the liquid. Owing to this the Ba-salts of the various hexose-phosphoric esters precipitate almost completely.

This precipitate was brought over on a Buchner-funnel and after removing the liquid, it was washed with 70 % alcohol, and afterwards treated for one night with boiling absolute alcohol to denaturalise the protein present, if any. The dried precipitate was weighed, and then extracted with the tenfold weight of cold water ; which brings about the solution of the Ba-salts of the hexose-*monophosphoric* ester, whereas nearly the whole of the much less soluble Ba-salt of the hexose-*biphosphoric* ester remains behind.

The Ba-salts of the esters prepared with the *A*-yeast and *B*-yeast show a very striking difference in the above-mentioned treatment, as appears from the following figures :

	<i>A</i> -yeast	<i>B</i> -yeast
Weight of the <i>Ba</i> -salts	4.055 gr.	4.050 gr.
" " " " after extraction with 40 cc. of water and subsequent drying	1.810 gr.	traces

Although not the whole of the dissolved substance may be looked upon as the Ba-salt of the mono-ester, yet it may be concluded with certainty from the behaviour of the esters obtained by *B*-yeast, that only a very small quantity of hexose-*biphosphoric* ester was present in them. That indeed the phosphate, when the fermentation was stopped, was present as a hexose-*monophosphoric* ester, was further proven by a purification of the Ba-salts which had been dissolved (by a conversion via the lead salt) and the preparation of the phenylosazone of the liberated acid. This osazone had, in accordance with ROBISON's statement, a melting-point of 139—141° C. In contradistinction to this, a hexose-phosphoric acid could be prepared from the remaining Ba-salts of the esters prepared with *A*-yeast by applying the usual purification-methods, which produced a phenylosazone which agreed in every respect with the wellknown LEBEDEFF-osazone (melting-point 151°).

These experiments were repeated a number of times and it appeared that special yeast-samples always behaved in accordance with the *B*-samples. In all these cases the relation of the hexose-*monophosphoric* ester to the hexose-*biphosphoric* ester appeared to have been altogether shifted to the first, this being contrary to ROBISON's experience and the experience obtained with other yeast-samples. Owing to these results it had been ascertained that, under the conditions chosen for special yeast-samples, the

hexose-monophosphoric ester might, from a relatively unimportant secondary product of the dissimilation, become the main-product.

How are we to explain the differences in the behaviour of the various maceration-extracts described above? In the light of the conception, advocated by us, of the catalytic agents active in the alcoholic fermentation of the hexoses, the following view seems satisfactory to us in every respect. Whereas it is usual to assume that the various successive reactions which take place in the fermentation proceed each under the influence of a specific catalyst, we place ourselves on the point that only two agents are active, viz :

- a. an esterifying agent, which is also able to split up the esters ¹⁾).
- b. an oxydoreducing agent.

Whereas in the living yeast cell both catalysts are present in a harmonious quantity, so that the different reactions of our scheme take place in succession, this situation is changed if it is tried to withdraw from the cell the active agents by preparing maceration-juices. In the normal maceration-juices, which among other things show the course of fermentation described by HARDEN and YOUNG, the first-mentioned agent has evidently been weakened in proportion to the second. This appears from the fact that the hydrolysis of the triosephosphoric ester formed has been weakened, owing to which the latter, under the influence of the abundantly present oxydoreducing agent, condenses to hexose-biphosphoric ester. It appears to us, that the phenomena observed with the abnormal maceration-juices described above, find an unconstrained explanation, if we assume that in these juices the relation of the phosphatase (-ase) and the oxydoreducing-agent has been shifted in favour of the first-mentioned agent. For, to be sure, then an accumulation of the triose-phosphoric ester which leads to the formation of hexose-biphosphoric ester will not take place, but it may be expected on the other hand that the first reaction incited by the oxydoreducing agent, i.e. the splitting-up of the hexose-monophosphoric ester, will fall behind to the production of the last-mentioned substance. So accumulation of this substance shall take place, which is in accordance with the facts observed. In accordance with the conception that the zymase function proper has relatively been weakened a great deal in case of the maceration juices prepared from our "abnormal" sample of yeast, there is also the fact that these extracts do not lend themselves to the co-enzyme experiments described in a former paper by us, because they are soon irreversibly inactivated on continued washing.

On the ground of the view developed above, the maceration-juices (which behave according to HARDEN and YOUNG's description) may with the same right be called "abnormal" as the maceration-juices with different

¹⁾ We leave open the possibility, that it would be desirable to assume the existence of a separate phosphatase and phosphatase, like VON EULER does. The undermentioned considerations become a little more complicated, owing to this, but need not be materially changed.

behaviour, and in the fitness of our scheme to explain these divergent phenomena, we find further indication in favour of its correctness.

§ 4. *The appearance of the triose-stage in the cell-free fermentation of the hexoses.*

Another point, by which experimental support might be given to the scheme proposed by us, would be the isolation of the triose-monophosphoric ester assumed by us as an intermediary product. As is known, L. IWANOFF reports as early as in 1907, so at the very beginning of the examination of the biochemical phosphorylation, the isolation of such an ester. Later investigators however, have never been able to confirm this result, with the exception of the fact that EULER and FODOR¹⁾ in 1911, make mention of this ester by the side of the hexose-biphosphoric ester. In our first paper on this subject we already pointed out that the different results of IWANOFF and the later investigators might be the consequence of the use of different phosphate concentrations in the esterification, and therefore we made up our mind to repeat the experiments strictly in accordance with IWANOFF's prescriptions. In spite of various attempts we have not succeeded in isolating the osazone described by IWANOFF, although we not only applied the not very clear prescription by IWANOFF but made as well manifold variations in the way of isolation of the triosephosphoric ester.

The negative result of these experiments made us consider whether the presence of triosephosphoric ester in the fermentation-medium, after esterification had set in, might not be ascertained by other means. In doing so we made use of the triose reaction given by NEUBERG and co-operators²⁾. These investigators were able to ascertain that various trioses and also dioxycetonephosphoric ester — in case of distillation with sulphuric acid and keeping the volume constant by addition of water — are converted to a very important degree into methylglyoxal volatile with steam. The latter substance can then be demonstrated in the distillate as p-nitrophenylosazone and hereupon a quantitative estimation, at least as regards the free trioses, can be established.

It appeared to us that 10 cc. of a yeast mixture, according to IWANOFF's receipt (40 grams of zymine, 50 grams of glucose, 10 grams of Na_2HPO_4 12 aq. in 1000 cc. of water) after a three-days' fermentation at room-temperature distilled in the above-mentioned way, produced with p-nitrophenylosazone a compact precipitate of the methylglyoxal-p-nitrophenylosazone. We had already settled before in accordance with NEUBERG that the reaction turns out practically negative with the various hexoses, and also with hexose-biphosphoric ester. The same could also be ascertained as regards ROBISON's mono-ester. A special blank-test, moreover, showed

¹⁾ H. VON EULER und A. FODOR, Biochem. Zeitschr., Bd. 36, pag. 401 (1911).

²⁾ C. NEUBERG, E. FÄRBER, A. LEVITE und E. SCHWENK, Biochem. Zeitschr., Vol. 83, p. 244 (1917).

us that a mixture of the said quantities of phosphate, glucose and zymine killed by boiling only gave a negligible triose reaction in the period of time under consideration ¹).

It seemed to us a matter of importance to ascertain how far the triose-reaction observed, was based on the appearance of free triose or rather of a triose-phosphoric ester in the fermentation medium. For this purpose the zymine of a second similar experiment was removed after two days by centrifugation. In the clear solution which we obtained, in which hardly any inorganic phosphate could be found, the quantity of triose was estimated by weighing the quantity of osazone obtained. After this it was slightly acidified with acetic acid, boiled a minute and removed by filtration from the protein coagulated. The precipitate was washed and the filtrates were at a low temperature evaporated to one seventh of the original volume in the Faust-Heim apparatus. Another triose-estimation was made in these concentrated filtrates. Thereupon the Ba-salts of the phosphoric esters were precipitated in the way described above and the triose was estimated once again both in the precipitate and in the filtrate.

The following quantities of osazone were isolated in this experiment (calculated for the entire quantity of centrifugate (200 cc.)) :

In the untreated liquid	620 mgr.
After boiling and concentration	425 „
In the Ba-precipitate	traces
In the filtrate of the Ba-precipitate	430 mgr.

From these experiments it appears in the first place that part of the triose when concentration is applied disappears, either by volatilization or by conversion. Although the entire quantity of triose present at the outset (about 150 mgr.) must be called rather small as regards the quantity of the glucose used, this quantity is not so small, however, as regards the quantity of esterified phosphate (about 800 mgr. of anhydrous Na_2HPO_4).

However, the negative result of the triose-reaction in the precipitated Ba-salts, does not make it probable that a triose-phosphoric ester should be present. As, however, the possibility did not seem to be excluded, that the Ba-salt of a similar ester would be soluble in water relatively well, the experiment was once more repeated while the phosphorus was simultaneously estimated. This was done according to the well-known method of NEUMANN, and as regards the small quantities according to the calorimetric method of MARTLAND and ROBISON ²).

The following results (also calculated for 200 cc. of centrifugate), were now obtained. (See next page.)

From these figures it is shown conclusively, that by far the greater part of the triose is found in the filtrate last mentioned, and certainly is not present in it as phosphoric ester. Moreover, it appears from the fact that the

¹) H. A. SPOEHR and P. C. WILBUR, *Journal of Biol. Chem.*, Vol. **69**, p. 421 (1926).

²) M. MARTLAND and R. ROBISON, *Biochem. Journ.*, Vol. **20**, pag. 847 (1926).

	Osazone in milligrams	P inorganic in milligr.	P bound in milligr.
In the untreated liquid	560	9.0	
After boiling and concentration . .	362	15.0	275
In the <i>Ba</i> -precipitate	40	—	269
In the filtrate of the <i>Ba</i> -precipitate .	335	—	9

quantity of free phosphate remains practically constant in boiling and concentration, that no splitting-up of a triosephosphoric ester, of which the presence at the outset might be deemed possible, occurs.

As in the case of the first experiment the phenomenon, however, appears that the triose-quantity is reduced to about two thirds in the evaporation of the solution. Whereas tests to check this showed that glycerine aldehyde does not disappear when exposed to our procedure of boiling and evaporation, there yet remains the possibility, that this decrease of the triose-reaction is a consequence of the condensation of an eventually present triosephosphoric ester. This possibility is further being investigated at present. Although a small quantity of the triose was found again in the *Ba*-precipitate on this occasion, this quantity is too small to attach any importance to it as yet.

The experiments were now continued in order to ascertain how far indications of the appearance of triose might be obtained in the case of esterification during a short time with the aid of maceration-juice, and by using a higher concentration of phosphate. It seemed plausible to us, that, immediately after the end of a typical "phosphate-period", a certain amount of triose-phosphoric ester would be present. We shall not enter into details of this series of experiments which have not yet been finished. It may only be stated here that, in using certain maceration-juices, very convincing triose-reactions, were again obtained. In using other maceration-juices, however, no or only weak reactions were met with. The most important thing in this, however, was, that those very juices which show a typical "phosphate-period" (as described by HARDEN and YOUNG), gave a strong reaction, whereas those extracts, of which it has been stated in the preceding section that they produce much hexose-*monophosphoric* ester, developed no reaction at all or but a very weak one. Experiments to ascertain whether the substance, which develops the triose reaction can be precipitated by direct preparation of the lead salts, yielded no positive result up till this time.

Summarizing all this, the conclusion must be drawn that we have not yet succeeded to prove the formation of a triosephosphoric ester in the dissimilation of the hexoses. That great difficulties will be connected with this follows from our scheme at first sight, as this ester is subject to two kinds of conversions; viz. a hydrolysis on the one hand and a condensation on

the other, so that here a weakening of one of the two catalytic agents does not lead to an accumulation, as is the case with the other intermediary products.

Nevertheless the results obtained did not seem to us to be altogether without importance, as practically no observations of the triose stage in the alcoholic fermentation of the sugars had been made with positive results ¹⁾. NEUBERG still writes in his summary in OPPENHEIMER's Handbuch der Biochemie, which has been quoted already before: "Wie mehrfach erwähnt worden ist, ist das intermediaire Auftreten von Triosen beim Gärungsprozesse noch nicht bewiesen" ²⁾. In this respect, the investigations described above, seem to fill up a void and, at the same time, they support the scheme drawn up by us.

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the Technical University of Delft.*

¹⁾ The old investigation of P. BOYSEN-JENSEN, Biochem. Zeitschr., Vol. 58, p. 451 (1914), is very little convincing in this respect.

²⁾ *l. c.*, p. 458.
