Biochemistry. — *The first phases of the chemistry of the dissimilation of the hexoses.* (2nd Part). By A. J. KLUYVER and A. P. STRUYK.

(Communicated at the meeting of October 27, 1928).

§ 1. *Introduction.*

In our first communication under the same title [1]) mention was made a.o. of some preliminary experiments which aimed at giving a clearer insight into the nature of the phosphoric esters formed in the so-called "cell-free" fermentation of the hexoses. It appeared from these experiments that the ratio in which hexose-*bi*phosphoric and hexose-*mono*phosphoric esters are present at the end of the phosphorylation period is greatly influenced by the nature of the dried yeast-sample used for the preparation of the maceration juice. The most remarkable result of these experiments was doubtless that in special cases the amount of hexose *mono*phosphoric ester largely exceeded that of hexose *bi*phosphoric ester.

Untill we had claimed a hexose *mono*phosphoric ester to be the first reaction product in the dissimilation of hexoses, investigators used to attribute exclusivily to the hexose *bi*phosphoric ester an important function in the biochemical degradation of the hexoses [2]). Thus the schemes representing the chemistry of the dissimilation of the hexoses drafted before 1926 did not include the hexose *mono*phosphoric ester.

It is obvious that the observation mentioned above as to the predominance of the hexose *mono*phosphoric ester under special conditions — observation published at a little earlier date also by NEUBERG and LEIBOWITZ [3]), who however did not derive important conclusions from it — was quite appropriate to support our theory developed at the end of 1925. But from the very beginning we were quite aware that we might only consider our theory as being confirmed if we should succeed in explaining why under special conditions the hexose *mono*phosphoric ester prevails, whereas under other conditions, as in the majority of the cases examined since HARDEN and YOUNG's classical investigations, the amount of the hexose *bi*phosphoric ester largely exceeds that of the hexose *mono*phosphoric ester. Of course at the same time the question rose in how far our conception of the first phases of the chemistry of the dissimilation of hexoses might offer certain advantages as compared with the other existing theories for the explanation of the signalled diversity in behaviour.

---

[1]) These Proceedings **30**, 871, (1927).

[2]) These Proceedings **29**, 322, (1926); c.f. too the communication cited above: § 2.

[3]) C. NEUBERG und J. LEIBOWITZ, Biochem. Z., Bd. **184**, 489, (1927).

A short survey will be given of the experiments which show that on the one hand it is possible to control the conditions determining the formation of each of the two esters mentioned and which on the other hand are appropriate to answer in the affirmative the question raised above. For a more detailed description of these experiments we refer to the thesis published in the meantime by one of us (Str.) [1]).

§ 2. *Further experiments on the appearance of a hexose monophosphoric ester in "cell-free" fermentation.*

In the course of the experiments of which some examples were already given in our previous paper many observations were made on the ratio of both phosphoric esters present at the end of the phosphorylation period. In doing so we used an improved method of analysis, which was largely due to NEUBERG and LEIBOWITZ (l.c.). As for some modifications introduced by us we refer to the thesis mentioned above.

Table I gives the results of four experiments. In all experiments we used a fermentation mixture composed of 1 part of maceration juice and 1 part of a 5 % solution of Na_2HPO_4; the initial concentration of the glucose was always 10 %. In the table *Vm* indicates the molecular ratio of hexose *mono*phosphoric and hexose *bi*phosphoric ester at the end of the phosphorylation period, viz. at the moment that the rate of fermentation had fallen down to the rate previous to the addition of the phosphate. At this moment the phosphate added appeared to be practically completely esterified. Moreover the maximum rate of production of carbonic acid, in ccm per 5 minutes, observed in the various experiments is included in the table.

TABLE I.

Number of experiment [2])	*Vm*	Maximum rate of production of CO_2 (ccm. per 5 min.)
7	4.6	—
8	3.3	24.9
10A	12.6	14.2
11A	9.8	17.2

These results strike us because in all cases the amount of the hexose *mono*phosphoric ester considerably exceeds that of the hexose *bi*phosphoric ester. Therefore it is necessary to revise our original conception in so far

[1]) A. P. STRUYK, Onderzoekingen over de alcoholische gisting, Diss., Delft 1928.

[2]) The numbers correspond to the numbers of the experiments reported in STRUYK's thesis.

that the hexose *mono*phosphoric ester of ROBISON may not be considered as the primary product of the phosphorylation of glucose, but has to be taken for a stabilised modification, as has already been earlier suggested by MEYERHOF [1]). For the assumption that ROBISON's *mono*-ester should be the primary reaction product does not go in with the observed downfall of the rate of development of carbonic acid at the end of the phosphorylation period in those cases in which nearly all the phosphate may be recovered as *mono*-ester.

The attention is drawn to the fact, already underlined in our previous communication, that the maximum rate of production of carbonic acid is higher in as much as the ratio of the two phosphoric esters changes in favour of the *bi*phosphoric ester. However even in experiment 8, the curve of which shows a pronounced top, the amount of the *bi*phosphoric ester is still far less than that of the *mono*phosphoric ester.

Nevertheless in trying to reproduce similar experiments under exactly the same conditions as chosen by ROBISON we came across various cases in which the hexose *bi*phosphoric ester predominated and in which the amount of the *mono*phosphoric ester corresponded with the amount estimated by ROBISON at about 15 % of the total phosphate esterified.

In examining the differences in the conditions underlying these various experiments we were struck first of all by the fact that, although in both cases the initial concentration of the phosphate was the same, ROBISON used the maceration juice in higher concentration than we did in our experiments. Thus the concentration of the catalytic principles in the experiments of ROBISON was higher.

Therefore we came to suppose that the same maceration juice would convert the inorganic phosphate added more or less in one of the two phosphoric esters depending on the concentration in which the juice was applied. A higher concentration of "active zymase" would increase the yield of *bi*phosphoric ester.

The experiments gave a complete confirmation of this view.

A maceration juice which applied at an earlier date in a concentration of

TABLE II.

Number of experiment	Concentration of the maceration juice	Vm	Indication of the corresponding curves
14	0.67	2.4	*C*
13	0.56	8.4	*B*
12	0.50	16.4	*A*

[1]) O. MEYERHOF, Die Naturwissenschaften, **14**, 1179, (1926); c.f. too: O. MEYERHOF und K. LOHMANN, Biochem. Z. **185**, 113, (1927).

0.81 led to a value for $Vm = 0.34$ gave the results reproduced in Table II when applied in lower concentrations.

The attention may be drawn to the fact — as appears from Fig. 1 — that the observed change in the ratio of both phosphoric esters is accompanied by a pronounced change in the nature of the curves which represent the rate of development of the carbonic acid during the phosphorylation period.

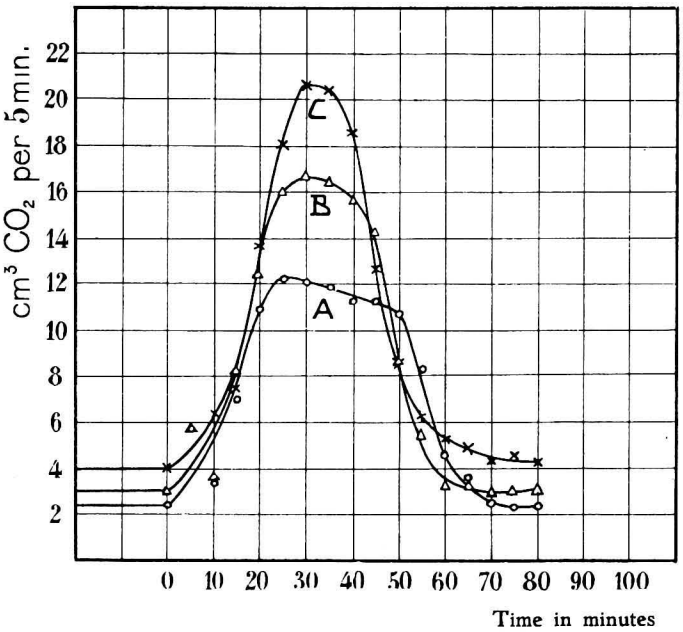


Fig. 1.

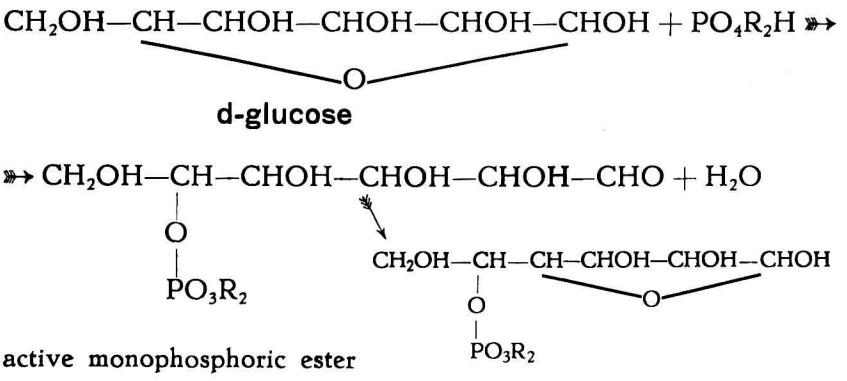
Apparently there is a close connection between the two phenomena mentioned in so far as the type with the pronounced top changes into the "flat type" when the *mono*phosphoric ester predominates.

§ 3. *Explanation of the observed phenomena on the basis of the scheme adopted for the chemistry of alcoholic fermentation.*

It was already pointed out in our previous communication that the scheme for the chemistry of alcoholic fermentation adopted by us seemed quite appropriate to explain the changes in ratio of both phosphoric esters during "cell-free" fermentation. Further down this matter will be subject to a closer consideration.

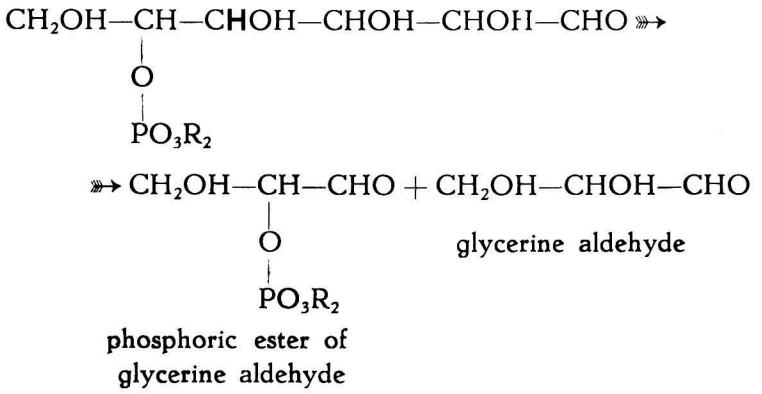
First of all the scheme developed in our earlier papers will be reproduced here once more as a whole. As for the position to be given to ROBISON's hexose *mono*phosphoric ester the remarks made in § 2 will be taken into account.

I. *Initiating phosphorylation.*

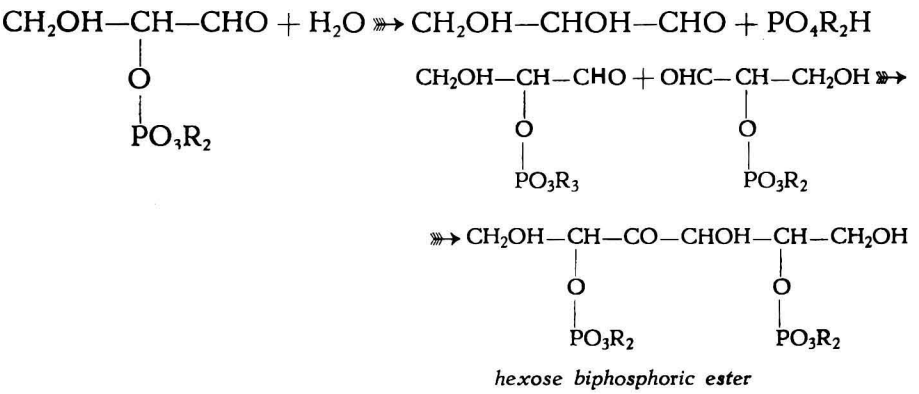


Robison's monophosphoric ester.

II. *Oxidoreduction of the hexosephosphoric ester.*



III. *Hydrolysis of the triosephosphoric ester.*



IV. *Final oxidoreductions.*

a. $CH_2OH—CHOH—CHO \rightarrow\!\!\!\!\rightarrow CH_2OH—CHOH—C\begin{matrix}H\\OH\\OH\end{matrix} \rightarrow\!\!\!\!\rightarrow$

$\rightarrow\!\!\!\!\rightarrow CH_2—CHOH—C\begin{matrix}H\\OH\end{matrix} \rightarrow\!\!\!\!\rightarrow CH_3—COH—C\begin{matrix}H\\OH\end{matrix} \rightarrow\!\!\!\!\rightarrow$

$\rightarrow\!\!\!\!\rightarrow CH_3—CO—C\begin{matrix}H\\OH\\OH\end{matrix}$

methylglyoxalhydrate

b. $CH_3—CO—C\begin{matrix}H\\OH\\OH\end{matrix} + CH_3—CHO$ (in general: acceptor) $\rightarrow\!\!\!\!\rightarrow$
acetaldehyde

$\rightarrow\!\!\!\!\rightarrow CH_3—CO—COOH + CH_3—CH_2OH$ (in general:
pyruvic acid **aethyl alcohol** hydrogenated acceptor)

c. $CH_3—CO—COOH \rightarrow\!\!\!\!\rightarrow CH_3—CHO + CO_2$
carbonic acid

The compounds printed in italics will be formed exclusivily in "cell-free" fermentation ; all the other compounds mentioned in the scheme are normal intermediate products in the fermentation caused by living yeast cells.

As for the normally occurring reactions two different types may be distinguished. The majority of the reactions may be characterised as oxidoreductions ; the reactions I and III however belong to the group of esterforming and estersplitting processes. This implies in all probability that in alcoholic fermentation only two catalytic agents are acting viz. an oxidoreducase and a phosphatase (= phosphatese) [1].

In the living yeast cell a harmonious succession of two reactions proceeding under the influence of the different catalytic agents will be assured. Thus phosphate bound in reaction I will be quantitativily freed by reaction III and will be ready to start a new cycle.

As soon as the catalytic agents will be separated from the cell one may expect a partial disturbance of this harmony and an accumulation of intermediate products at these critical stages will result.

A closer examination of the scheme leads to the insight that the phenomena typical for the "cell-free" fermentation fully correspond to this expectation.

---

[1]) It is interesting to note that a quite similar conception was given as early as 1909 by L. IWANOFF. This assumption was however quite overtaken by the modern trend of thought in enzymology.

The first case of transference of an intermediate product from one agent to the other is met with when the primary product of phosphorylation formed under the influence of the phosphatese (reaction I) must be split under normal conditions by the oxidoreducase according to reaction II. Any falling back of the reducase in this direction will result in an accumulation of the primary product of reaction. This will then be stabilised as ROBISON's hexose *mono*phosphoric ester. Thus a part of the phosphate will be withdrawn from the normal cycle of fermentation.

The second case of transference of an intermediate product from one agent to the other is offered when the triosephosphoric ester formed under the influence of the oxidoreducase will have to be split into triose and phosphoric acid by the phosphatase according to reaction III. Any shortcoming of the latter agent will primarily lead to an accumulation of the triosephosphoric ester. On the basis of the arguments given in our previous communication a continued contact of this ester with the oxidoreducase will result in a condensation of the ester to hexose *bi*phosphoric ester. This too means a deviation of a part of the phosphate from the normal cycle.

However another part of the phosphate will continue its normal course in "cell-free" fermentation as well and start a new cycle. Thus during the phosphorylation period the fermentation will proceed with increased rate as long as the total quantity of added phosphate has not yet arrived at the side-ways mentioned [1]).

It needs no further explanation that the results obtained with the maceration juices of different origin are in excellent agreement with the above considerations. If in the process of isolation of the catalytic agents from the cells one of the agents has been more damaged than the other, one may expect that in some instances one stabilised product will prevail, in other instances the other. Furthermore it may logically be derived from the following that a decrease of the concentration of "active zymase" in a fermentation medium will give a higher yield of ROBISON's *mono* ester. A more pronounced disturbance of the co-operation of both agents will cause in first instance slower transference of the intermediate products. This implies for the primary product of hexose dissimilation an increased chance for stabilisation in the form of ROBISON's *mono*-ester. As the intermediatily formed triose phosphoric ester has no chance of stabilising as such, this ester will as yet — may be retarded — remain subject to the action of the phosphatase. This process will even be favoured, in comparison with what occurs in media with more concentrated zymase, because the greater dilution will promote the escape of the triose phosphoric ester from the sphere of action of the oxidoreducase which tends to bring about a condensation of this ester to hexose *bi*phosphoric ester.

---

[1]) The rate of fermentation at the end of the phosphorylation period will thus be conditioned by the rate at which phosphate is split off from the stabilised esters present.

§ 4. *Quantitative examination of the scheme adopted and of other theories concerning the chemistry of the dissimilation of the hexoses.*

It seemed desirable to look out for a quantitative experimental test of the scheme adopted and of the other existing theories concerning the chemistry of the dissimilation of hexoses. This being possible may be derived from the following very condensed survey of the views of other investigators.

1°. *The classical theory of* HARDEN *and* YOUNG. As has been observed more than once in our previous communications this theory can be summarized by the following equations :

$$2\,C_6H_{12}O_6 + 2\,PO_4HR_2 = 2\,CO_2 + 2\,C_2H_6O + \\ + C_6H_{10}O_4\,(PO_4R_2)_2 + 2\,H_2O \quad . \quad (I)$$

$$C_6H_{10}O_4\,(PO_4R_2)_2 + 2\,H_2O = C_6H_{12}O_6 + 2\,PO_4HR_2 \quad . \quad . \quad (II).$$

Although it is difficult to grant any reality in chemical direction to these symbols, it must be acknowledged that the requirements in quantitative respect, drawn up by the authors some 20 years ago for the course of "cell-free" fermentation, are duly laid down in these equations. At the one hand it is expressed clearly that at the end of the phosphorylation period the whole of the inorganic phosphate may be recovered as hexose *bi*phosphoric ester, at the other hand the production of one molecule of alcohol and of one molecule of carbonic acid is inevitably bound up with the conversion of one molecule of inorganic phosphate into the *bi*phosphoric ester.

It is obvious that the simple fact of the appearance of a hexose *mono*-phosphoric acid demonstrates the inadequacy of the formulation mentioned above. As long as the amount of this ester is small in comparison with that of the *bi*phosphoric ester it is easy to understand that there will be a tendency to ascribe the formation of the *mono*phosphoric ester to secundary factors.

2°. *The modified view of* HARDEN *and* HENLEY [1]). In a recent publication these authors have tried to reconcile the new points of view with the classical theory. In the first place they are inclined to ascribe the appearance of the hexose *mono*phosphoric ester partly to a direct esterification of the hexose, a process occurring independently from the main process. In the second place the authors are willing to accept the possibility that part of the *mono*phosphoric ester will be NEUBERG's ester and thus will originate from a hydrolysis of the *bi*phosphoric ester.

As for the first point it must be observed that then in the cases studied by us, in which the value for Vm was generally very high (till 16), the by-process has grown into the main process. Anyhow it will be clear, that

[1]) A. HARDEN and F. R. HENLEY, Biochem. J. **21**, 1216, (1927).

in the supposition made the requirement CO_2/esterified P = 1 is no longer in need of fulfilment, but that the requirement CO_2/biphosph. ester = 2 still must be maintained.

As for the second point experiments have shown conclusivily that the hydrolysis of the *bi*phosphoric ester during the phosphorylation period is so small that only small deviations of the ratio CO_2/biphosphoric ester can be accounted for in this way. Moreover NEUBERG and LEIBOWITZ (l.c.) succeeded in identifying a *mono*phosphoric ester produced under similar conditions for no less than 90 % as ROBISON's ester.

Thus even in accepting the view of HARDEN and HENLEY the requirement may be maintained that the ratio CO_2/biphosphoric ester will be equal to, or only slightly larger than, 2.

3°. *The theory of* MEYERHOF. It was already observed in our previous paper that medio 1926 the above author drew up a new representation of the chemistry of the first phases of the dissimilation of the hexoses. Soon after this MEYERHOF [1]) gave the following formulation of his views :

$$2\,C_6H_{12}O_6 + 2\,H_3PO_4 = 2\,C_6H_{11}O_5\,(H_2PO_4)^* \text{ (*active)} + 2\,H_2O =$$
$$2\,C_3H_6O_3 + C_6H_{10}O_4\,(H_2PO_4)_2 + 2\,H_2O.$$

At this occasion MEYERHOF expressed the opinion — which was taken over in our scheme — that ROBISON's *mono*phosphoric ester was a stabilised form of the primary reaction product of glucose.

It is obvious that this mode of view in comparison with that of HARDEN and YOUNG has the advantage that arbitrary quantities of *mono*phosphoric ester can be formed along with the *bi*phosphoric ester. Thus there is no necessity for a fixed proportion CO_2/esterified P. In contrary to this it must be observed that according to MEYERHOF's scheme too in alcoholic fermentation — where the $C_3H_6O_3$ of the equation will be split quantitativily into CO_2 and C_2H_6O — the proportion CO_2/biphosphoric ester must be equal to 2.

4°. *The theory of* VON EULER *and* MYRBÄCK [2]). In recent time the latter authors have advanced a new view concerning the first phases of the process under consideration. The most essential part of their theory is doubtless that the primary reaction of hexose dissimilation "eine Reaktion darstellt die von derselben Natur ist wie die Aldehyddismutation" [3]).

The principal argument raised by the Swedish investigators is to be found in their observations that firstly the co-enzyme of alcoholic fermentation acts in a phase preceding that of the phosphorylation and that secondly this very principle is identical with the "co-mutase", a principle indispensable in the mutation of aldehydes by dried yeast preparations.

[1]) O. MEYERHOF, Die Naturwissenschaften, **14**, 1175, (1926).

[2]) C.f. the summary of H. VON EULER, K. MYRBÄCK und R. NILSSON, Ergebnisse der Physiologie, **26**, 531, (1928).

[3]) H. VON EULER und K. MYRBÄCK, Zeitschr. f. physiol. Chemie, **165**, 38, (1927).

It will suffice to refer to our communication on the heterogeneous nature of the principles indicated by various investigators as "co-enzyme" [1]) for explaining why we cannot accept this argumentation as fully convincing. However a closer analysis of the considerations of VON EULER and MYRBÄCK can be regarded as premature, since the recent investigation of NEUBERG and SIMON [2]) has made the identity of "co-enzyme" and "co-mutase" very doubtful. Moreover NEUBERG and SIMON rightly criticize the equalization of a process as the dismutation of aldehydes and of the introductory phase of hexose dissimilation in the scheme of VON EULER and collaborators, since in the last process no oxidoreduction occurs.

For these various reasons a further discussion of the views of VON EULER and MYRBÄCK may be omitted here.

From the preceding survey it results that the three former theories all require the occurrence of a definite ratio between the carbonic acid produced in surplus during the phosphorylation period and either the amount of phosphate esterified or the amount of *bi*phosphoric ester formed.

In contrary to this a reference to § 3 will suffice to show that our mode of view does not bring with it any requirements regarding the ratio of carbonic acid and total phosphate esterified. As for the ratio of carbonic acid and *bi*phosphoric ester formed the only requirement is a minimum value of 2, but much higher values may be reasonably expected.

In Table III a summary is given of the results of a series of experiments

TABLE III.

	Ratio $\frac{\text{monophosph.ester}}{\text{biphosph. ester}}$	Ratio $\frac{\text{carbonic acid}}{\text{esterified P.}}$	Ratio $\frac{\text{carb. acid}}{\text{biphosph. ester}}$
HARDEN and YOUNG	0	1	2
HARDEN and HENLEY	indefinite	< 1	2
MEYERHOF	indefinite	≤ 1	2
KLUYVER and STRUYK	indefinite	$\gtreqless 1$	$\geqq 2$
Experiment 12	16.4	0.60	11.4
„ 13	8.4	0.71	7.5
„ 14	2.4	0.72	3.2
„ 16	0.34	0.89	2.2
„ 17	3.6	0.67	4.0
„ 18	2.1	0.71	2.9
„ 19	0.93	0.82	2.4

[1]) These Proceedings **30**, 569, (1927); Biochem. Z. **201**, 212, (1928).

[2]) C. NEUBERG und E. SIMON, Biochem. Z. **199**, 232, (1928).

which aimed at testing whether the requirements derived from the different theories did hold good. To render the results more easily accessible the meant requirements are put at the head of the table.

For the details of the method of determination of the figures given in Table III we refer to the thesis of STRUYK. It must be kept in mind however that the figures of the carbonic acid produced in the phosphorylation period have been subject to a post-experimental correction as the changes of the concentration of hydrogen ions during the process had been neglected.

Even when taking into account the small inaccuracy of the results owing to this circumstance, there can be no doubt that in many cases the ratio between carbonic acid produced in surplus and the *bi*phosphoric ester formed is considerably larger than 2. In addition attention may be drawn to the fact that also in the paper of HARDEN and HENLEY instances may be found where this ratio exceeds 2. In one case even a value as high as 3.45 was found, a result which HARDEN and HENLEY themselves characterize as inexplicable [1]).

These results are quite incompatible with the different theories advanced by the various investigators. Now we are quite aware that the foregoing does not yield a direct proof for the correctness of our scheme, but we want to emphasize here that our mode of view is up till now the only one which is in perfect agreement with all observations made on the chemistry of "cell-free" fermentation.

Moreover it may be observed that our theory is the only one which brings a logical nexus between "cell-free" fermentation and the fermentation caused by living yeast cells. For in the latter case the ratio of carbonic acid produced and the amount of phosphate esterified — which equals zero — is infinite large, a possibility anticipated in our scheme and in the requirements derived from it.

§ 5. *Final remarks.*

Although the preceding paragraphs only deal with observations on the chemistry of alcoholic fermentation of the hexoses there can be no doubt — taking into consideration the experiences of EMBDEN and MEYERHOF — that the considerations given regarding the first phases of this process apply as well to the first phases of the dissimilation of hexoses by the muscular tissue of animals. The same opinion is expressed in many of the papers of VON EULER. Moreover it seems highly probable that the same holds good for the dissimilation of hexoses by sugar fermenting bacteria of various groups.

The fact that in all these processes the conversion of intermediately

---

[1]) C.f. l.c. p. 1222. The fact, that HARDEN and HENLEY did not get the high values obtained by us, finds its cause in the circumstance that these authors applied higher concentrations of active zymase (zymin-preparations).

formed triose takes different courses does not alter at all the point of view given.

For these reasons we felt justified to give this publication — as well as the preceding one — the general title chosen.

Finally it may be emphasized that this paper only brings a preliminary rounding off of the views given in the two preceding communications in which many arguments may be found which could not be repeated here. A more ample discussion of all problems concerned is given in the thesis of STRUYK.

From the Laboratory for Microbiology of the Technical University.

Delft, October 1928.