GLUCOSE METABOLISM OF CLOSTRIDIUM PERFRINGENS: EXISTENCE OF A METALLO-ALDOLASE

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Iron has been demonstrated to play an important role in the metabolism of the genus *Clostridium*. The essentiality of iron is indicated by its influence on growth (Pappenheimer and Shaskan, 1944), on riboflavin production (Hickey, 1945; Rodgers, Henika, and Hanson, 1946), and on toxin production (Tamura *et al.*, 1941; Feeney, Mueller, and Miller, 1943; Bernheimer, 1944). These results, however, leave undefined the site and mechanism of action of iron.

An approach to an understanding of the function of iron in *Clostridium perfringens* was made by Pappenheimer and Shaskan (1944). Growth in complex carbohydrate-containing media yielded the usual mixture of fermentation products: acetic, butyric, and lactic acids, ethanol, hydrogen, and carbon dioxide, as previously reported by Ris (1936) and Friedemann and Kmieciak (1941), whereas growth in iron-deficient media resulted in diversion to a homolactic fermentation. Glucose fermentation by cell suspensions harvested from media containing 0.6 mg or more iron per liter yielded mixed products, whereas cells from media containing 0.1 mg or less iron per liter yielded principally (more than 80 per cent) lactic acid. No growth was obtained in completely iron-free media or in media containing α,α'-dipyridyl, a divalent metal-trapping agent. In addition, glycolysis by cell suspensions from iron-sufficient and iron-deficient media was inhibited by dipyridyl. From these results Pappenheimer and Shaskan (1944) concluded: (1) that a considerable amount of an iron-containing enzyme is required for the mixed fermentation yielding acids and gases, and (2) that iron is involved in some step in glucose dissimilation to lactic acid, as indicated by the dipyridyl inhibition of the glycolysis even of the iron-deficient cells.

Other investigators, using several species of the genus *Clostridium*, have observed an influence of iron on glucose fermentation. Hanson and Rodgers (1946) reported a shift to a predominantly lactic fermentation in iron-deficient *Clostridium acetobutylicum* cultures. Lerner and Pickett (1945) observed an increase in the rate of the alcoholic fermentation by *Clostridium tetani* cell suspensions harvested from deferrized medium to which increasing amounts of iron had been added. Recently Lerner and Mueller (1949) reported that glutamine stimulates glucose fermentation by iron-deficient *C. tetani*.

Attempts to determine the function of iron in glycolysis have been made by the use of metal-complexing agents such as carbon monoxide, cyanide, dipyridyl, etc. Employing cell suspensions of *Clostridium butyricum*, which produce a butyric acid type of fermentation, Kempner (1933), Kempner and Kubowitz (1933), and Kubowitz (1934) observed inhibition of gas (hydrogen and carbon dioxide) production both by carbon monoxide and by cyanide, with a shift to a

lactic fermentation. Simon (1947) was able to inhibit the growth of *C. acetobutylicum* by bubbling a stream of carbon monoxide through a corn mash medium; similar treatment of glycolyzing cell suspensions resulted in a diversion from an acetone-butanol type fermentation to a homolactic fermentation. With *C. tetani* Lerner and Pickett (1945) obtained almost complete inhibition of gas production by α,α'-dipyridyl and 58 per cent inhibition with 0.02 M cyanide, but carbon monoxide did not affect the rate of gas production. Bacon (1949) reported a shift to a lactic fermentation by *C. perfringens* in an atmosphere of carbon monoxide.

These data do not support a clear-cut interpretation of the function of iron in the metabolism of the genus *Clostridium*. The inhibitory action of carbon monoxide and of cyanide upon the fermentation of several members of the genus *Clostridium* suggests the participation of iron-hematin catalysts (Warburg, 1948), whereas the inhibitory action of α,α'-dipyridyl—known to form complexes with inorganic iron but not with hematin iron (Sherman, Elvehjem, and Hart, 1934)—suggests an inorganic iron function. Thus it appears that both forms of iron are involved in clostridial fermentation.

To determine the site of iron function in the fermentation of the genus *Clostridium* an understanding of the intermediary glycolytic steps is required. Although it has been tacitly assumed that a glycolytic system similar to the Embden-Meyerhof scheme exists in these organisms (Prescott and Dunn, 1949; Stephenson, 1949), the actual data are neither abundant nor specific enough to support such a hypothesis. The available information consists of three types: (1) Pett and Wynne (1932) reported the fermentation of fructose diphosphate by toluene-treated, desiccated *C. acetobutylicum*, yielding methylglyoxal and traces of pyruvate; (2) Lerner and Pickett (1945) found complete inhibition of *C. tetani* glycolysis by 0.0001 M monoiodoacetate, suggesting the existence of triosephosphate dehydrogenase; and (3) Stone and Werkman (1937) were unable to isolate phosphoglyceric acid as a product of fructose diphosphate breakdown in the presence of sodium fluoride by *C. butylicum*, *C. histolyticum*, and *C. sporogenes*. These limited data suggest the need for additional experimental evidence before conclusions can be drawn concerning the mechanism of glycolysis in *Clostridium*.

The present study was undertaken to determine the site of action of iron in the glycolysis of iron-deficient *C. perfringens*. By this means the additional function of iron in the gaseous fermentation was eliminated as a complicating factor. Evidence for the existence of a metallo-aldolase is presented, indicating a specific enzymatic site of action for iron. In subsequent reports data will be presented to demonstrate the presence of several other enzymes of the Embden-Meyerhof system in *C. perfringens*.

METHODS

Clostridium perfringens, strain BP6K, from the departmental collection, was maintained by serial transfer in heart infusion broth containing tryptone, 1 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; heart infusion, 50 ml; distilled water, 50 ml. The medium was tubed over desiccated heart particles and sterilized in the auto-

clave at 120 C for 20 minutes. Glucose, sterilized by filtration through sintered glass, was added aseptically to a final concentration of 1 per cent. To obtain active cells for metabolic study, a 1 per cent inoculum of an actively gassing (4 to 8 hours) heart infusion broth culture was employed.

Media. Iron-sufficient cells were grown in medium A: tryptone, 1 g; yeast extract, 1 g; K_2HPO_4, 0.5 g; distilled water, 100 ml; and glucose added aseptically to 1 per cent concentration. This medium contained about 2 mg inorganic iron per liter. A deferrized medium was prepared from medium A as follows: 5 ml of 10 per cent $CaCl_2$ were added per liter, and the medium was brought to boiling, allowed to cool, and filtered through folded paper. The treatment was repeated 4 to 6 times until the iron concentration was less than 0.1 mg per liter. The volume was readjusted with iron-free distilled water, sufficient solid K_2HPO_4 (about 3 g per liter) added to bring the pH to 7.2 to 7.4, and the medium sterilized in iron-free pyrex glassware, fitted with nonabsorbent cotton plugs wrapped in cheesecloth. Sterile glucose was added to 1 per cent concentration. The following salt solution (sterilized at 120 C for 15 minutes) was added at a level of 5 ml per liter: $MgSO_4 \cdot 7H_2O$, 2.0 g; $MnSO_4 \cdot 4H_2O$, 0.05 g; NaCl, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.05 g; $CuSO_4 \cdot 5H_2O$, 0.05 g; $CoSO_4 \cdot 7H_2O$, 0.05 g; iron-free distilled water, 100 ml.

Iron-free pyrex glassware was prepared by treatment with chromic acid solution, followed by rinsing with tap water and finally with iron-free distilled water. Glass containers intended to hold deferrized medium were filled with iron-free distilled water and heated in the autoclave for 30 minutes at 120 C. Pipettes were sterilized in glass containers by dry heat.

Cells suspensions. Metabolically active, iron-sufficient cells (termed *Fe+ cells*) were obtained in medium A after 5 to 6 hours' incubation in a 37 C water bath. The cells were harvested by centrifugation, washed with sterile saline (one-half culture volume), and suspended in distilled water. Iron-deficient cells (termed *Fe− cells*) were obtained by adding 1 per cent inoculum from an actively gassing medium A culture to the deferrized medium and harvesting as for Fe+ cells. The quantity of cells in a given suspension was determined by measuring the turbidity in an Evelyn colorimeter with a 660-mμ filter and converting turbidity to dry weight by means of a previously calibrated curve.

Chemical methods. Iron was determined by the following modification of the method of Snell and Snell (1936): 1.0 ml of sample (less than 5 μg iron) was placed in an 18-mm colorimeter tube containing 1.0 ml of 1 per cent hydroquinone in acetate buffer, pH 4.5 (100 ml 2 per cent CH_3COOH and 100 ml 3 per cent $CH_3COONa \cdot 3H_2O$), and 1.0 ml of 0.5 per cent α,α'-dipyridyl in the same buffer. After standing 1 hour, the mixture was diluted to 5.0 ml with iron-free distilled water and the red color measured in an Evelyn colorimeter with a 515-mμ filter. A standard curve, accurate from 0.1 μg to 5 μg iron, was prepared with $Fe(NH_4)_2(SO_4)_2$.

All pH values reported were determined with a Beckman glass electrode pH meter.

Chemical analyses were performed on Warburg flask contents after the metabolic activity had been stopped by the addition of 0.1 ml 3 N H_2SO_4 per ml flask

contents and the cellular debris removed by centrifugation. Glucose and lactic acid analyses were run on Somogyi (1930) filtrates—glucose by the method of Nelson (1944) and lactic acid by the method of Barker and Summerson (1941).

Inorganic phosphorus was determined by a modified Fiske and SubbaRow (1925) method employing 1 per cent "elon" in 3 per cent $NaHSO_3$ as a reducing agent.

Aldolase activity was determined by the method of Sibley and Lehninger (1949). From the color (optical density) produced in the assay procedure the equivalence of alkali-labile phosphorus formed was calculated—optical density $\times$ 10.8 equals μg alkali-labile phosphorus (figure 1). Aldolase activity was ex-

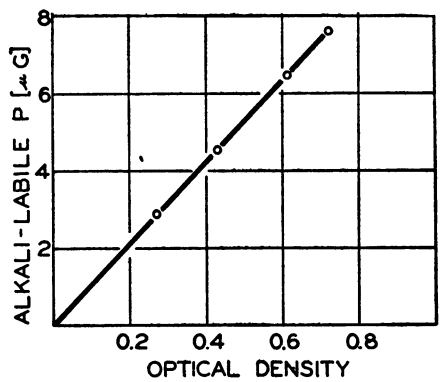

Figure 1. Formation of triose phosphate from HDP: relationship of alkali-labile phosphorus to color produced by 2,4-dinitrophenylhydrazine.

Protocol: 1.50 ml veronal buffer, pH 6.5 (Michaelis, 1931)
0.25 ml 0.56 M hydrazine, pH 6.5
0.10 ml 1:10 dilution of cell-free enzyme extract
H_2O or other additions to 2.25 ml

Equilibrate in 37 C water bath and add 0.25 ml 0.05 M HDP, pH 6.5. Stop reaction in 15 min with 2.0 ml 10 per cent trichloroacetic acid and centrifuge. Transfer 1.0 ml supernatant to colorimeter tube, add 1.0 ml 0.75 N NaOH, incubate 10 min at 25 C. Add 1.0 ml 2,4-dinitrophenylhydrazine reagent (0.1 per cent in 2 N HCl), incubate 10 min at 37 C. Dilute to 10 ml with 0.75 N NaOH and read after 10 min in Evelyn colorimeter using 540 filter against duplicate to which HDP is added after TCA. Optical density $\times$ 10.8 = μg alkali-labile phosphorus.

pressed as the quantity of alkali-labile phosphorus formed per hour per mg protein in a cell-free extract. Protein content was determined by the quantitative biuret method of Robinson and Hogden (1940).

As substrate in the aldolase assay, barium fructose-1,6-diphosphate (HDP), purified by reprecipitation (Neuberg, Lustig, and Rothenberg, 1943, p. 40) and containing only a trace of inorganic phosphorus and about 68 per cent Ba_2-HDP, was used. Barium was removed with sulfate and the HDP concentration adjusted to 0.05 M on the basis of fructose analysis (Roe, 1934).

Manometric methods. Gas measurements were performed at 37 C with the standard Warburg apparatus (Umbreit *et al.*, 1945). Hydrogen and carbon di-

oxide formation during glycolysis was measured in a nitrogen atmosphere using phosphate buffer at pH 6.5. Acid production, usually lactic acid, was followed in $NaHCO_3$:CO_2 buffer at pH 6.5, in an atmosphere of 70 per cent N_2:30 per cent CO_2.

EXPERIMENTAL RESULTS

Influence of iron on growth and glycolysis. As reported by Pappenheimer and Shaskan (1944), only restricted growth of *C. perfringens* occurs in deferrized medium (table 1). Cells from iron-deficient medium were not found, however, to be "elongated, curved and atypical" as described by these workers. More recently Webb (1948) has reported an altered morphology of *C. perfringens* in magnesium-deficient medium. Since in our experiments a mineral salts mixture was added to the $Ca_3(PO_4)_2$ deferrized medium, it seems possible that the atypi-

TABLE 1

Influence of iron content of medium on growth and fermentation of C. perfringens

IRON CONCENTRATION	GROWTH*	GLUCOSE FERMENTATION BY RESTING CELLS†		
		Lactate	H_2	CO_2
mg per L	*optical density*	*μM per 100 μM C_3 fermented*		
<0.1	0.15	89	3	3
0.1	0.27			
0.2	0.31			
0.4	0.36			
0.6	0.38			
1.0	0.41			
2.0	0.41	16	103	91

* Ten ml cultures in 18-mm tubes incubated 6 hr at 37 C; turbidity measured in an Evelyn colorimeter using 660-mμ filter.

† Analyses on Warburg flask contents, containing 10 μM glucose as substrate, after more than 80 per cent was fermented.

cal morphology which they described resulted from deficiencies other than iron in their medium.

As is also shown in table 1, cells harvested from medium containing less than 0.1 mg iron per liter converted more than 90 per cent of the glucose fermented to lactic acid with only traces of H_2 and CO_2, whereas cells from medium containing 2 mg iron per liter yielded less than 20 per cent of the glucose fermented as lactic acid with the quantities of H_2 and CO_2 approximating one mole each per mole of triose fermented. The glycolytic rates of Fe+ and Fe− cells were approximately equal, as indicated by the rate of acid and gas production in $NaHCO_3$:CO_2 buffer. The addition of KCN at 0.001 M to cell suspensions did not inhibit glycolysis nor shift the type of fermentation with either Fe+ or Fe− cells.

Thus, as first demonstrated by Pappenheimer and Shaskan (1944), Fe+ cells produce a gaseous fermentation, whereas Fe− cells show a nongaseous fermentation. These experiments do not, however, indicate whether the higher iron con-

centration during growth is required for the formation, or the action, of the gas-producing enzyme system. The addition of ferrous iron to Fe− cells during glycolysis did not significantly increase gas production, suggesting the absence of the gas-producing enzyme(s). The mechanism of iron action may not be direct; e.g., it is possible that iron in excess of the amount necessary for growth is required for the synthesis of flavins (Rodgers, Henika, and Hanson, 1946), which may be involved in the gas-producing enzyme system.

Iron requirement in the glycolysis of Fe− cells. In addition to the function of iron in the gaseous fermentation of *C. perfringens*, there is evidence for another function(s) that is indispensable to the organism as indicated by two lines of evidence: (1) *C. perfringens* does not grow in completely iron-free medium and (2) growth and fermentation even of Fe− cells are inhibited by trapping agents

TABLE 2

Inhibition and restoration of glycolysis

Per Warburg flask:
2.0 ml 0.017 M $NaHCO_3$
0.3 ml cell suspension (≏ 2 to 3 mg dry wt Fe- cells)
Inhibitor, metal solutions, or water to 2.8 ml
Side arm, 0.2 ml 0.05 M glucose
Atmosphere: 30 per cent CO_2; 70 per cent N_2.
Final pH: 6.5.

	Q_G		
	No additions	With 0.001 M Fe^{++}	With 0. 01 M Co^{++}
Cells	125	145	130
Cells + 0.001 M dipyridyl	0	90	135
Cells + 0.001 M phenanthroline	3	140	165
Cells + 0.01 M pyrophosphate	45	—	—

Q_G = μl CO_2 (H^+) formed per mg dry wt per hour.

such as α,α′-dipyridyl, which complex frees ferrous iron. The site of this function of iron has been studied with Fe− cells, which lack the gas-producing mechanism.

The inhibition of the homolactic glycolysis by Fe− cells with the divalent metal-trapping agents, α,α′-dipyridyl, *o*-phenanthroline, and sodium pyrophosphate, is shown in table 2. The inhibition by dipyridyl and phenanthroline is relieved by Fe^{++} or Co^{++}, but not by Zn^{++}, Cu^{++}, Mg^{++}, Mn^{++}, or ferric iron. Relief of pyrophosphate inhibition could not be studied because the addition of metal ions to pyrophosphate-bicarbonate solutions caused liberation of CO_2, probably due to the hydrolysis of the pyrophosphate.

Glycolysis by Fe− cells was also completely inhibited by 0.001 M Fe^{+++}, Zn^{++}, or Cu^{++}; Ni^{++} inhibited about 60 per cent, whereas Mg^{++} and Mn^{++} caused no significant changes in glycolytic rate. Cysteine (0.01 M and 0.02 M) stimulated the glycolysis slightly.

The results with metal inhibitors indicated a function of ferrous iron, and ap-

parently cobalt, in the glycolytic mechanism. It seemed likely that these ions might serve as activators for an enzyme, or enzymes, in the glycolytic scheme. In view of the report of Warburg and Christian (1943) that yeast aldolase is a metallo-protein, this enzyme seemed a logical one to study. To test this hypothesis a cell-free extract containing aldolase was prepared from *C. perfringens* and the effect of trapping agents and metal ions on aldolase activity was determined.

Aldolase

Cell-free extracts were prepared by sonic oscillation of heavy cell suspensions in 0.03 M $NaHCO_3$. Approximately 1 g of frozen cell paste was suspended in 50 ml 0.03 M $NaHCO_3$ and subjected to oscillation for 30 minutes in a 9,000-cycle, 50-watt Raytheon magnetostriction oscillator. This treatment ruptured virtually all the cells, as indicated by microscopic examination. The cell debris was removed by centrifugation at 4 C for 15 minutes at 12,000 rpm; the clear cell-free supernatant was collected and either stored in the refrigerator at 4 C or in the "deep freeze" at -20 C.

Properties of aldolase. Using the aldolase assay of Sibley and Lehninger (1949) to measure the conversion of hexose diphosphate to triose phosphates, the cell-free extract was diluted within the range of the color reaction for linear response to enzyme concentration. The time course of activity was found to plot as a straight line from 15 to 60 minutes. The units of aldolase activity—μg alkali-labile phosphorus formed per hour per mg protein—were calculated from the linear portion of the curve for enzyme dilution.

The effect of substrate (HDP) concentration on the reaction rate as indicated by alkali-labile phosphorus (triose phosphate) formation is shown in figure 2. The Michaelis-Menten constant, obtained by inspection of the curve, is approximately 0.001 M. This value agrees with the reports of Herbert *et al.* (1940) for rabbit muscle aldolase and of Sibley and Lehninger (1949) for aldolase from various animal tissues, as well as the value reported by Stumpf (1948) for pea aldolase. However, Dounce and Beyer (1948) reported a constant of 0.009 M for crystalline rabbit muscle aldolase.

The optimum pH for the aldolase activity of *C. perfringens* is about 7.5 (figure 3). For these measurements the reaction mixtures were adjusted to the desired pH values, HDP serving as a buffer. Until recently reports of the optimum pH for the activity of aldolase from most sources have been above this value. The optimum pH for purified rabbit muscle aldolase was reported by Herbert *et al.* (1940) to be about pH 9. In contrast to this, Dounce and Beyer (1948) reported pH 6.7 to be the optimum for crystalline rabbit muscle aldolase. Sibley and Lehninger (1949), using extracts from several animal tissues, reported the optimum for aldolase activity to be pH 8.5 to 9.0. With partially purified pea aldolase, Stumpf (1948) found an optimum of 8.5. Recently, however, Baranowski and Niederland (1949) have redetermined the optimum pH for crystalline rabbit aldolase activity and reported a value of 7.0 with barely noticeable diminution of activity at pH 6.5 or 7.5. The higher pH optima previously reported were attributed to a less effective binding by cyanide, used to trap the triose

phosphates, at pH values below 9, so that only part of the aldolase activity was measured at the lower pH values.

Effect of metal-binding agents and metals on aldolase activity. In order to determine whether a metallic ion, such as Fe^{++} or Co^{++}, was required for aldolase activity, cell-free extracts were tested at pH 6.5 in the absence and presence of ferrous iron inhibitors (table 3). The results with aldolase were similar to those on the over-all glycolytic rate, i.e., dipyridyl, phenanthroline, and pyrophosphate

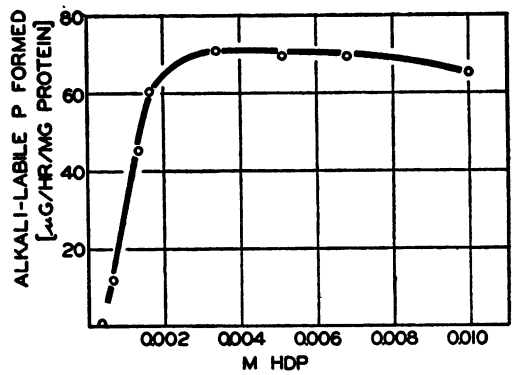

Figure 2. Effect of substrate (HDP) concentration on adolase activity.

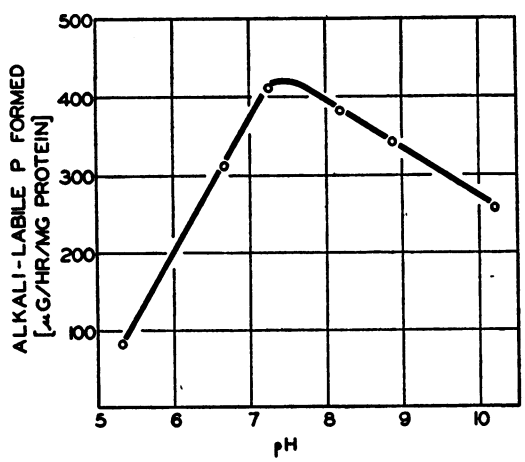

Figure 3. Effect of pH on aldolase activity.

inhibit aldolase activity and the inhibition is relieved by Fe^{++} and Co^{++}. The addition of Fe^{+++}, Zn^{++}, Cu^{++}, Mg^{++}, and Mn^{++} did not relieve the inhibition due to dipyridyl. In the case of pyrophosphate, relief with Fe^{++} or Co^{++} was not satisfactory. Warburg and Christian (1943) also found that pyrophosphate-inhibited yeast aldolase was not reactivated by the addition of metallic ions unless the ions were added before the pyrophosphate. This phenomenon is attributed to an irreversible binding of the enzyme protein by pyrophosphate, a reaction that requires a few minutes and can be prevented by the simultaneous presence of metallic ions.

In this experiment and those following, aldolase activity was assayed at pH 6.5 and not at the optimum pH of about 7.5 for the following reasons: (1) dipyridyl and phenanthroline are more active at acid reaction—apparently less dissociation of the complex occurs (Warburg and Christian, 1943); (2) in glycolytic measurements with live cells, the effects of inhibitors and metals were determined at pH 6.5; and (3) ferrous iron is converted to ferric iron less rapidly

TABLE 3

Inhibition and restoration of aldolase activity

	TRIOSE PHOSPHATE FORMED (μG ALKALI-LABILE P/HR/MG PROTEIN)		
	No additions	With 0.005 M Fe^{++}	With 0.005 M Co^{++}
	μg	μg	μg
Enzyme extract	69	238	231
+ 0.005 M dipyridyl	12	103	72
+ 0.005 M phenanthroline	4	120	73
+ 0.01 M pyrophosphate	12	6	27

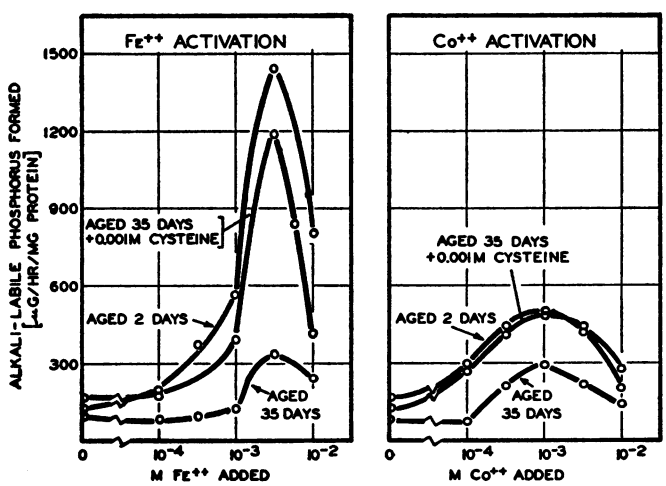

Figure 4 (left). Aldolase activation by ferrous ions.
Figure 5 (right). Aldolase activation by cobaltous ions.

at pH 6.5 than at 7.5, a factor of considerable importance since ferrous iron is an aldolase activator whereas ferric iron is strongly inhibitory (see below).

From table 3 it may also be noted that the addition of Fe^{++} or Co^{++} to the enzyme extract stimulated aldolase activity about fourfold, indicating partial resolution with respect to metal. This situation permitted study of the specificity of various metals.

Aldolase activation by metals and reducing agents. Cell-free aldolase extracts, prepared by sonic oscillation, were activated by ferrous and cobaltous ions (figures 4 and 5). Other metallic ions, tested at concentrations of 0.0005 M and 0.005

M, did not activate aldolase: Fe^{+++}, Cu^{++}, and Ni^{++} caused almost complete inhibition at both concentrations; Zn^{++} did not affect activity at 0.0005 M but inhibited about 70 per cent at 0.005 M; Mn^{++} inhibited about 50 per cent at both concentrations, whereas Mg^{++} did not alter activity.

As the aldolase preparations were aged, activation by the metal component decreased. It was soon found, however, that full activation occurred if a reducing agent was also present. As shown in figure 4, ferrous iron at 0.005 M activated fresh aldolase extracts, or those stored for only 2 days at 4 C, maximally, whereas preparations stored 35 days at 4 C were only slightly activated. However, the addition of 0.001 M cysteine to the aged preparations along with ferrous iron restored the activity to approximately the level found in fresh preparations.

Similar data for cobalt activation are shown in figure 5. The maximum activa-

TABLE 4

Effect of aging on the cysteine and Fe^{++} activation of aldolase

DAYS AGED AT 4 C	TRIOSE PHOSPHATE FORMED (μg ALKALI-LABILE P/HR/MG PROTEIN)			
	Without additions	+ 0.005 M Fe^{++}	+ 0.005 M Fe^{++} + 0.001 M cysteine	+ 0.001 M cysteine
0	300	1,300	—	—
2	150	1,200	—	—
13	115	730	1,225	213
35	100	300	1,400	130
(13-day-old enzyme, aged at 4 C)				
MINUTES AT 37 C	WITHOUT ADDITIONS	+ 0.005 M Fe^{++}	+ 0.005 Fe^{++} + 0.001 M CYSTEINE	+ 0.001 M CYSTEINE
0	115	730	1,230	210
30	23	460	1,050	220
60	13	168	1,030	210

tion by cobalt occurred at 0.001 M. It is noteworthy that the maximum activation by cobalt is only one-third the level obtained with ferrous iron. In both cases fairly sharp optima were found in the metal concentration required for maximum activation—higher concentrations being inhibitory.

The influence of aging cell-free aldolase preparations upon the activators required was followed to study the effect of ferrous iron and reducing agents. As shown in table 4, aldolase activity decayed gradually at 4 C and much more rapidly at 37 C. The degree of activation by ferrous iron decreased at about the same rate, i.e., fresh preparations showed approximately fourfold stimulation, and some of the aged preparations as high as tenfold stimulation, in the presence of 0.005 M Fe^{++}. The addition of cysteine alone maintained the aldolase activity during aging at 37 C but did not, by itself, materially increase the activity. The addition of both cysteine and ferrous iron did, however, restore the aldolase

activity of all preparations to approximately the level found in freshly prepared extracts. Thus, the aldolase of *C. perfringens* requires both a metallic ion and a reducing agent for activity.

The requirement for cysteine is not specific since other substances including glutathione, thioglycolate, and ascorbic acid are of approximately equal effectiveness. The activation by these compounds varies with concentration, apparently depending partially upon their effectiveness as metal-complexing agents. Thus at higher concentrations, 0.01 M or more, some inhibition of aldolase activity is noted. From these data it appears that cysteine serves to maintain essential sulfhydryl groups of aldolase in the reduced state. Lynen and Hoffmann-Walbeck (1948), working with a cell-free aldolase from *Penicillium notatum*, reported a loss in activity in 4 hours by aging at 18 C. The aldolase of *P. notatum* may be similar to the *C. perfringens* enzyme, i.e., the activity of the *P. notatum* enzyme too may be reversed by reducing agents.

The metal requirement for aldolase activation, however, is specific. As previously indicated, of the metals tested, only ferrous and cobaltous ions served as

TABLE 5

Fe^{++}, Co^{++}, and cysteine activation of aldolase

ADDITIONS	TRIOSE PHOSPHATE FORMED (μG ALKALI-LABILE P/HR/MG PROTEIN)	
	Without cysteine	+ 0.001 M cysteine
None	115	210
0.005 M Fe^{++}	730	1,230
0.0005 M Co^{++}	550	506
0.005 M Fe^{++} + 0.0005 M Co^{++}	506	980

activators, and, as indicated in figures 4 and 5, ferrous ions are superior to cobaltous ions as activators. As a further clarification of the nature of the metallic ion activation of aldolase, ferrous and cobaltous ions were added simultaneously to determine whether they fulfill the same or different requirements. The effect of iron and cobalt, singly and in combinations, with and without reducing agent, is shown in table 5. Again ferrous iron gave greater stimulation than cobalt. Furthermore, the addition of both metals did not show an additive effect but reproduced the effect of cobalt. Cysteine did not increase the activation by cobalt, and the simultaneous addition of cobaltous and ferrous ions in the presence of cysteine gave a value intermediate between the ferrous and cobaltous ion activation values. On the basis of these data and those previously shown in figures 4 and 5, it is concluded that the aldolase of *C. perfringens* requires but one metal for activation and that ferrous iron is the natural activating agent. The inhibitory effect of cobalt on the maximum activation obtainable with ferrous iron may result from a competition with ferrous iron for the active site on the enzyme.

DISCUSSION

The inhibition of glycolysis by metal-complexing agents such as dipyridyl, phenanthroline, and pyrophosphate and the absence of any effect by cyanide indicate that the glycolysis of *C. perfringens* requires participation of *free* metallic ions, of which ferrous iron is the most effective. The effect of the metallic ions has been traced to the activation of aldolase, which has been prepared in cell-free but not purified form. Iron appears to be indispensable to the growth of, and to glycolysis by, the organism. A second function of iron, that is, in the gaseous fermentation, also exists, as reported previously by Pappenheimer and Shaskan (1944). Gaseous fermentation, however, requires a considerably larger amount of iron than the aldolase function and appears to be dispensable, since the organism grows satisfactorily, yielding a lactic fermentation when only small amounts of iron are present in the medium. The form and mechanism of the function of iron in gaseous fermentation remain to be elucidated.

The extreme sensitivity of aldolase to ferric iron and the ease with which ferrous iron is converted to ferric iron bring to mind Warburg's suggestion that this effect is a mechanism in the control of the glycolytic rate during aerobic reactions of yeast. In the case of *C. perfringens* the inhibition of aldolase action by ferric iron may be a contributory factor in the inhibitory effect of oxygen on clostridial growth.

The demonstration of the existence of an aldolase in *C. perfringens* is positive evidence for the occurrence of at least a partial Embden-Meyerhof glycolytic scheme, i.e., to the triose stage. The presence of triose phosphate dehydrogenase is further indicated by the reduction of diphosphopyridine nucleotide in the presence of hexose diphosphate as substrate. Evidence to be presented elsewhere has also been obtained for the existence of isomerase and ethanol dehydrogenase in the aldolase-containing extracts of *C. perfringens*. From these observations it is suggested that previous conclusions to the effect that an Embden-Meyerhof system does not exist in clostridia should be re-evaluated.

SUMMARY

The homolactic fermentation of iron-deficient *Clostridium perfringens* is inhibited by α,α'-dipyridyl and *o*-phenanthroline, an effect that can be reversed by ferrous and cobaltous ions but not by Zn^{++}, Cu^{++}, Ni^{++}, Mg^{++}, Mn^{++}, or ferric iron.

Aldolase, the enzyme converting hexose diphosphate to triose phosphate, was prepared in cell-free form from *C. perfringens* and shown to be inhibited by the same agents that inhibit glycolysis. Furthermore the inhibition is reversed by ferrous, and less completely by cobaltous, ions. The cell-free aldolase also required a reducing agent, such as cysteine, for maximum activation.

The essentiality of iron for the function of aldolase offers a possible explanation of the indispensable requirement of iron for clostridial growth and for homolactic fermentation.

The occurrence of aldolase as the key enzyme for the transformation of hexose

diphosphate to triose phosphate suggests the occurrence of the Embden-Meyerhof system in this organism.

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