

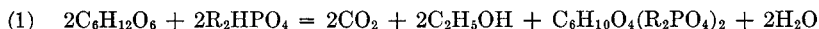
THE ORIGIN OF THE REACTION OF HARDEN AND YOUNG IN CELL-FREE ALCOHOLIC FERMENTATION*

By OTTO MEYERHOF

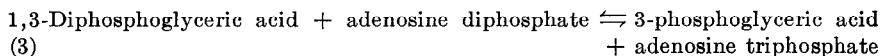
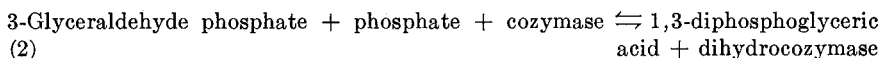
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Equation (1) of Harden and Young (1) states that 1 sugar molecule is fermented to CO₂ and alcohol, while a 2nd one is esterified to hexose diphosphate (HDP).



The reason for this chemical balance was found in the "coupling reaction" of fermentation (Meyerhof, Ohlmeyer, and Möhle (2)). The coupling itself is caused by the intermediary formation of a 1,3-diphosphoglyceric acid and the following transphosphorylation with the adenylic system (Warburg and Christian (3), Negelein and Brömel (4)).



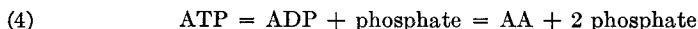
Since adenosine triphosphate (ATP)¹ transfers its labile phosphate to glucose, for every mole of triose phosphate oxidized 1 mole of inorganic phosphate is taken up by the fermenting system. Besides this uptake, an internal cycle exists for the second phosphate group, which is already present in the triose phosphate and after oxidation appears finally in the phosphopyruvic acid. This phosphate is also transferred to glucose or hexose monophosphate and reappears therefore in a new molecule of triose phosphate. This internal cycle does not add new phosphate to the system in the stationary state and can therefore be omitted from the discussion.

However, another aspect of the reaction of Harden and Young is less obvious. In cell-free fermentation two phases follow each other. First there is a rapid phase, "phosphate period," for which equation (1) applies;

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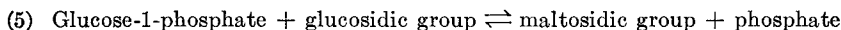
¹ Throughout this paper the following abbreviations are used: HDP for hexose diphosphate, ATP for adenosine triphosphate, ADP for adenosine diphosphate, AA for adenylic acid, and apyrase for adenylypyrophosphatase. In conformity with Kalekar (5), I use the name adenylypyrophosphate for ATP and ADP, and apyrase for those dephosphorylating enzymes, which do not distinguish between both, while ATP-ase and ADP-ase may be used for the one-step enzymes.

then a second slower phase, when either the inorganic phosphate or the free sugar is exhausted, dependent on which compound was added in excess at the start. During this period the fermentation rate is controlled by the fermentation of HDP (6). In the absence of a phosphate acceptor ATP, resulting from reaction (3), can be split only by means of the enzyme adenylypyrophosphatase (apyrase).



Since the highest rate obtained in cell-free sugar fermentation may approach the fermentation rate obtained with an equivalent amount of living yeast (7), and since the rate of fermentation of HDP is generally only a tenth of this or less, one may attribute the origin of the two phases to a destruction of the greater part of the apyrase of living yeast during the procedures of drying, macerating, and extracting. The apyrase would be the most sensitive and least extractable of all enzymes of fermentation.

This hypothesis, made many years ago by the author (8), was never strictly proved and while it has met with some approval (Harden (9), Lynen (10)), others have disagreed. I will not deal here with proponents of different pathways of sugar breakdown in living and non-living yeast, either non-phosphorylating fermentation in the former (Nord (11)) or a partial phosphorylation (Nilsson (12)). More serious objections could be raised against the theory of "apyrase destruction" from other observations. The complicated way in which HDP in the presence of arsenate loses its phosphate groups in the absence of apyrase (Meyerhof and Junowicz-Kocholaty (13)), simultaneously maintaining the high rate of sugar fermentation, could favor the idea that an analogous way may exist in the living cell, and that it could be destroyed by disorganization or by the various procedures for obtaining the yeast preparations. Or, one may assume the presence of a special labile enzyme in the living yeast which splits off the carboxyl phosphate without any interference of phosphate transfer (*cf.* (14) for splitting off acetyl and succinyl phosphate). Finally in the living cell transphosphorylation may be connected with the formation of higher carbohydrates and the phosphate may be split off by a reaction similar to the Cori reaction (15).



It appeared worth while to settle this matter experimentally.

The investigation was extended in two directions: (1) a study of the behavior of apyrase of yeast and its distribution between the liquid and solid components of the cell in connection with the fermentation rate of HDP, as well as the means for preserving and separating this enzyme; (2) addition of apyrase of foreign origin to fermenting yeast preparations. Such an addition, if made in sufficient amount, should raise the rate of

fermentation of HDP to the level of fermentation of free sugar, or to the "arsenate level."

Both series have led to complete verification of the hypothesis that destruction of apyrase is responsible for the two phases of cell-free fermentation.

Methods

Yeast preparations were made from Schmidt's brewers' yeast.²

For destruction of the yeast a modified Peirce magnetostriction oscillator with a frequency of about 8900 cycles per second, constructed and described by Chambers and Flosdorf (16), was used.³ The apparatus has a cooling system of running water. 6 gm. of well washed, fresh yeast in 16 cc. of distilled water (or 13 cc. of distilled water + 3 cc. of 0.1 N glutathione) were exposed to sonic vibrations for 90 to 100 minutes. About three-fourths of the yeast cells were destroyed in this period of time. Cell débris was removed by centrifugation in an angle centrifuge in the cool room and the cloudy supernatant solution used.

Frozen yeast was prepared by keeping lumps of yeast in liquid air for a period of 12 to 15 hours. After melting, the mass was either diluted with water and used as such or it was centrifuged, the supernatant liquid separated, and the cell fragments washed several times with ice water. Juice and fragments served for different experiments.

ATP was prepared from rabbit muscles by B. D. Polis and myself,⁴ according to Kerr's procedure (17), which gives somewhat higher yields than that of Lohmann (18). We contented ourselves with one reprecipitation of the barium salt in weakly acid solution. The air-dried salt had a total P content of 10 to 11 per cent, 63 to 67 per cent of which was 7 minute P.

Fermentation was measured with Warburg's manometric technique. Flasks of 35 cc. capacity were used with two side bulbs. Sugar or HDP was introduced in one bulb and special reagents, such as potato apyrase, in the other. The gas space was usually filled with air. If sugar is fermented at pH 6, there is practically no retention of CO₂. But this is different with HDP, in which, with the splitting off of phosphate, basic equivalents are developed (Meyerhof and Suranyi (19)). The retention of CO₂ amounts to 1.24 and all measured values of CO₂ formation must be multiplied by this factor.

Apyrase from potatoes was recently described by Kalckar (5). He puri-

² I thank C. Schmidt and Sons, Inc., Philadelphia, for an ample supply of fresh brewers' yeast.

³ I thank Dr. Leslie A. Chambers of the Johnson Foundation for Medical Physics for permission to use the oscillator, and Dr. J. Conway and Miss J. Clawson for their help.

⁴ Further studies on purification of apyrase will be published by us.

fied the enzyme starting with a potato extract, which was cleared of most of the proteins by 0.6 saturation with ammonium sulfate. Probably owing to differences in the extracting procedure this method failed in my hands. I precipitated a large part of the inert protein by 0.4 saturation (30 gm. of solid salt to 100 cc. of solution). The solution was concentrated after addition of a further amount of solid salt (20 gm. per 100 cc.), until it reached full saturation. The precipitate was dissolved in water, dialyzed for 2 hours, and centrifuged. The solutions used for the manometric experiments contained 5 to 12 mg. of protein per cc., 0.1 cc. of which splits 200 to 300 γ of 7 minute P from ATP in 5 minutes at 30° at pH 6.

Protein content was determined by the biuret method (Robinson and Hogden (20)). For measuring the apyrase activity of the different preparations, the properly diluted enzyme was incubated with ATP, containing 350 to 450 γ of 7 minute P, in 1.4 cc. total volume, and the turnover measured at 30° after 5, 10, and 15 minutes. 0.1 to 0.2 cc. of 0.1 M glutathione was always added, because of its beneficial effect on all kinds of apyrase. For the yeast enzyme, veronal buffer in the optimal range of pH 9 was used; for potato apyrase, succinate-borax at pH 6.

Results

Apyrase from Yeast. Distribution in Connection with Fermentation Rate— Generally the separation of the two phases of sugar fermentation is more distinct with yeast extracts (press juice, maceration juice) than with solid preparations (dried yeast, acetone yeast, frozen yeast, etc.). According to the proposed hypothesis this should result from the unequal distribution of apyrase between the solid elements and the juice of the cell. The structural elements, containing more of the enzyme, would ferment HDP quicker, which would render the transition from the first to the second phase of sugar fermentation less marked.

This is actually the case. A good material for demonstration is yeast frozen in liquid air. When the thawed yeast is centrifuged and the solid elements removed, the juice ferments glucose easily but reacts on HDP extremely slowly. On the other hand, the solid elements washed several times with ice water, unable to ferment alone because of lack of coenzymes, show a high rate of fermentation of HDP after addition of the pure coenzymes or of boiled yeast juice.

The same volume of washed fragments splits ATP 6 times as fast as the juice, as *e.g.* in Table I, Protocol M177, where the HDP fermentation rate of the juice is 19.0 per cent that of the fragments (with boiled juice), the apyrase activity 15.5 per cent (without boiled juice). In Table I a number of experiments are reproduced with different yeast preparations. The mole turnover of HDP, measured by CO₂, and of ATP measured by 7 minute P

split off in a parallel sample under similar conditions, is very nearly the same in all cases.

It follows from the above experiments that apyrase of yeast is an enzyme bound to the cell structure. Supersonic vibration often brings such enzymes into solution; the same is true for apyrase. After 90 to 100 minutes oscillation of yeast, two layers are obtained in an angle centrifuge, a supernatant cloudy solution and a precipitate consisting of cells and débris. This precipitated fraction is rather inactive, nearly all activity being found in the cloudy suspension. But here again it is not the suspended particles,

TABLE I

Fermentation Rate of HDP and Dephosphorylation of ATP by Same Yeast Preparations

Protocol No.	Kind of preparation	Material used	Temperature	Time	HDP fermentation		7 minute P from ATP	Micromole turnover		
								HDP		ATP
								CO ₂	P	P
			°C.	min.	c.mm. CO ₂	split mg. P	mg. P			
M165	Dry yeast	1 cc. total	25	60	155	0.20	0.23	6.9	6.5	7.4
				190	481	0.675	0.60	21.5	21.8	19.5
M177	Frozen in liquid	0.4 cc. washed fragments	22	15			0.44			
				30	460		0.76	20.5		24.5
	air	0.4 cc. juice	22	30	88		0.118	3.9		3.8
M178	" "	0.4 cc. total solution	22	60	532		0.556	23.8		18.0
		0.6 cc. washed fragments	22	60	393		0.444	16.8		14.2
M182	Super-sonic	0.7 cc. total	22	60	372		0.52	16.6		16.8
		0.7 cc. supernatant	22	60	361		0.51	16.1		16.5
M189	" "	0.6 cc. total	22	15	200		0.258	8.9		8.3
		0.6 cc. supernatant	22	15	160		0.251	7.2		8.1

but the solvent which contains the enzymatic activities of fermentation. The particles can be removed by centrifuging at 10,000 R.P.M., with little change in fermenting power. However, since high speed centrifuging was generally not available, the cloudy supernatant was used directly in most of the experiments.

The rate of fermentation of HDP is about 20 per cent of the maximal rate of glucose. The rate of this fermentation agrees with the speed of dephosphorylation of ATP. Often a complete quantitative agreement is obtained (Table I, Protocols M182 and M189).

Some Purification Steps of Dissolved Apyrase from Yeast—The enzyme

dissolved by supersonic treatment is highly unstable. Even if kept in the ice box, the apyrase activity diminishes in some hours to a fraction of the initial activity. Various means were found to stabilize the enzyme in connection with some purification. The beneficial influence of glutathione has already been mentioned. Glutathione was added to the yeast before vibration and again to the test solution, so that the final concentration was about 2×10^{-2} M. The optimum pH is 9, outside the range of most other fermentation enzymes of yeast. By making use of this fact, a clear solution of relatively high activity could be obtained by mixing the supersonic solution with the same amount of 10 per cent bicarbonate, and centrifuging. But the stability of this cleared solution was not very much improved. A better procedure is the addition of ethyl alcohol to a concentration of 10 to 15 per cent. This forms a precipitate of the suspended particles and part of the dissolved protein and leaves the enzyme in solution. With 15 per cent alcohol nearly 50 per cent of the total protein is removed and only 10 per cent of the total activity lost. Adding to the solution a tenth of its volume of a concentrated suspension of $\text{Al}(\text{OH})_3$ and centrifuging remove a further 30 to 40 per cent of the remaining protein.⁵ This solution is now sufficiently stable for use during 24 hours with a small loss of activity. Even if kept for a week in the ice box, only a third of the activity is lost. Such a solution has an activity of $Q_P = 250$ at 30° ,⁶ compared with 50 to 100 for the supersonic solution under optimal conditions, or with 20 to 30 in the pH range of fermentation.

Some Properties of Dissolved Apyrase from Yeast—Apyrase from yeast exhibits the same pH optimum as apyrase from muscle and also an activation by some bivalent cations. But Ca, which strongly activates ATP-ase of myosin (21), has here a depressing effect. Mn, however, activates apyrase of yeast, while Mg seems ineffective. Since the solution already contains the metal components of the yeast, an exact picture of the activating ions cannot be obtained in this way. An especially interesting feature is the high sensitivity to sodium azide, while KCN is ineffective. This inhibition is much stronger at pH 9 than at pH 6, at which the inhibition of hemin catalysis by azide occurs. 2×10^{-4} N azide inhibits about 60 per cent, 1×10^{-4} 40 per cent. The inhibition is not quite the same with different preparations, but is of the same order of magnitude. On the other hand, the inhibition is not complete even with much higher concentrations. Absence of glutathione does not change the inhibition by azide, and changing the concentration of Mn also has no appreciable effect. Since KCN is ineffective, it is improbable that iron is the responsible metal

⁵ Similar steps were used by Kalckar at a higher level of purity for potato apyrase.

⁶ Q_P calculated like Q_{CO_2} (31 γ of P = 22.4 c.mm.), Q = c.mm. turnover per 1 mg. of dry weight in 1 hour.

for the azide inhibition. Neither myosin nor potato apyrase is affected by azide (Table II).

Increase of Fermentation Rate of Hexose Diphosphate to "Arsenate Level" by Addition of Apyrase from Potatoes—If the slow rate of fermentation of HDP compared with sugar is due to a preferential destruction or removal of apyrase in the yeast preparations, then addition of enough apyrase from other sources must raise the fermentation rate to that of sugar or to the arsenate level. In 1934 it was shown (6) that under optimal conditions the arsenate rate (with 2×10^{-3} M arsenate) equals or surpasses the maximum rate of sugar fermentation in the phosphate period. Some time later it was found (22) that addition of creatine together with the enzyme fraction of muscle, containing the transphosphorylase for creatine, raises

TABLE II
Inhibition of Adenylpyrophosphatase from Yeast by Azide

Protocol No.	Purification step	Gluta-thione added	Mn added	Time	Azide	P split	Per cent inhibition
				<i>min.</i>	<i>M</i>	γ	
P449	15% alcohol	+	+	10		148	
		—	+	10		140	
		—	+	10	5.5×10^{-4}	57	59
		—	+	10	5.5×10^{-3}	20	86
P455	Al(OH) ₃	+	+	5		123	
		—	+	5	1.0×10^{-4}	77	38
		—	+	5	1.8×10^{-4}	35	71
		+	+	5	1.8×10^{-4}	40	67
P467	"	+	+	5		127	
		+	—	5		109	
		+	—	5	1.8×10^{-4}	30	72

the speed of fermentation of HDP in maceration juice to the arsenate level. This experiment proves that the turnover of HDP in the presence of a phosphate acceptor conforms to that necessary for a true intermediary of sugar breakdown. Now it will be shown that the same happens in the absence of a stoichiometric amount of phosphate acceptor by a sufficiently high concentration of apyrase. This result was achieved with the help of purified apyrase from potatoes. The advantage of this enzyme compared with others, especially with myosin, is its high stability and concentration, its favorable optimal pH range at 6 (close to the optimum of fermentation), and its easy solubility in water. Kalekar has obtained purified preparations, which split 5 to 7 γ of P in 5 minutes at 30° per microgram of protein. My own less purified preparations with an activity of only 10 per cent of this, are nevertheless, per cc., at least 10 times more active than the most

concentrated solutions of myosin apyrase described in the literature or obtained in this laboratory by Dr. Polis.

When added directly to HDP, this fraction brings about a very slow dephosphorylation, because of contamination with an ordinary phosphatase, but if added to a suitable yeast preparation, it increases enormously the rate of fermentation of HDP, and if added in sufficient amount raises it to

TABLE III
Maximal Rates of Fermentation of Glucose (G.) and HDP (H.)

Protocol No.	Preparation	Temperature	Sugar	Addition	Potato apyrase 10^{-3} units	CO ₂ in 3 min.	Multiple of direct fermenta- tion	Q _{CO₂}
		°C.				<i>c.mm.</i>		
M284	Supersonic, 22 mg. dry weight	25	H.			27		
			"	ATP		27		
			"	AA		56		
			"	ATP	160	96	3.5	
			"	AA	160	84		
M285	" "	27	"	Arsenate		120	4.5	
			G.			54		
			"	Arsenate		96		
			H.	ATP		31		
			"	"	130	134	4.3	130
M287	" "	27	"	Arsenate		143		
			"	"	130	150	4.9	
			"			22		
			"	ATP	310	175	8	158
			"	"	130	125		
M290a	Frozen yeast, total 41 mg. dry weight	29	"	Arsenate		172	8	
			"			81		
M290b	Frozen yeast juice, 39 mg. dry weight	29	"	ATP	240	225	2.8	110
			"	Arsenate		194	2.4	
M290b	Frozen yeast juice, 39 mg. dry weight	29	G.			114		
			"	Arsenate		112		
			H.			11		
			"	ATP	240	160	15	82
"	"	120	112					
"	Arsenate		133	12				

the arsenate level or somewhat above. If added together with arsenate, it has no effect (Table III, Protocol M285). This result is more regularly obtained if some ATP or AA is added at the same time. Addition of ATP alone has not the slightest effect, but in the presence of a large amount of apyrase it gives a marked increase in speed (Fig. 1, Curves II and III). These experiments prove our hypothesis. The results must be expected if the factor which is lacking in the system is apyrase and nothing else.

In this case, addition of ATP alone cannot have an effect because the enzyme is saturated with it. But if a great excess of enzyme is added, the situation is different. Now the enzyme is no longer necessarily saturated by the preexisting amount of adenine nucleotide. It is moreover interesting to note that the highest possible rate obtained with an excess of enzyme is a little more than the arsenate level (Figs. 2 and 3, and Table III). Now another enzymatic system, and not the apyrase, controls the speed.

In order to obtain this result, the added amount of apyrase, measured in terms of 7 minute P split at 30° under optimal conditions, must be about 3 times as high as the amount of HDP fermented at the arsenate level.

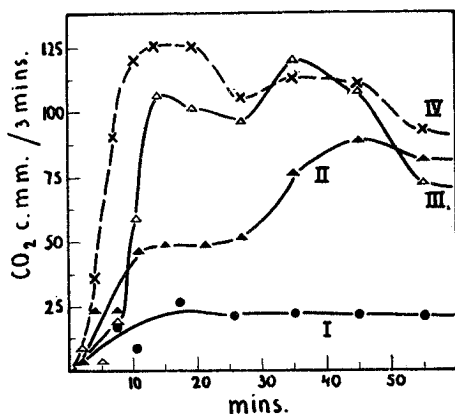


FIG. 1. Rate of fermentation of HDP in supersonic solution (Protocol M285). Curve I, without additions; Curve II, + 0.7 cc. of potato apyrase; Curve III, + 0.7 cc. of potato apyrase + 0.2 cc. of ATP (280 γ of 7 minute P); Curve IV, + arsenate (2.5×10^{-3} M). All samples contain 0.5 cc. of supersonic yeast solution, 0.4 cc. of boiled juice, 0.1 cc. of 0.1 N glutathione, 1×10^{-3} M CaCl_2 , 5×10^{-4} M MnSO_4 , 0.7 cc. of HDP with 3.0 mg. of P, introduced from one side bulb in the beginning; the above additions made 6 minutes later from the other side bulb. Volume of fluid 2.6 cc., 27°.

Besides the somewhat lower temperature of the fermentation experiments (28° and 25°), this apparently is due to a lowered activity of the apyrase in the fermenting mixture. Especially the Ca ion concentration is here suboptimal for potato apyrase, since otherwise it would inhibit the fermentation.

Example—In Protocol M287 (Fig. 2) the maximal speed is 172 c.mm. of CO_2 per 3 minutes or 4.9 mg. of P per hour. The arsenate level is reached by 0.7 cc. of potato apyrase, which splits under optimal conditions 19 mg. of 7 minute P per hour. But with 0.3 cc. of an apyrase solution of similar strength, which splits 8 mg. of 7 minute P per hour, the curve does not reach the arsenate level, but remains at 125 c.mm. of CO_2 per minute. The

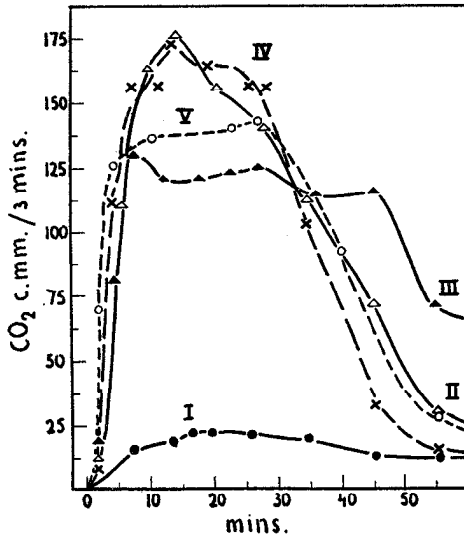


FIG. 2. Rate of fermentation of HDP and glucose in supersonic solution (Protocol M287). Curve I, HDP without additions; Curve II, HDP + 0.7 cc. of potato apyrase + ATP; Curve III, HDP + 0.3 cc. of potato apyrase + ATP; Curve IV, HDP + arsenate (2.5×10^{-3} M); Curve V, glucose (12 mg.) + arsenate. Other components are like those in Fig. 1.

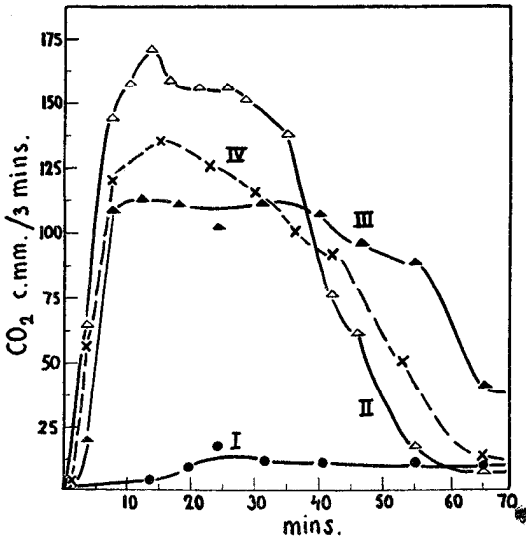
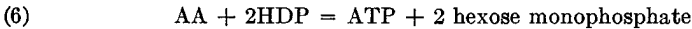


FIG. 3. Rate of fermentation of HDP in juice of frozen yeast (Protocol M290b). Temperature 29°. Curve I, HDP, without additions; Curve II, + 0.6 cc. of potato apyrase + ATP; Curve III, + 0.3 cc. of potato apyrase + ATP; Curve IV, + arsenate. All samples contain 0.6 cc. of yeast juice and the other components as in Fig. 1.

same is true in the other experiments. In Protocol M290 (Fig. 3) the arsenate level corresponds to 136 c.mm. of CO₂ per 3 minutes or 3.76 mg. of P per hour. With potato apyrase splitting 14.2 mg. of 7 minute P per hour, 170 c.mm. of CO₂ per 3 minutes are obtained, a figure appreciably above the arsenate value. However, with half the amount of apyrase, only 116 c.mm. of CO₂ per 3 minutes are obtained, which is below the arsenate level. For comparison the content of apyrase in the solution is expressed in units, 1 unit being the amount of enzyme which splits 1 mg. of P per minute at 30° under optimal conditions (Table III).

While added ATP alone is without effect on the rate of fermentation of HDP, AA, as shown by Ohlmeyer (23), increases the rate because it serves as P acceptor. Its effect corresponds in amount and duration to the phosphorylation of AA to ATP. Simultaneously some of the HDP is split to hexose monophosphate, in the reaction



This reaction, in going to the right, requires energy and is coupled with the oxidation-reduction step of fermentation. But the hexose monophosphate generated in this way has apparently no great influence on the fermentation speed. If potato extract is added together with AA, the system, after complete phosphorylation of the AA, is identical with that formed by addition of ATP and potato extract and behaves similarly (Table III, Protocol M284).

Very suitable preparations to demonstrate these effects are the yeast suspensions after supersonic vibration, cleared from cells and débris by centrifuging, or yeast frozen in liquid air and centrifuged after thawing to obtain the clear juice. With supersonic solutions the arsenate level is about 5 to 8 times the rate of HDP fermentation in the absence of arsenate, and the increase with excess of potato apyrase corresponds to this relation. With frozen yeast, in which most of the yeast apyrase remains bound to the solid particles, the arsenate level of the full suspension (particles + juice) is only 3 times the ordinary rate. But after the solid cell material is removed by centrifuging, the rate of HDP fermentation is very low, and now a spectacular increase of speed of about 15 times occurs with the potato enzyme as well as with arsenate (Fig. 3).

These ratios must not be taken as rigidly fixed. They depend somewhat on the dilution of the extract and on the amount of coenzymes added for "fortifying" the enzymatic system. In the supersonic solutions the yeast was diluted during the vibration and again afterwards, so that the concentration of the soluble substances became one-twentieth of that in the living yeast. Most often some boiled juice was added in the fermentation experiments, but even with this addition the dilution was still about one-twelfth that of living yeast. Addition of cozymase had little effect and was

generally omitted. The absolute speed would be considerably increased if the dilution were less, but it was not advisable to have such high speeds for exact measurements; nor was it feasible to have higher concentrations for effective destruction of the yeast by vibration. Even under these relatively unfavorable conditions the maximal fermentation rate of HDP in the presence of potato apyrase amounts to 40 to 50 per cent of that of free sugar for the same amount of living yeast (Q_{CO_2} about 150 at 28° , instead of 300 to 350 for living yeast). For cell-free juice of frozen yeast the figure is about $Q_{CO_2} = 80$ and for total frozen yeast $Q_{CO_2} = 100$. In

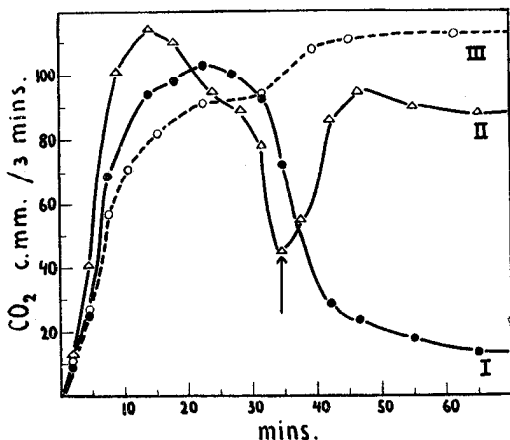


FIG. 4. Rate of fermentation of glucose in juice of frozen yeast (Protocol M290b). Temperature 29° . Curve I, without additions; Curve II, the same, but at arrow (36 minutes) 0.5 cc. of potato apyrase added; Curve III, arsenate (2.5×10^{-3} M). All samples contain 12 mg. of glucose, with HDP (0.2 mg. of P) and 0.5 mg. of acetaldehyde. Other components are like the experiments in Fig. 1. The peak of Curve II comes earlier than that of Curve I and is higher, because the enzyme mixture of Curve II is somewhat more concentrated before the addition of 0.5 cc. of potato extract. Afterwards the concentration of both samples is the same.

all samples some acetaldehyde (about 1 mg.) was introduced together with the HDP to shorten the induction period. For the same reason the glucose solution added contained acetaldehyde as well as a trace of HDP (24).

For ascertaining that transphosphorylation plays no part in the high rate of HDP fermentation, changes of inorganic phosphate were determined simultaneously with CO_2 in several experiments. The ratio, mole of H_3PO_4 split to mole of CO_2 , was always close to 1, with the high turnover in the presence of potato apyrase. A great deviation from this ratio occurs only at a very slow fermentation because of higher carbohydrates (preexisting in yeast juice), which can be phosphorylated by means of the

Cori reaction, thus diminishing the amount of free phosphate. For example, in Protocol M287 (Table III) the microequivalents of phosphate split in 1 hour were 91, 93, and 3, for HDP + potato apyrase, HDP + arsenate, and HDP without addition, while the respective CO₂ microequivalents were 89, 86, and 15.

The high rate of fermentation is maintained until the HDP is nearly exhausted. The amount added was about 90 microequivalents (2.8 mg. of P).

In general it is difficult to carry out the same experiments with glucose instead of HDP. Here, too, it could be presumed that when potato enzyme would be added from the start of the fermentation the system would behave as in the presence of arsenate; so that the maximum rate attained during the phosphate period would be upheld until all glucose is fermented. But apparently on account of the impurities which accompany the potato apyrase this does not happen. The extract interferes considerably during the phosphate period with the fermentation of glucose. Theoretically only half of the amount of apyrase should be sufficient for the maximal fermentation rate of glucose as compared with that of HDP, since for 2 sugar molecules only 2 phosphates have to be removed by apyrase. This expectation cannot be tested for the reasons given above. But if one waits until the peak of the fermentation rate of glucose is passed before introducing the potato extract, then the rate of fermentation rises again to the arsenate level, because in this second period the splitting of HDP controls the rate (Fig. 4).

All these facts show conclusively that the two phases have originated from a relative deficit of apyrase in the dead yeast preparations.

DISCUSSION

The glucose molecule passes anaerobically through twelve stable intermediary stages before becoming alcohol and CO₂; at least three dissociable organic coenzymes, twenty or more enzyme proteins, and some bivalent metals (Mn, Mg) are necessary for the breakdown. Since every single reaction concerned may be varied in speed according to the dilution of the coenzymes and other reactants and to the activity of the enzyme proteins, it is small wonder that many variations occur in yeast preparations in regard to the speed with which glucose and HDP ferment, or in regard to the amount in which the mono- or diesters of sugar accumulate. But in spite of this, the separation of the cell-free fermentation of sugar into two more or less distinct phases of very different speed, the phosphate period and the HDP period are a customary feature.

The most extensive work devoted to this problem is that of Nilsson (12), who, between 1936 and 1942, investigated yeast preparations which either

showed the usual break when half of the sugar was fermented or did not show it (*intakte Trockenhefe*⁷).

In the latter case addition of cytolytic agents, dyestuffs, etc., induced the appearance of this break. Summarizing his numerous papers in a lecture (25) given in Heidelberg at the same place where the modern scheme of sugar fermentation was developed, Nilsson rejected this scheme because of these findings. The break according to this author proves the decomposition of the hexose into one phosphorylated and one unphosphorylated half which ferment in the living yeast with equal speed. After an organizing principle in the living yeast is destroyed, only the unphosphorylated half ferments in yeast preparations, while the other half "abnormally" condenses to HDP.⁸

In the present paper it is shown that this accumulation of HDP is due to a relative deficit of apyrase, which is the most sensitive enzyme of fermentation in yeast and therefore more damaged than the others. All observations of Nilsson are easily explained by the greater or smaller destruction of the yeast apyrase in his different preparations. Of course fermentation of glucose, and HDP in the absence of glucose, cannot be compared quite strictly. Very small differences of the concentrations of coenzymes, of the pH, and of the total salt present can have a great influence on the speed of fermentation. In glucose fermentation the phosphate diminishes, in fermentation of HDP it rises, and the pH changes in both cases differently. Nevertheless, in all systems investigated here, the highest rate obtained with HDP and excess of potato apyrase was at least the same, but mostly higher, than the highest rate obtained with glucose. A better comparison is given by the fermentation of HDP in presence of arsenate, because this fermentation is independent of apyrase. With addition of enough apyrase this arsenate level is always reached or exceeded.

SUMMARY

The two phases of cell-free alcoholic fermentation of yeast, the fast "phosphate period" and the slow "hexose diphosphate period," result from a destruction of the sensitive and structurally bound adenylypyrophosphatase of the living yeast by the drying and extracting procedure.

⁷ A sharp break just after fermentation of half of the sugar is to be expected if, at the time when all sugar has disappeared by fermentation and esterification, inorganic phosphate is still available. If, on the contrary, all of the inorganic phosphate is esterified before the free sugar is exhausted, two breaks occur, one at the time when the phosphate disappears and a second one when the free sugar disappears. Then for two phosphate groups liberated from the ester only one hexose ferments, instead of two. Actually for many reasons, including a partial formation of hexose monophosphate besides diphosphate, the breaks are often rather indistinct; so that from the mere aspect of the curves, which is the sole criterion used by Nilsson, it cannot always be decided what caused the break.

⁸ For further discussion, refer to the author's review (26).

Two series of experiments prove this thesis. (1) In different preparations of yeast the adenylypyrophosphatase remains mainly with the solid elements. Fermentation of hexose diphosphate runs parallel to this distribution and the rate of fermentation agrees closely with the activity of the adenylypyrophosphatase of the same preparation. (2) By addition of concentrated adenylypyrophosphatase of foreign origin (potatoes) to fermenting yeast juice, the fermentation rate of hexose diphosphate is increased to the maximal rate of sugar fermentation, or to or above the "arsenate level."

Some properties of adenylypyrophosphatase of yeast are described. Supersonic vibration brings a large part of the enzyme into solution. The dissolved enzyme can be partially purified, and is stabilized by purification. It is highly sensitive to sodium azide, 2×10^{-4} N inhibits over 50 per cent but it is insensitive to KCN.

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