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A survey of the glycolytic enzyymes of Bordetella pertussis

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A SURVEY OF THE GLYCOLYTIC ENZYMES OF BORDETELLA PERTUSSIS

by

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CHAPTER I

INTRODUCTION AND HISTORICAL

The first substantiated whooping cough epidemic occurred in Paris in 1578 and was described by Guillaume Baillou. In earlier times whooping cough often assumed a more serious character than it does today. More than 120,000 died from it in England and Wales between 1858 and 1865, and during a five year period from 1875 to 1880, 85,000 people died from whooping cough in Prussia (Madsen, 1925). While mortality from this disease has reduced steadily, its relative importance as a cause of death in children under five years of age has increased progressively, and now it is exceeded only by gastro-enteritis, pneumonia and tuberculosis (Burrows, 1959). In Bulgaria, whooping cough, among the so-called "children's infections", is second only to measles in incidence and is first in mortality (Donchev and Stoyanova, 1961).

The etiological agent of whooping cough, Bordetella pertussis, was demonstrated positively, for the first time, in 1906 by Bordet and Gengou at the Pasteur Institute, Brussels, Belgium. This organism had been isolated in 1900, but due to difficulties with the isolation and successful sub-cultivation of B . pertussis upon suitable media, they were unable to report their data until six years later. The first difficulty lay in the primary isolation of a pure culture of the organism from the sputum and exudate of a patient diagnosed as having

whooping cough. It was observed (Bordet and Gengou, 1906) that, in cases favorable for the recovery of the organism, an almost pure culture of the whooping cough bacillus could be obtained from a bronchial exudate, rich in leucocytes, which was eliminated by a cough. However, it was also observed that this held true only at the start of the paroxysmal stage of the disease, and, if the leucocytic exudate was examined on successive days, although the coughs remained numerous and characteristic, the number of specific organisms recovered became visibly less and phagocytosis was found more frequently. The problem of recovering B. pertussis from pharyngeal exudates or swabs was further complicated by the presence of innumerable bacterial rivals which would quickly overgrow the etiological agent on almost all artificial media (Bordet and Gengou, 1906; Lacey, 1954).

In 1900 Bordet and Gengou recovered an organism from the bronchial exudate of a six month old infant, diagnosed as having whooping cough. Since the child had no previous history of any respiratory infections, they believed this organism to be the true causative agent of the disease, and were thus disappointed when they were unable to culture it upon any of the customary media. However, by the time they made a second recovery of these bacilli under similar circumstances, a new medium, the Bordet-Gengou potato extract-blood-agar medium, had been developed which was suitable for le microbe de la coqueluche (Bordet and Gengou, 1906).

As soon as the problem of cultivation of B. pertussis was solved

another problem took its place. Although freshly isolated strains of B. pertussis are immunologically homogeneous, i.e., the virulent form occurs only as a single antigenic type (Leslie and Gardner, 1931; Lawson, 1939; Ungar and Stevens, 1951; Breed et al., 1957), all strains undergo a slow degradation even on blood media. Leslie and Gardner (1931) found four successive stages of degradation in pertussis organisms which they termed Phases I, II, III and IV. Later, Lawson (1939) demonstrated all of these phases except Phase II. It was suggested in 1935 by Toomey et al. and later by Standfast (1951) and Burrows (1959) that the stages should not be arbitrarily limited to phases, but divided instead into broader groups such as "virulent" and "avirulent", corresponding to the smooth-rough dissociation observed in other bacteria. This degradation occurred on most artificial media, but most rapidly on media which do not include blood or a blood substitute.

This need for fresh, defibrinated blood in the culture medium, together with a morphological resemblence of B. pertussis to Pfeiffer's bacillus (Haemophilus influenzae)originally led to the incorporation of the Bordet-Gengou bacillus into the genus Haemophilus. Later discoveries that neither the X nor V factor was required for growth (Breed et al., 1957; Rowatt, 1957(b)), that metabolic differences existed between B. pertussis and the other Haemophilus species (Meyer and Cameron, 1957; Cowan and Steele, 1961) and that there were strong morphological, cultural and immunological similarities between H.

pertussis, Brucella bronchiseptica and the parapertussis bacillus (Ferry and Klix, 1918; Ferry and Noble, 1918; Bradford and Slavin, 1937; Eldering and Kendrick, 1938; Eldering and Kendrick, 1952; Moreno-Lopez, 1952; Proom, 1955) led eventually to the formation of a new genus, Bordetella, which included all three species: B. pertussis, B. parapertussis, and B. bronchiseptica.

The necessity for incorporation of fresh blood in the Bordet-Gengou (B-G) medium was found to be due to an adsorptive rather than a nutritive quality, serum albumin being the most important constituent (Pollock, 1949; Dawson et al., 1950). Blood can be replaced in agar and semi-synthetic fluid media by starch (Hornibrook, 1940; Cohen and Wheeler, 1946; Pollock, 1949; Ungar et al., 1950), charcoal (Ensminger et al., 1934; Ungar et al., 1950; Mishulow, Sharpe and Cohen, 1953; Abe, 1953; Turner, 1961; Holt, 1962), and glass beads or anion exchange resins (Kuwajima, Matsui and Kishigami, 1958). Unsaturated fatty acids have been shown to be highly inhibitory toward B. pertussis (4 µg/ml by Pollock, 1945; 20 µg/ml by Rowatt, 1957(a)) and it was felt that the protective action of these substances lay in their ability to adsorb from the medium an oleic acid-like substance produced by the cells during growth (Pollock, 1949; Kuwajima, Matsui and Kishigami, 1958).

Growth of B. pertussis in liquid media is also inhibited by autoclaved cysteine, sulfides and colloidal sulfur (Rowatt, 1957(a); Rowatt, 1957(b); Schuhardt et al., 1952). Inhibition due to auto-

claved cysteine may be overcome by Seitz filtration, addition of serum albumin or the substitution of glutathione for cysteine in the medium. The action of another growth inhibitor of B. pertussis, a peroxide-like substance, may be counteracted or overcome by the addition of certain substances to the medium, such as red blood cells, haematin, cobalt, charcoal, cupric, ferrous and ferric ions, serum fractions, cysteine and several surface-active agents (Gilder and Granick, 1948; Rowatt, 1957(a); Barry et al., 1956).

The difficulties frequently encountered in growing B. pertussis are probably due to the presence of these inhibitors in the medium, since the nutritional requirements of the organism are not elaborate. Apparently the only essential nutrients for this organism are nicotinic acid and amino acids.(Jebb and Tomlinson, 1955; Turner,1961) Nicotinic acid was required by all species of Bordetella (Fukimi et al., 1953; Jebb and Tomlinson, 1955) and appeared to serve the same function as yeast dialysate or cozymase. Apparently, given the nicotinic acid residue, B. pertussis was capable of synthesizing the cozymase molecule (Hornibrook, 1940; James, 1949). Purines, haemin and biotin all enhance growth but were not essential (Jebb and Tomlinson, 1955).

The choice of a sulfur-source for B. pertussis appears to be somewhat limited. It is generally agreed that cystine and cysteine are suitable for this purpose (Fukimi et al., 1953; Abe, 1953; Proom, 1955; Imamura, 1952; Jebb and Tomlinson, 1957) and in some cases it

was felt that they were essential for the growth of the organism (Proom, 1955; Imamura, 1952). Abe (1953) and Jebb and Tomlinson (1957) also found glutathione to be utilizable as a sulfur-source. Reports on other substances are varying and contradictory. S_2O_5 was found satisfactory by Fukimi et al. (1953) and Abe (1953). Abe also found that thioglycollate and Na_2S supported good growth. Imamura (1952) confirmed this for sodium thioglycollate, but obtained only slight growth with sulfide. Jebb and Tomlinson (1957) reported no growth with sodium sulfate, thiosulfate, taurine, sodium hydrosulfate, sulfite, sodium sulfide, homocystine, methionine, mercaptoacetic acid or cysteic acid. Abe reported good growth with methionine, and Imamura (1952) slight growth with both methionine and taurine. Negative results were obtained for methionine by Fukimi et al. (1953) and for taurine by Abe (1953). It would appear to be fairly conclusive that B. pertussis is incapable of utilizing sulfate, most inorganic and many organic sulfur compounds as sources of sulfur.

Amino acids serve as a source for carbon, nitrogen and energy in B. pertussis. The nitrogenous elements in the potato extract of B-G agar appear to be the main source of nutrition for B. pertussis; the extract probably furnishes optimal concentrations of amino acids and peptides for growth (Dawson et al., 1950; Dawson et al., 1951). The major metabolic activity of B. pertussis is the oxidation of amino acids (Rowatt, 1957(b)). Glutamic acid is the amino acid most readily oxidized by resting cell suspensions (Jebb and Tomlinson,

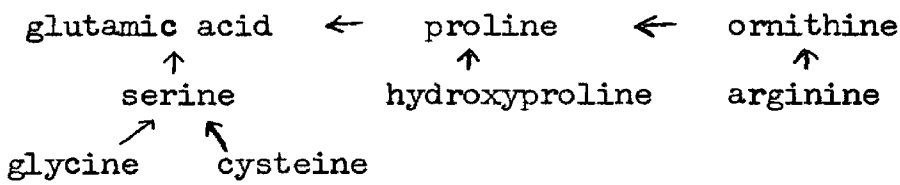
1951; Abe, 1953; Meyer and Cameron, 1957) and best able to support growth in semi-defined media (James, 1949; Dawson et al., 1950; Imamura, 1952; Fukimi et al., 1953; Rowatt, 1955, Rowatt, 1957(a)). Aspartic acid, proline, alanine and serine follow glutamic acid in metabolic importance. Proline, or, to a lesser degree, aspartic acid could replace glutamic acid for growth in the Medium D of Fukimi et al. (1953) but not arginine, alanine, glycine, citrulline or histidine. Imamura (1952) reported satisfactory growth (10-12 billion organisms/ml) in synthetic medium containing only two amino acids, cystine and glutamic acid. The glutamic acid could be replaced in this medium by proline, but not histidine, arginine, glycine or phenylalanine. Glutamic acid, aspartic acid, serine, glycine and proline are completely removed from the medium by both virulent and avirulent strains in 12-15 days, whereas threonine and alanine are only partially used (James, 1949).

Jebb and Tomlinson (1955) found that omission of glycine prevented growth whereas the omission of glutamic acid or cystine restricted growth markedly. They felt that although glutamic acid is not essential for growth, it is the amino acid present in the highest concentrations, is most rapidly oxidized by resting suspensions and is probably a principal energy source. When glutamic acid was replaced with aspartic acid, proline and lactate only 30-60% of the growth attained with glutamic acid was observed.

Inhibition due to valine could be overcome by the addition of

leucine or isoleucine, but preferably by both together. Conversely, methionine, proline and serine were found to be inhibitory singly or in pairs, but especially when all three were together; an inhibition which was overcome by the addition of glycine (Jebb and Tomlinson, 1955).

Since B. pertussis mainly utilizes amino acids for growth, particularly glutamic acid, it is most probable that new substances enter the energy-rich tricarboxylic acid (TCA) cycle in the form of α-ketoglutaric acid. The first step in the oxidation of glutamate by B. pertussis is deamination to α-ketoglutaric acid since it has been demonstrated that cells oxidizing glutamic acid in the presence of arsenate produced α-ketoglutarate.(Jebb and Tomlinson, 1951; Rowatt, 1957(b)). In simple amino acid mixtures, glutamic acid was replaced only by an alternate source of energy, namely α-keto-glutaric acid (Proom, 1955). Fukimi et al. (1953) suggested that the amino acids capable of replacing glutamic acid in media are those which can be converted to it in the metabolic pathway:



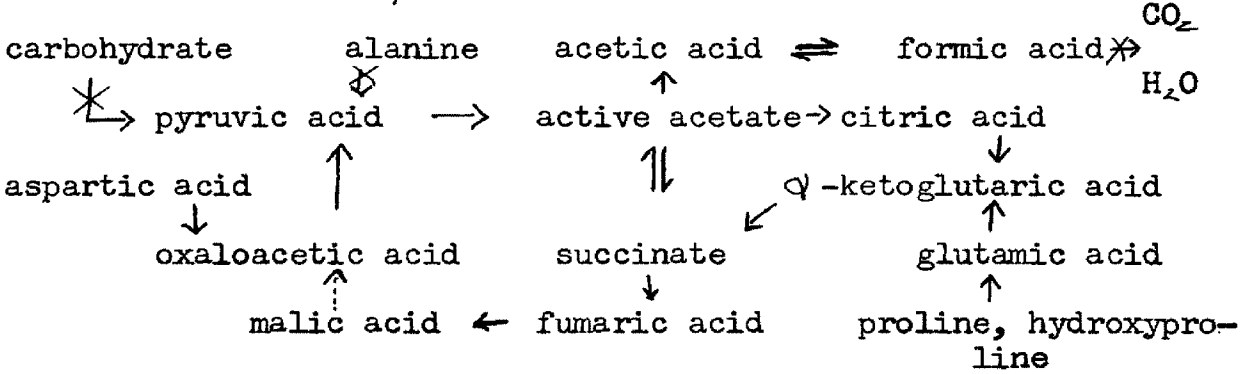
Several workers have demonstrated the probablity of an operative TCA cycle in B. pertussis. Fukimi et al. (1953) reported that pyruvic acid, acetic acid, citric acid, α-ketoglutaric acid, fumaric acid and malic acid were readily oxidized by each member of the pertussis-

parapertussis-bronchiseptica group of organisms. Jebb and Tomlinson (1951) confirmed oxidation with lactate, succinate, α-ketoglutarate and pyruvate, but found no oxidation with oxaloacetate, fumarate, malate, propionate, acetate and formate. Succinic acid, α-keto-glutaric acid, fumaric acid and citric acid were shown by Abe (1953) to be the TCA cycle intermediates most readily oxidized by washed cell suspensions of B. pertussis.

Fukimi et al. (1953) demonstrated the presence of aconitase and the production of α-ketoglutaric acid from citric acid. From his work, he postulated that a full tricarboxylic acid cycle is operative in the genus Bordetella, but he denied the reversal of the glutamate to α-ketoglutarate reaction on the grounds that glutamate is required for growth. Seim (1963) also demonstrated the enzymes of the TCA cycle in cell-free extracts of B. pertussis. Most notable was the demonstration of a fumarase and a diphosphopyridine nucleo-tide-linked malic dehydrogenase activity, since, both Jebb and Tomlinson (1951) and Abe (1953) showed little or no oxidation of these substrates (fumarate and malate) with washed cell suspensions of B. pertussis.

Although B. pertussis possesses a TCA cycle and is capable of metabolizing pyruvic acid, it does not utilize any of the common sugars aerobically or anaerobically (Ungar et al., 1950; Jebb and Tomlinson, 1951; Fukimi et al., 1953; Jebb and Tomlinson, 1955; Abe, 1953; Rowatt, 1957(b); Breed et al., 1957). Jebb and Tomlinson (1951)

found that glucose, maltose, sucrose, lactose and starch were not oxidized or fermented by washed suspensions of B. pertussis nor did any fermentation occur during growth with 1% (w/v) solutions of glucose, fructose, maltose, galactose, lactose, sucrose, glycerol, adonitol, arabinose, rhamnose, mannitol, dulcitol, sorbitol or raffinose. Abe (1953) reported that B. pertussis showed no oxygen uptake with solutions of sucrose, lactose, trehalose, glucose, galactose, rhamnose, inositol, xylose, arabinose, mannitol, sorbitol, dulcitol, inulin or salicin. He did find, however, that a small amount of oxygen consumption occurred with glucosamine. He (Abe, 1953) maintained that a carbohydrate to triose phosphate to pyruvic pathway can not exist in B. pertussis because sugars are not oxidized and alanine did not stimulate growth, and, because of the high oxidation activity of B. pertussis on succinate and α-keto-glutarate, suggested a C_4 metabolic cycle according to the scheme:



In spite of the fact that B. pertussis is remarkably inactive toward carbohydrates and is apparently unable to utilize them either for growth or energy, the organism does contain polysaccharides and

nucleic acids (Eldering, 1942; Overend et al., 1951; Smolens and Vogt, 1953) and must be capable of metabolizing carbohydrates sufficiently for incorporation into the cell substance, although the major metabolic activity of the cell may be the oxidation of amino acids. The question therefore arises as to what pathway is utilized by B. pertussis for the synthesis of these cellular polysaccharides. Although a pathway from external carbohydrates to pyruvic acid might not exist, it is probable that a pyruvic acid to cellular carbohydrate pathway does exist. The Embden-Meyerhof pathway of glycolysis, one of the major mechanisms for the metabolism of carbohydrates in living cells, is capable of operating either toward or from pyruvic acid. In an attempt to further clarify the problem of carbohydrate metabolism in B. pertussis, cell-free extracts of this organism were examined to determine whether or not they possessed all or any of the enzymes of glycolysis.

CHAPTER II

STATEMENT OF PROBLEM

Amino acids are deaminated by Bordetella pertussis and a tricarboxylic acid cycle is presumed to be operative in it. Apart from this, very little work has been done to elucidate the various metabolic pathways of this organism.

Although B. pertussis does not utilize any of the common sugars for energy or growth, it does contain polysaccharides and nucleic acids. It was felt that an examination of cell-free extracts for the enzymes of the Embden-Meyerhof pathway of glycolysis might shed some light upon the possible mechanism of carbohydrate synthesis in B. pertussis.

CHAPTER III

METHODS AND MATERIALS

I. GENERAL METHODS AND MATERIALS

(1) Organisms employed:

Three strains of Bordetella pertussis were used throughout these experiments and 1 strain of Escherichia coli was occasionally used as a control organism.

Laboratory strains #1, #2 and #4 of B. pertussis were kindly donated by Dr. John J. Munoz, formerly Director of the Stella Duncan Memorial Research Institute, Montana State University, and presently at the Rocky Mountain Laboratory, Hamilton, Montana. Strains 1, 2 and 4 were originally designated as strains 2927, 5375L45 and 5373L-2 respectively.

Strain ATCC 9637 of E. coli was used in those experiments requiring a control organism.

(2) Culture media:

For the maintenance of stock cultures, preliminary growth and primary isolation, the blood-potato extract-agar medium described by Bordet and Gengou (1906) still remains the solid medium most suited to the needs of B. pertussis. The Bordet-Gengou (B-G) medium was prepared as follows:

30	g	B-G agar base	Difco
10	g	proteose peptone	Difco
1	l	1% solution of glycerol	Allied Chemicals

Heat to boiling to dissolve completely; dispense in 170 ml lots into 500 ml Erlenmeyer flasks; autoclave 15 minutes at 15 pounds pressure; cool to 45-50°C and add aseptically 30 ml of sterile, defribinated, fresh rabbit blood. Dispense aseptically into sterile, screw-capped, 16 x 125 mm test tubes.

Medium CM-211, a modification of the semi-synthetic fluid medium of Cohen and Wheeler (1946), was used to obtain large cell crops of B. pertussis. It was prepared in the following manner:

Casamino Acids	Bacto tech., Difco	14.0	g
Yeast extract	Difco	1.5	g
Niacin	Nutritional Biochemicals Co.	0.02	g
Glutathione(reduced)	Nutritional Biochemicals Co.	0.01	g
$MgCl_2 \cdot 6H_2O$	Merck, Reagent	0.1	g
KH_2PO_4 (anhyd)	Baker, Reagent	0.5	g
Soluble starch	Fisher, Reagent	1.0	g
$CaCl_2 \cdot 2H_2O$, 1.00% sol.	Fisher, Reagent	1.0	ml
$FeSO_4 \cdot 7H_2O$, 0.50% sol.	Allied Chemicals, Reagent	2.0	ml
$CuSO_4 \cdot 5H_2O$, 0.05% sol.	Mallinckrodt, Reagent	1.0	ml
Distilled water		1.0	l

Adjust pH to 6.8 with 1 N NaOH. Heat the starch in a small amount of distilled water until it is dissolved and clear, then add it to the rest of the medium. Dispense the medium in 200 ml aliquots into 1 liter Blake bottles, then autoclave for 20 minutes at 15 lbs.

(3) Chemicals:

The chemicals employed in these experiments were:

(a) Substrates:

Glucose	anhydrous	Allied Chemicals
Fructose-6-phosphate	Barium	Nutritional Biochemicals
Glucose-6-phosphate	Barium	Sigma
Fructose-1,6-diphosphate	Sodium	Sigma
Glyceraldehyde-3-phosphate	Barium	Nutritional Biochemicals
3-Phosphoglyceric acid	Barium	Nutritional Biochemicals
Phosphoenolpyruvic acid	Barium	Sigma
dl-Malic acid	anhydrous	Nutritional Biochemicals

(b) Purified enzymes:

Glucose-6-phosphate dehydrogenase (Zwischenferment)	Sigma
α-Glycerophosphate dehydrogenase/triosephosphate isomerase	Sigma
α-Glycerophosphate dehydrogenase	Sigma
Triose phosphate isomerase	Sigma
Glyceraldehyde-3-phosphate dehydrogenase	Sigma

(c) Coenzymes, etc.:

Triphosphopyridine nucleotide	(TPN)	Nutritional Biochemicals
Diphosphopyridine nucleotide	(DPN)	Nutritional Biochemicals
Coenzyme I (reduced)	(DPNH)	Nutritional Biochemicals
Adenosine triphosphate	(ATP)	Nutritional Biochemicals
Adenosine diphosphate	(ADP)	Sigma

The insoluble barium ion of the carbohydrate substrates was exchanged for sodium ion by the general method of DeMoss (1955). A certain weight of the substrate (depending upon the total number of μmoles of substrate desired in the final volume of solution) was dissolved in approximately 5 ml of 0.1 N HCl. The barium ion was removed by the addition of saturated Na_2SO_4 solution. The solution was centrifuged to settle the precipitate, and a few more drops of saturated sodium sulfate solution were added to the supernatant fluid to insure the complete removal of barium in the form of insoluble barium sulfate. The supernatant fluid was removed by means of a

capillary pipette and its pH was adjusted to a range of 7.0-8.0 with 0.2 N NaOH. The substrate solution was then diluted to the desired final volume (usually 10 or 20 ml) with distilled water.

(4) Buffer solutions:

A 0.15 M phosphate buffer, pH 7.0, was used for washing the cells and suspending the extracts (Jyssum, 1959), since any of the organic buffers used in the experiments would interfere with the nitrogen determinations of the extracts. This buffer was prepared by mixing 611 ml of a 0.15 M solution of disodium phosphate (Fisher certified reagent, $Na_2HPO_4 \cdot 7H_2O$) with 389 ml of a 0.15 M solution of monopotassium phosphate (Baker analyzed reagent, KH_2PO_4) to make 1 liter of buffer, pH 7.0.

Barbital and Tris buffers, 0.05 M, were prepared according to the method of Gomori (1955).

(a) Barbital buffer

A: 0.2 M solution of sodium barbital (veronal) (41.2 g in 1 l)
B: 0.2 M HCl

50 ml of solution A plus x ml of B, diluted to 200 ml.

x	pH
9.0	8.4
12.7	8.2
17.5	8.0
22.5	7.8

(b) Tris (hydroxymethyl) aminomethane (Tris) buffer

A: 0.2 M solution of Tris (24.2 g in 1 l)
B: 0.2 M HCl

50 ml of solution A plus x ml of B, dilutied to 200 ml.

x	pH
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

Sterile physiological saline solution (8.5 g NaCl in 1000 ml) was used to transfer growth from B-G slants to liquid CM-211 in Blake bottles.

(5) Maintenance of cultures:

Reserves of the stock cultures were kept in the freezer in the lyophilized state. When needed, new vials were opened and the contents transferred aseptically by means of sterile capillary pipettes and sterile saline, to slants or petri dishes containing B-G agar. They were allowed to incubate at 37°C for 3-4 days before a second transfer onto B-G slants was made.

Stock cultures were grown on B-G slants, incubated for 2 days at 37°C, then kept in the refrigerator for a week for subculturing.

Cells for experiments were grown on B-G slants for 2 days at 37°C, then they were transferred in sterile saline to 2 Blake bottles, each of which contained 200 ml of CM-211. The Blake bottles were then incubated for 2 days in a nearly horizontal position on a shaker kept in a warm roon at a temperature of about 35°C. The growth in these was checked for purity by a Gram stain; then a 30 ml inoculation

was made from 1 of these bottles into each of 4 fresh bottles of CM-211. The bottles containing the secondary transfer were incubated for 24 hours on the shaker in the warm room, at the end of which time the cells were harvested.

E. coli stock cultures were kept in the refrigerator on nutrient agar slants until needed. For experiments they were incubated for 24 hours at 37°C, for 2 successive subcultures on nutrient agar; then they were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of nutrient broth. The cells were incubated on the shaker for 24 hours and then harvested in the same manner as the B. pertussis cells.

(6) Cell preparations:

After Gram staining to check for purity of cultures, the contents of the Blake bottles were poured into 250 ml, screw-capped, Nalgene centrifuge bottles and centrifuged in a refrigerated International centrifuge (5°C) at 3500 rpm for 30 minutes.

The harvested cells were washed with 0.15 M phosphate buffer, pH 7.0, transferred to 50 ml celluloid centrifuge tubes and centrifuged at 5000 rpm for 10 minutes in a Super Speed Servall centrifuge which was kept in a cold room. The cells were then washed and centrifuged twice in the same manner.

After the final washing, the crude enzyme preparation was extracted from the cell paste in accordance with the method of Gunsalus (1955). In a chilled mortar, the cell paste, 1 or more parts

of alumina and 5 ml of phosphate buffer were subjected to vigorous hand grinding for 2-5 minutes. The mixture was diluted with more buffer, transferred to stainless steel, 50 ml centrifuge tubes and centrifuged at 13,000 rpm for 20 minutes in a Super Speed Servall centrifuge. This was all done in the cold room. The supernatant fluid containing the crude enzyme preparation was transferred to a test tube and used on the same day it was prepared. Unused extract was stored in screw-capped tubes in the refrigerator for later nitrogen determinations.

(7) Determination of nitrogen:

The nitrogen content of the enzyme preparations was determined by the colorimetric method of Johnson (1941). A sample containing 10 to 40 micrograms of nitrogen was pipetted into an 18 x 150 mm Pyrex test tube. One ml of 2 N H_2SO_4 containing 0.2 g per liter of $CuSeO_3$ was added, and the tube was covered with a stainless steel cap. A glass bead was added to the tube to prevent excessive splattering while the contents were digested overnight in an electrically controlled digestion rack kept at 105°C. To the tube, after digestion, were added in order: 2 ml of water, 2 ml of color reagent and 3 ml of 2 N NaOH. The color reagent contained per liter: 4 g of KI, 4 g of HgI_2, and 1.75 g of gum ghatti. After standing 15 minutes, the tube was placed in a Coleman Junior spectrophotometer and a reading taken at a wavelength of 490 mμ. Blanks and nitrogen

standards were used each time a determination was done, and the nitrogen determinations of the enzyme preparations were always done at least in duplicate.

(8) Spectrophotometric methods:

For all reactions involving a pyridine nucleotide-linked enzyme a Beckman spectrophotometer, Model DB, was used to measure the enzyme activity by the rate at which the reduced form of the coenzyme used appeared or disappeared from the reaction mixture. DPNH and TPNH absorb light strongly at a wavelength of 340 mμ. This wavelength lies in the upper limits of the visible light range and in the lower limits of the ultraviolet range, hence either a tungsten or a hydrogen lamp may be used as a light source in these experiments.

Pyrex cuvettes with a 3 ml volume and a 1 cm light path were used to contain the reaction mixtures and the reaction rates were measured at a wavelength of 340 mμ with a tungsten lamp light source.

II. EXPERIMENTAL METHODS AND MATERIALS

(1) Glucose-6-phosphate dehydrogenase:

This enzyme catalyzes the conversion of glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone and is coupled with the reduction of either TPN or DPN. The method used was that of Jyssum, Borchgrevink and Jyssum (1961).

The reaction mixture contained 100 µmoles barbital buffer, pH 8.0; 0.5 ml extract; 10 µmoles G-6-P; and, the reaction proper was started at zero time with the addition of 0.25 µmole of TPN.

Endogenous (without substrate) and reagent (without extract) blanks were run for each strain. All were read against a blank containing extract, buffer and water to a final volume of 3 ml.

In certain experiments 0.02 ml of purified G-6-P dehydrogenase was added to the reaction mixture, and in one experiment 0.25 µmole of DPN was substituted for the same amount of TPN.

(2) Hexokinase:

Mg ions and ATP are necessary for the hexokinase reaction to proceed (Berger et al., 1946). Hexokinase phosphorylates glucose to G-6-P. To demonstrate this enzyme it was necessary to couple this reaction with the TPN-linked G-6-P reaction and proceed as above (Jyssum, Borchgrevink and Jyssum, 1961).

The reaction mixture contained 100 µmoles barbital buffer, pH 8.0; 0.5 ml cell-free extract; 20 µmoles glucose; 2 µmoles ATP; 5 µmoles $MgCl_2 \cdot 6H_2O$; 0.02 ml G-6-P dehydrogenase from yeast; and, after these reagents were incubated in the cuvette for 5 minutes at room temperature, the reaction proper was started with the addition of 0.25 µmole of TPN. Readings were made every 15 seconds after zero time for from 1 to 5 minutes.

(3) Phosphohexoisomerase:

Two methods were used in an attempt to demonstrate the presence of a phosphohexoisomerase in B. pertussis. The first method (a), as in the case of hexokinase, was coupled with the spectrophotometric demonstration of the formation of G-6-P (Jyssum, Borchgrevink and Jyssum, 1961). The second method (b) was based upon both the method of Racker (1947) for the demonstration of hexokinase and phosphofructokinase and the method of Jyssum, Borchgrevink and Jyssum (1961) for the demonstration of phosphofructokinase and aldolase activity.

(a) This enzyme catalyzes the isomerization of G-6-P to fructose-6-phosphate (F-6-P) and vice versa, resulting in an equilibrium mixture containing 70% of the glucose isomer (Axelrod, 1960(a)).

The reaction mixture contained 100 µmoles barbital buffer, pH 8.0; 20 µmoles F-6-P; 0.5 ml cell-free extract; and 0.02 ml of added G-6-P dehydrogenase. After incubating the reagents for 5 minutes at room temperature, the reaction was started with the addition of 0.25 µmole TPN.

In one experiment the cells were washed and the extract suspended in barbital buffer, pH 7.0, rather than the customary phosphate buffer.

(b) This method is dependent upon the previous demonstration by similar means of both phosphofructokinase and aldolase.

100 µmoles barbital buffer, pH 8.0; 20 µmoles G-6-P; 0.2 ml

cell-free extract; 5 µmoles $MgCl_2 \cdot 6H_2O$; 2 µmoles ATP; 10 µmoles niacin; 3.2 µmoles KCN; and 0.01 ml of a mixture of α-glycerophosphate dehydrogenase and triosephosphate dehydrogenase (GDH/TIM) were incubated for 30 minutes in test tubes in a water bath set at 37°C. At the end of this time, the contents of the tube were transferred to a cuvette and 0.5 µmole of DPNH was added to start the reaction.

(4) Aldolase:

The method chosen for the demonstration of aldolase in B. pertussis was that of Jyssum, Borchgrevink and Jyssum (1961) which couples the aldolase reaction, a cleavage of fructose-1,6-diphosphate (FDP) to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GA-3-P), with the α-glycerophosphate dehydrogenase-DPNH reaction. Cyanide and hydrazine favor the formation of triose phosphate (Axelrod, 1960(a); Taylor, 1951) and cyanide probably inhibits the reoxidation of reduced coenzyme (Jyssum and Borchgrevink, 1960).

In a water bath held at 37°C 100 µmoles barbital buffer, pH 8.0, 0.1 ml cell-free extract, 10 µmoles niacin and 3.2 µmoles KCN were incubated for 7 minutes. Then 20 µmoles FDP and 0.01 ml GDH /TIM were added and the reaction mixture incubated for a further 20 minutes. At the end of this time, the contents were transferred to a Pyrex cuvette and the reaction started by the addition of 0.5 µmole DPNH.

There is an alternative procedure for the demonstration of

aldolase activity (Christian, 1955). However, this method, utilizing the DPN-linked GA-3-P dehydrogenase system, is impracticable with crude extracts, since the presence of triosephosphate isomerase and α-glycerophosphate dehydrogenase in the extract will interfere greatly, the equilibrium between the triose phosphates being far in favor of DHAP and glycerophosphate formation (Racker, 1947; Ling, Byrne and Lardy, 1955).

(5) Phosphofructokinase:

This enzyme also requires $MgCl_2$ and ATP for activity, since it catalyzes the phosphorylation of F-6-P to FDP. The method is the same as method (b) for phosphohexoisomerase except that 20 μmoles of F-6-P are used as substrate instead of an equal amount of G-6-P.

100 μmoles barbital buffer, pH 8.0; 20 μmoles F-6-P; 0.2 ml cell-free extract; 2 μmoles ATP; 5 μmoles $MgCl_2 \cdot 6H_2O$; 10 μmoles niacin; 3.2 μmoles KCN; and 0.01 ml GDH/TIM are incubated in test tubes for 30 minutes in a water bath held at 37°C. The contents are then transferred to a cuvette and the reaction started with the addition of 0.5 μmole DPNH at zero time.

(6) Triose phosphate isomerase:

An adaptation of the method of Jyssum, Borchgrevink and Jyssum (1961) for aldolase was used to demonstrate the presence of this isomerase which establishes an equilibrium between DHAP and GA-3-P.

The mixture contained 100 μmoles barbital buffer, pH 8.0; 0.5 ml cell-free extract; 10 μmoles GA-3-P and 0.001 or 0.0001 ml of a purified α-glycerophosphate dehydrogenase, and the reaction was started with the addition of 0.5 μmole DPNH.

(7) α-glycerophosphate dehydrogenase:

Basically the same method was used to demonstrate this enzyme, which causes the reduction of DHAP to α-glycerophosphate, as was used for the triose phosphate isomerase. However, the purified α-glycerophosphate dehydrogenase was omitted and the system was used either without any added enzyme, or with 0.01 ml of purified triose phosphate isomerase.

(8) Phosphoglycerokinase:

In the presence of ATP and Mg ions this enzyme will catalyze the formation of 1,3-diphosphoglyceric acid from 3-phosphoglyceric acid (PGA). This system is coupled with the enzyme GA-3-P dehydrogenase which, when in the presence of DPNH, will form GA-3-P from 1,3-diPGA.

The reaction mixture contained 75 μmoles barbital buffer, pH 8.0; 5 μmoles $MgCl_2 \cdot 6H_2O$; 5 μmoles glycine; 1μmole ATP; 3.2 μmoles KCN; 0.5 ml cell-free extract; 20 μmoles 3-PGA and 0.02 ml purified GA-3-P dehydrogenase. These reagents were incubated for 7 minutes at room temperature and then the reaction was started by the addition of 0.5 μmole DPNH.

Glycine is used to bind traces of copper which strongly inhibit the activity of GA-3-P dehydrogenase (Christian, 1955).

Both 0.02 and 0.2 ml aliquots of purified GA-3-P dehydrogenase were used in these experiments with no significant differences appearing in the results.

(9) Glyceraldehyde-3-phosphate dehydrogenase:

The method used for the demonstration of this enzyme was the same as that used to demonstrate phosphoglycerokinase, except the addition of the purified enzyme was omitted.

(10) Pyruvate kinase:

This demonstration was based upon the formation of pyruvic acid from phosphoenolpyruvic acid by the enzyme pyruvic kinase. The incubation mixture was a modification of that used by Jyssum, Borchgrevink and Jyssum (1961). It contained 10 μmoles of phosphoenol-pyruvic acid (PEPA); 3 μmoles $MgCl_2 \cdot 6H_2O$; 10 μmoles ADP; 1 ml extract in Tris buffer, pH 7.4 and 10 μmoles KCl. Potassium ion appears to be required for this reaction (Axelrod, 1960 (a)). Reagent and endogenous blanks were made up for each strain and all were brought up to a final volume of 3 ml with distilled water. The tubes were incubated for a suitable length of time (the exact time varied; usually being 1 hour); then the samples, together with 2 or 3 blanks containing 3 ml of 5% acetic acid, were analyzed for pyruvic acid

content by the method of Friedmann (1957).

All tubes were incubated 10 minutes in a water bath held at 25° ± 2° C. At accurately timed intervals (30") 1.0 ml of a 0.1% solution of 2,4-dinitrophenylhydrazine in 2 N HCl was added to each tube. Incubation was continued at 25° C for 5 minutes, at which time 3.0 ml of benzene were added. The contents were mixed immediately for 2 minutes by aeration with a rapid stream of air through a capillary pipette in each tube. The tubes were centrifuged at low speed to facilitate separation of the phases. The benzene phase was transferred to another set of tubes. Six ml of 10% $NaCO_3$ solution were added and mixed as above, and, if necessary, centrifuged. Five ml of the lower carbonate layer were transferred to a colorimeter tube held in the water bath at 25° C. At definite intervals (30") 5 ml of 1.5 N NaOH solution were added in turn to each tube. At the end of 5 minutes the densities were read (having first set the blanks to 100% T) at a wavelength of 435 mμ.

A standard curve of pyruvic acid, with a range from 0.1 μmole to 1.0 μmole of pyruvic acid, was established by this same method.

Friedmann's method is based upon the separate determination of keto acids due to differences in the rates of reaction with 2,4-dinitrophenylhydrazine and the distribution of the hydrazones in the aqueous phase and various solvents. At 25° C the monocarboxylic acids (such as pyruvic acid) react completely with the hydrazine in about 4 minutes; the dicarboxylic acids react within about 20

minutes (Friedmann, 1957).

(11) Enolase and mutase:

Mutase catalyzes the conversion of 3-PGA into 2-PGA and enolase converts this latter substance into phosphoenolpyruvic acid. The activity of these two enzymes was demonstrated by the formation of pyruvic acid from 3-PGA by cell-free extracts of B. pertussis.

The incubation method was almost the same as that for pyruvic kinase, but 40 µmoles of 3-PGA were substituted for the PEPA and 0.4 ml of Tris buffer, pH 7.4 was substituted for the 10 µmoles KCl.

The test for pyruvic acid was the Friedmann method for the determination of α-keto acids.(See above for details).

(12) TPN-linked malic dehydrogenase:

A "malic" enzyme exists, which, in the presence of TPN, converts malic acid to pyruvic acid. A spectrophotometric method (Ochoa, 1955) is used to demonstrate the activity of this enzyme.

The reaction mixture contained 100 µmoles barbital buffer, pH 8.0, 0.5 ml cell-free extract and 20 µmoles dl-malic acid. The reaction proper was started with the addition of 0.5 µmole TPN.

CHAPTER IV

RESULTS

In the experiments involving the use of the Beckman DB spectrophotometer, a unit of enzyme activity was defined as that amount which would cause an initial rate of change in absorbance (ΔA_{340}) of 0.01 per minute under the specified conditions of the experiment at room temperature (Wolff and Kaplan, 1955). The absorbancy change in the 15 to 30 second interval was taken as the initial change and multiplied by 4 to give the change per minute (DeMoss, 1955; Jyssum and Borchgrevink, 1960). This appears to be the most widely used method of recording enzymatic activity. Since most enzyme reactions tend to slow down with time as a result of many factors, it is felt that only the first part of the reaction curve, which is usually a straight line, should be regarded as indicative of the reaction rate (Gray, 1961). However, this method may also give rise to inaccurate representations in certain cases, notably those in which a reagent or endogenous blank shows a change in absorbancy during the 15-30 second interval and, although no further change may occur in the reaction, this ΔA_{340} is multiplied by 4 to give an erroneously high blank.

For those experiments in which the formation of pyruvic acid from PGA or PEPA was taken as the endpoint, a unit of enzyme activity was defined as that amount which caused the formation of 0.10 µmoles

of pyruvic acid in one hour of incubation at 37°C.

In all cases the specific activity is recorded as the number of units per mg nitrogen (N).

(1) Glucose-6-phosphate dehydrogenase:

Extracts of E. coli showed high G-6-P dehydrogenase activity, but extracts of strains 1, 2 and 4 of B. pertussis were not active (Fig. 1). No significant activity was demonstrable when DPN was used as the coenzyme instead of TPN.

All of the strains were tested in the presence of purified G-6-P dehydrogenase since it was felt that the added enzyme might be inhibitory toward this reaction in B. pertussis and toward those reactions which were coupled with it (hexokinase and phosphohexoisomerase). The reaction proceeded under these circumstances, but the activity demonstrated was in no case significantly higher than the reagent blank (Fig. 1).

The nitrogen contents and absorbancy changes for all strains, under all conditions, are recorded in Table I.

(2) Hexokinase:

Since B. pertussis extracts exhibited no G-6-P dehydrogenase activity of their own, it was necessary to add 0.02 ml of purified Zwischenferment in order to demonstrate hexokinase by a spectrophotometric method. Even in the presence of added enzyme, the B. pertussis

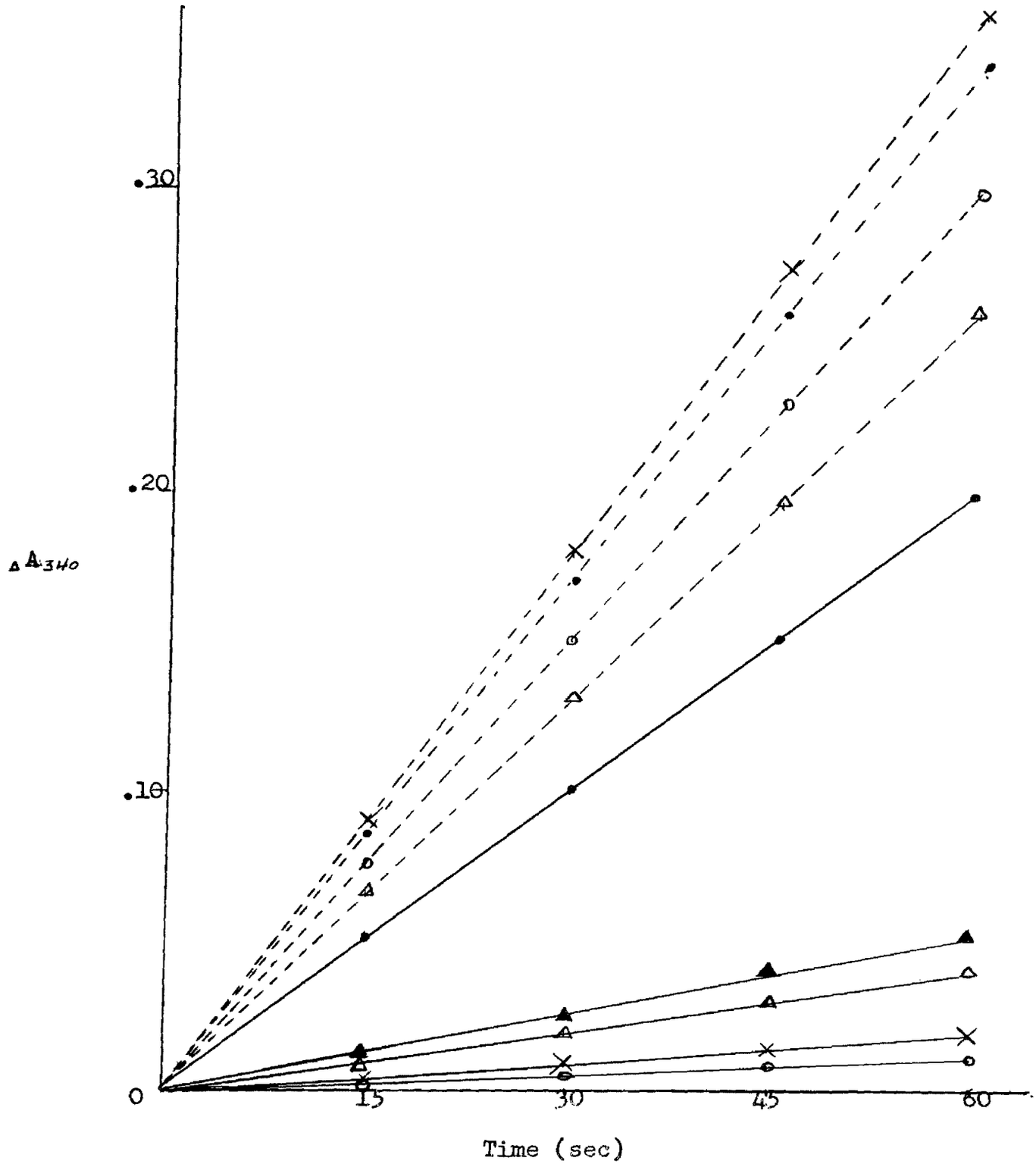


Figure 1. Glucose-6-phosphate dehydrogenase activity in Bordetella pertussis (strains 1,2 and 4) and E. coli extracts with TPN or DPN as coenzyme.

x--x #1 with Zwischenferment(Z) ●--● Reag. with Z o—o #2 & reag without Z
o--o #2 " " ●—● E. coli without Z △—△ #1 & 4 with DPN
△--△ #4 " " x—x #1 & 4 " " ▲—● Endog. " "

Table I. Glucose-6-phosphate dehydrogenase activities of Bordetella pertussis (strains 1,2 and 4) and Escherichia coli extracts.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec.Act U/mg N
E. coli	0.75	0.035	0.14	25	373
E. coli	0.69	0.05	0.20	40	58
E. coli	0.26	0.035	0.14	28	104
#1	1.09	0.005	0.02	0*	-
#2	0.80	0.003	0.012	0*	-
#4	1.05	0.005	0.02	0*	-
Reagent**	-	0.085	0.34	0*	-
#1**	0.75	0.09	0.36	0*	-
#2**	1.01	0.075	0.30	0*	-
#4**	0.73	0.065	0.26	0*	-
#1***	2.50	0.01	0.04	0*	-
#4***	1.35	0.01	0.04	0*	-

* not significantly higher than endogenous or reagent blanks
** plus added glucose-6-phosphate dehydrogenase
*** with DPN as coenzyme instead of TPN

extracts exhibited no activity that was significantly higher than the blanks.

In Figure 2 it can be seen that E. coli extracts, with or without added enzyme possess hexokinase activity, with the G-6-P dehydrogenase apparently the rate limiting factor in the over-all reaction.

The nitrogen content and absorbance changes of all strains, and the specific activities of the E. coli extracts are shown in Table II.

(3) Phosphohexoisomerase:

Due to the contamination of the added G-6-P dehydrogenase with phosphoglucoisomerase, the reagent blanks in this reaction were very high. The relative phosphohexoisomerase activities of the extracts, E. coli and B. pertussis, and the reagent blanks are shown in Figure 3.

It was noted (Table III) that in all cases where the B. pertussis extract was suspended in phosphate buffer the reaction was consistently lower than that of the blank. However, when these extracts were suspended in barbital buffer, the change in absorbance equalled that of the blank.

The results from method (b) for phosphohexoisomerase showed high enzyme activity in the E. coli extracts, while the B. pertussis extracts never exceeded the blanks (Fig. 4) although the nitrogen contents were similarly high in all extracts (Table IV).

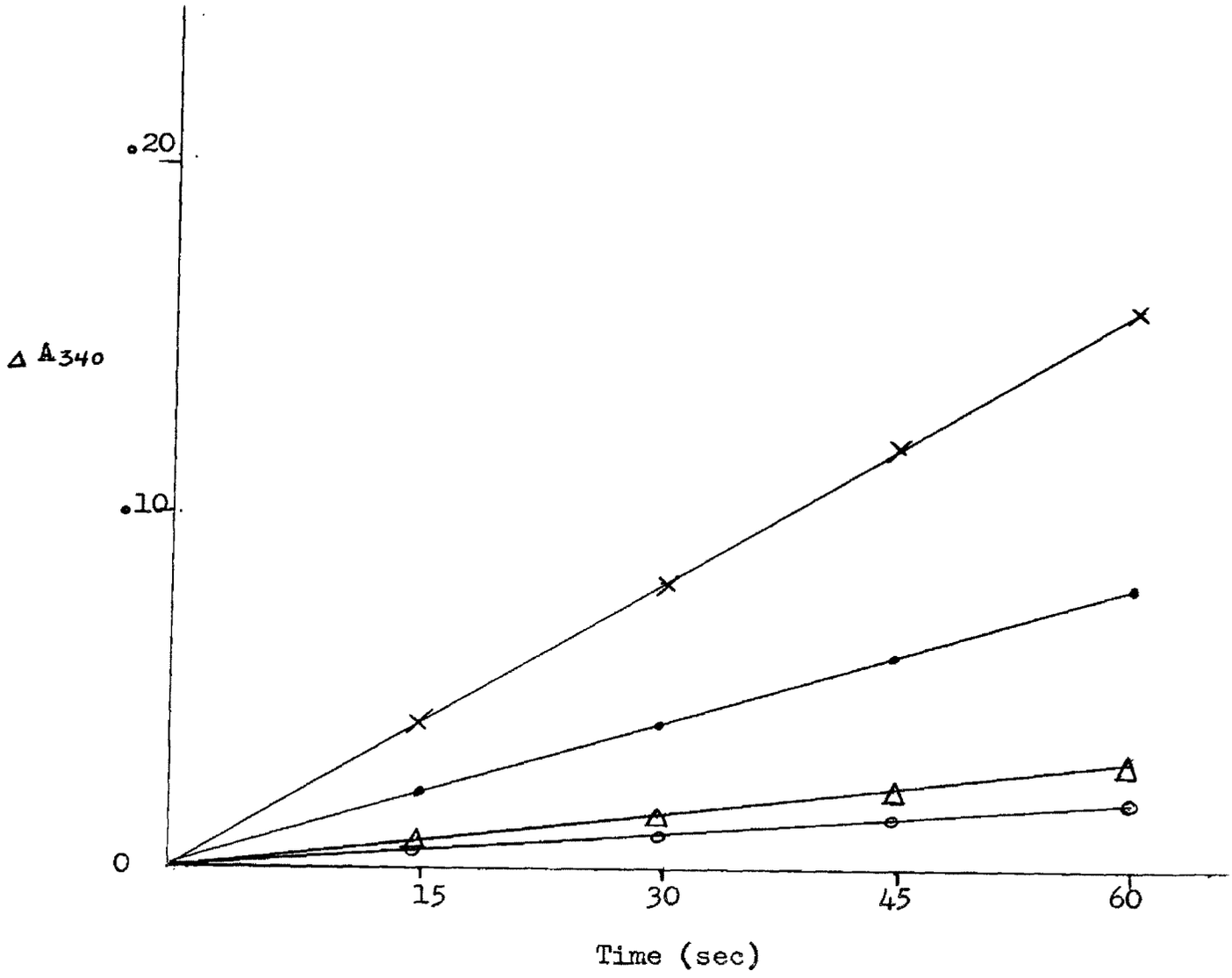


Figure 2. Hexokinase activity of Bordetella pertussis (strains 1,2 and 4) and Escherichia coli extracts.

X—X E. coli with Zwischenferment
•—• E. coli without Zwischenferment
△—△ #1 with Zwischenferment
o—o #2, #4, reagent and endogenous with Zwischenferment

Table II. Hexokinase activities of extracts of Bordetella pertussis (strains 1, 2 and 4) and Escherichia coli.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
E. coli	0.75	0.02	0.08	16	21.3
E. coli	0.69	0.015	0.06	12	17.3
E. coli*	0.41	0.04	0.16	32	78
#1*	0.01	0.008	0.032	0**	-
#2*	0.17	0.005	0.02	0**	-
#4*	0.87	0.005	0.02	0**	-

* plus added glucose-6-phosphate dehydrogenase
** not significantly higher than reagent or endogenous blanks

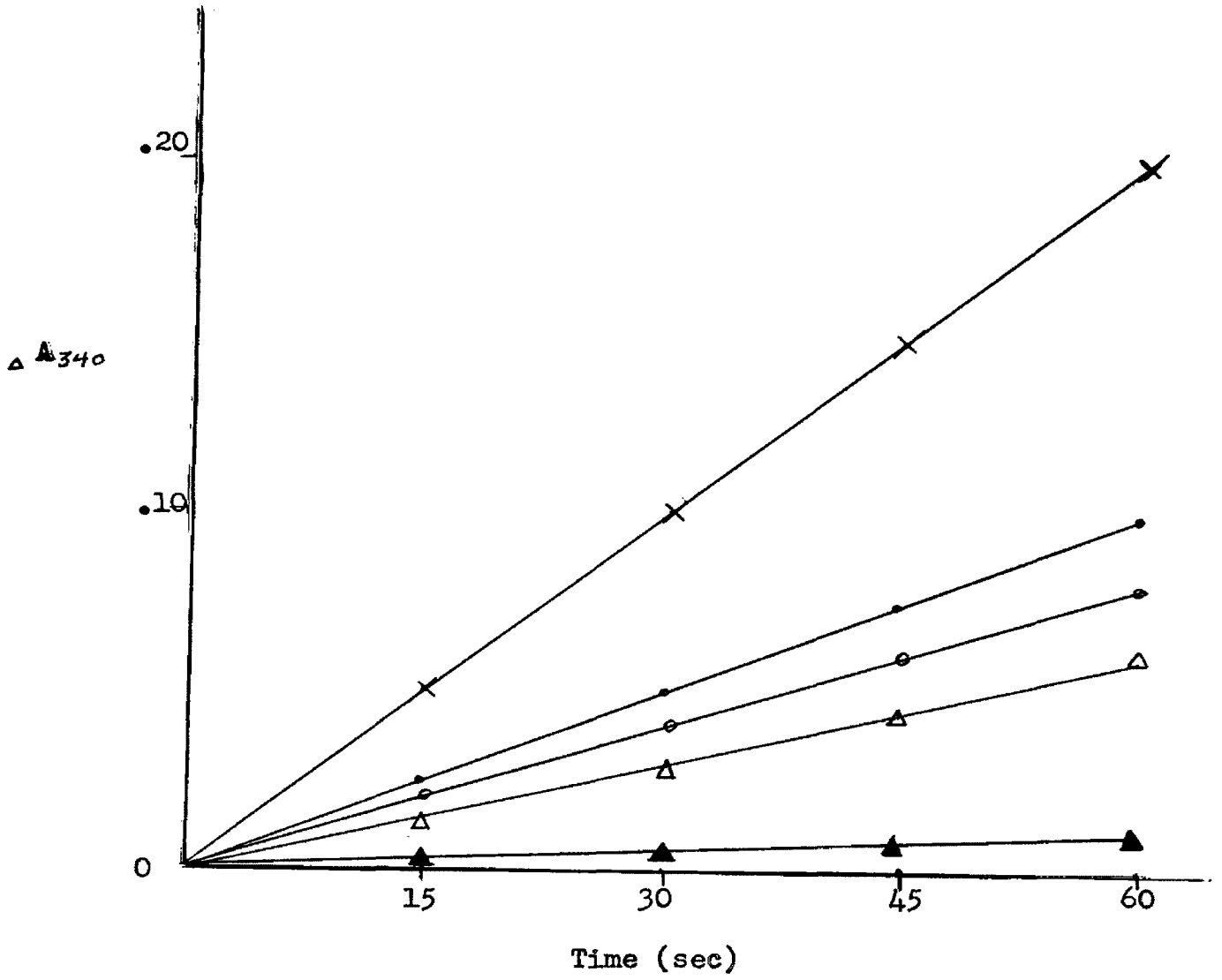


Figure 3. Phosphohexoisomerase activity of Bordetella pertussis (strains 1, 2 and 4) and Escherichia coli extracts in the presence of added Zwischenferment.

X—X E. coli
•—• Reagent average and #1, #2 and #4 in barbital buffer
o—o #1 and #4 in phosphate buffer
Δ—Δ #2 "
▲—▲ Endogenous "

Table III. Phosphohexoisomerase activities of extracts of Bordetella pertussis (strains 1,2 and 4) and Escherichia coli in the presence of added glucose-6-phosphate dehydrogenase.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340} /min	Units/ml***	Spec. Act.*** U/mg N
E. coli	0.42	0.05	0.20	20	47.6
Reagent*	-	0.025	0.10	-	-
#1	0.75	0.02	0.08	-	-
#2	1.01	0.015	0.06	-	-
#4	0.75	0.02	0.08	-	-
#1	0.01	0.02	0.08	-	-
#2	0.26	0.017	0.068	-	-
#4	0.11	0.018	0.072	-	-
#2	0.17	0.01	0.04	-	-
#2	0.10	0.013	0.052	-	-
#4	0.87	0.02	0.08	-	-
#1**	0.61	0.025	0.10	-	-
#2**	0.35	0.025	0.10	-	-
#4**	0.02	0.025	0.10	-	-

* average of 7 runs
** cells washed and extracted with barbital buffer, pH 7.0, instead of the customary phosphate buffer
*** corrected for reagent blank.

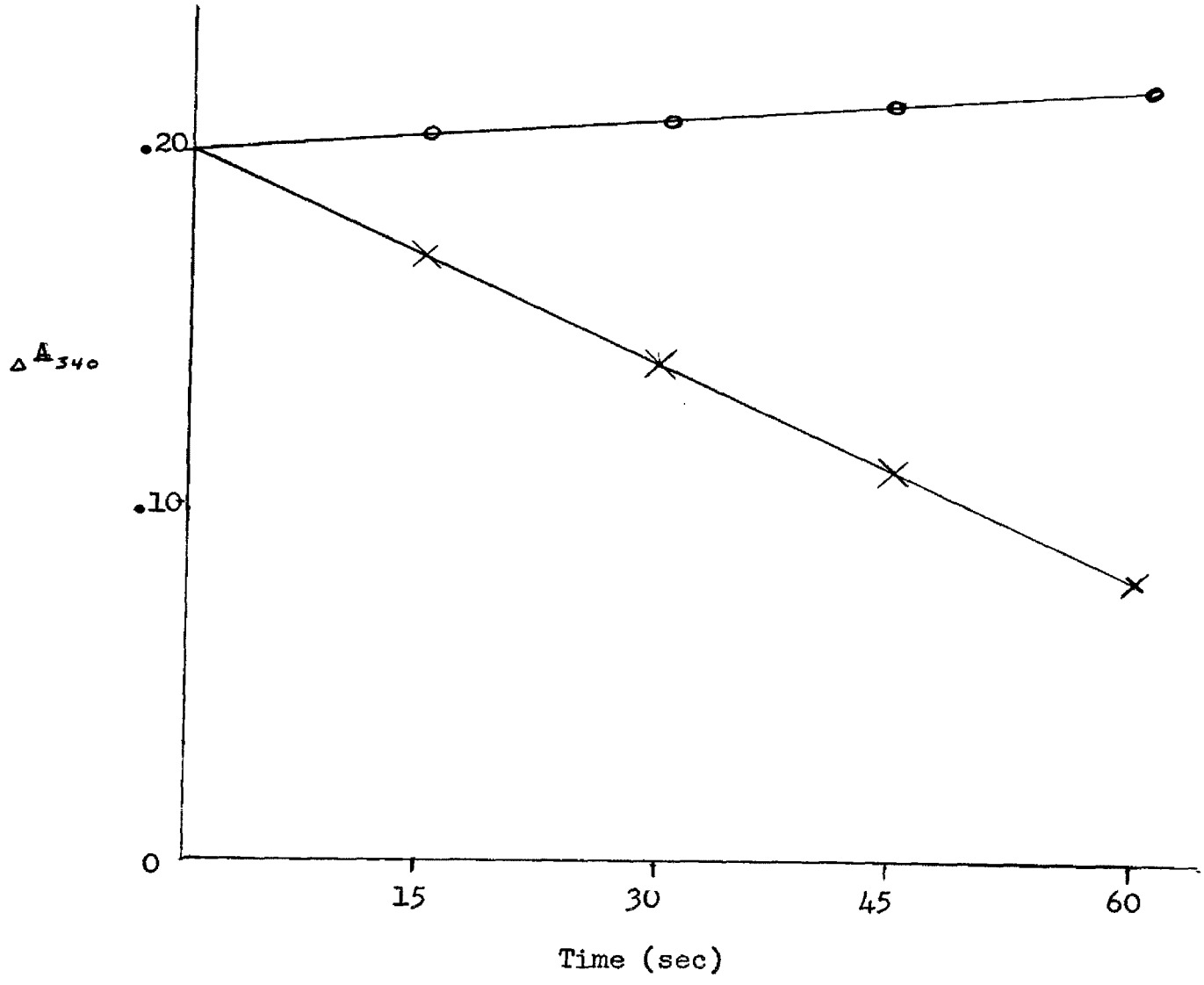


Figure 4. Phosphohexoisomerase activity of extracts of Bordetella pertussis (strains 1, 2 and 4) and Escherichia coli. Coupled with the aldolase-α-glycerophosphate dehydrogenase system.

o—o #1, #2, #4, reagent and endogenous
x—x E. coli

Table IV. Phosphohexoisomerase activities of Bordetella pertussis (strains 1, 2 and 4) and Escherichia coli extracts.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
E. coli	0.80*	0.03	0.12	60	750
#1	1.01	0	0	-	-
#1	1.35	+ 0.005	+ 0.02	-	-
#2	1.00	+ 0.005	+ 0.02	-	-
#4	1.26	+ 0.005	+ 0.02	-	-
#4	0.65	0	0	-	-

* 0.2 ml of a 1:10 dilution of E. coli extract was used in the experiment

(4) Aldolase:

Strains 1, 2 and 4 of B. pertussis all demonstrated aldolase activity (Fig. 5). The specific activities for these strains are recorded in Table V.

(5) Phosphofructokinase:

The relative phosphofructokinase activities of the three strains of B. pertussis employed are shown in Figure 6. All strains exhibited a very high PFK activity (Table VI).

(6) Triose phosphate isomerase:

No evidence was presented to indicate that B. pertussis possessed this enzyme. This was a case where the method for recording enzymatic activity presented a fallacious picture. In Figure 7 it appears that strain #4 of B. pertussis shows some slight isomerase activity, whereas in fact, the final change in absorbance, after six 15-second readings, was less than half that of the reagent blank.

From the high reagent blanks it would appear that the added α-glycerophosphate dehydrogenase was contaminated with triose phosphate isomerase. Experiments were run using both 0.001 ml and 0.0001 ml of the added enzyme. (Table VII).

(7) α-glycerophosphate dehydrogenase:

Figure 8 and Table VIII show that no α-glycerophosphate

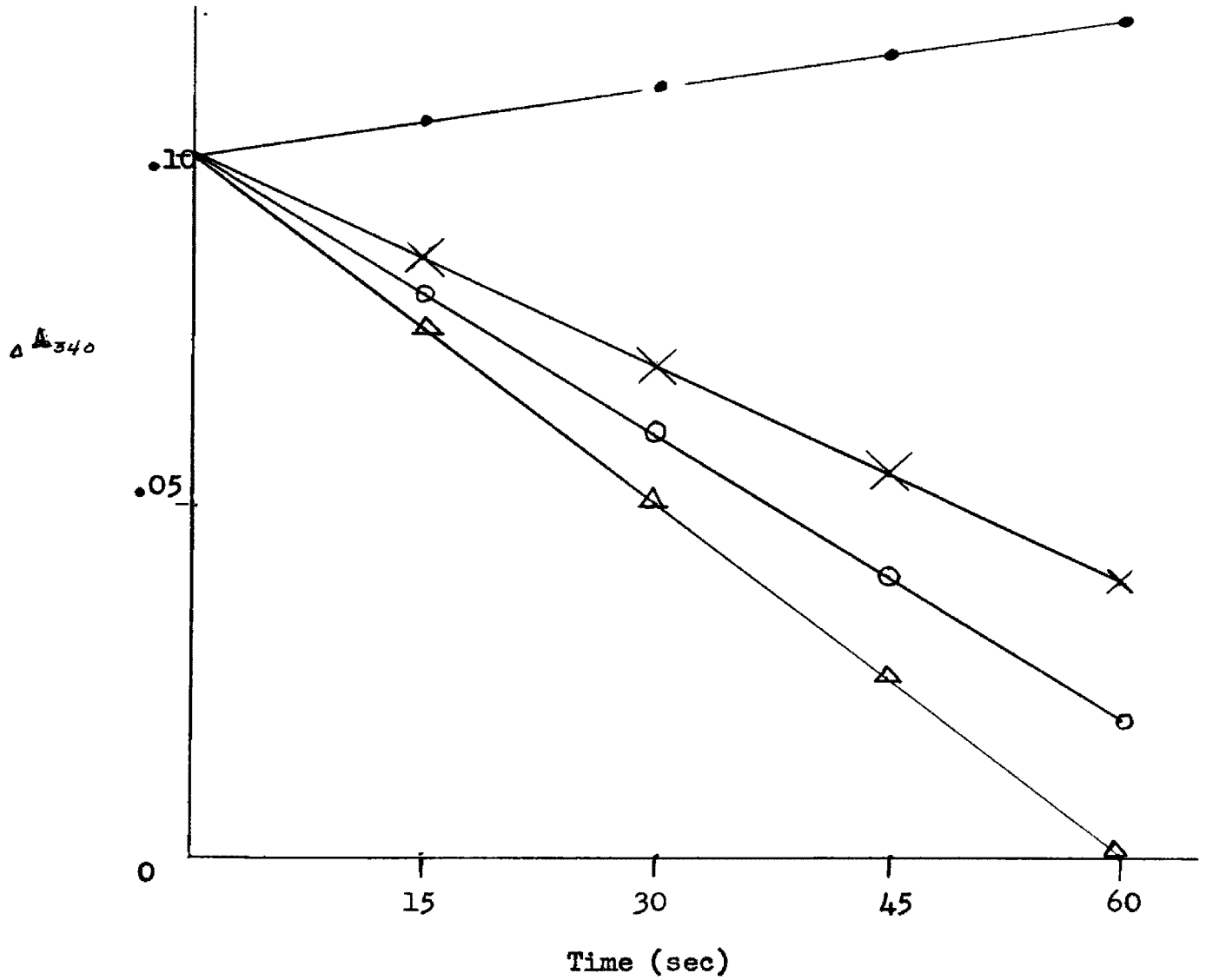


Figure 5. Aldolase activity of extracts of Bordetella pertussis, strains 1, 2 and 4.

- •—• Reagent and endogenous
- x—x #1
- o—o #2
- Δ—Δ #4

Table V. Aldolase activity of Bordetella pertussis extracts.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
#1	0.74	0.015	0.06	60	81
#2	0.75	0.02	0.08	80	106.6
#4	0.77	0.025	0.10	100	129.8
#4	0.44	0.015	0.06	60	136.3
#2*	0.63	0.02	0.08	40	63.5
#1*	0.72	0.02	0.08	40	55.5

* 0.2 ml extract used instead of 0.1 ml.

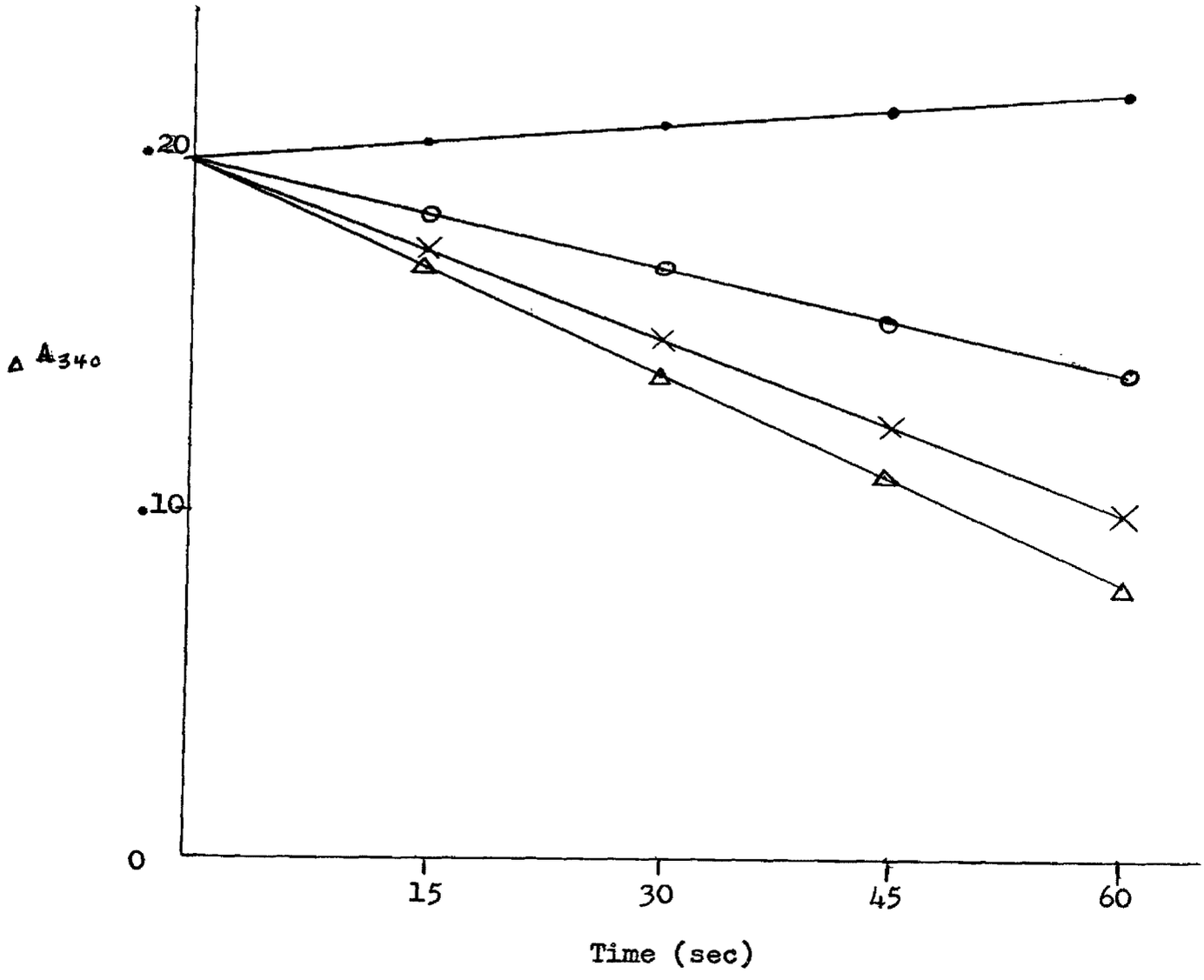


Figure 6. Phosphofructokinase activity of extracts of Bordetella pertussis, strains 1, 2 and 4.

●—● Endogenous and reagent
o—o #2
x—x #1
Δ—Δ #4

Table VI. Phosphofructokinase activity of Bordetella pertussis extracts.

Strain	mg N/ml extract	ΔA_{340} 15-30'	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
#1	1.01	0.025	0.10	50	49.5
#4	0.65	0.03	0.12	60	92.3
#2	0.86	0.015	0.06	30	34.8
#2	0.84	0.02	0.08	40	47.6
#2	1.00	0.055	0.22	110	110.0
#1	2.50*	0.03	0.12	60	240.0
#4	1.35*	0.03	0.12	60	444.0

* 0.2 ml of a 1:10 dilution of extract used.

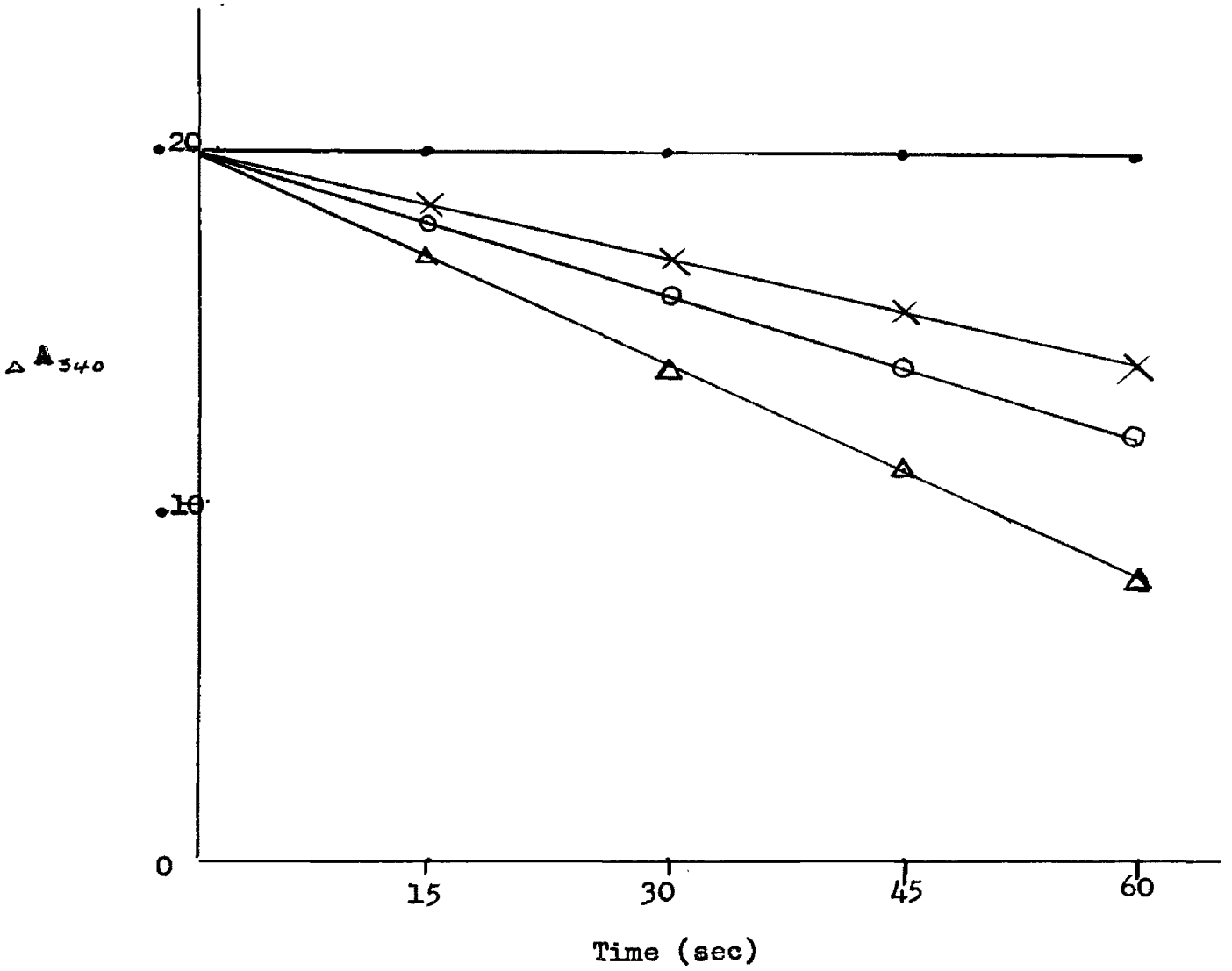


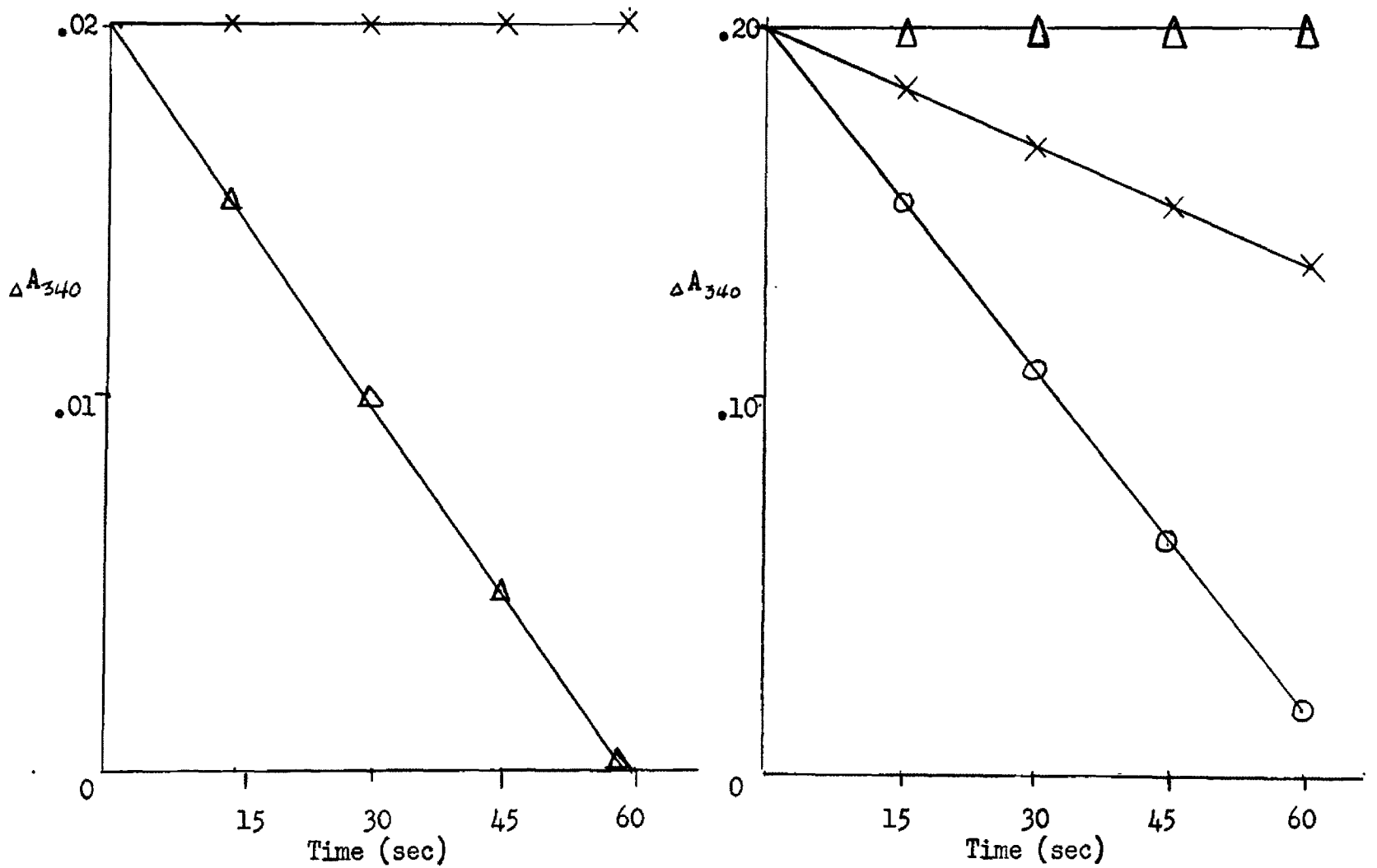
Figure 7. Triose phosphate isomerase activity in extracts of Bordetella pertussis (strains 1,2 and 4) and Escherichia coli.

x—x Reagent
Δ—Δ E. coli
o—o #4
•—• Endogenous

Table VII. Triose phosphate isomerase activities of extracts of Bordetella pertussis (strains 1, 2 and 4) and E. coli.

Strain	mg N/ml extract	ΔA_{340} 15-30'	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
plus 0.001 ml α-glycerophosphate dehydrogenase					
Reagent	-	0.12	0.48	-	-
#1	1.75	0.13	0.52*	-	-
#2	1.53	0.10	0.40*	-	-
#2	1.53	0.12	0.48*	-	-
#4	1.35	0.10	0.40*	-	-
#4-Endog	1.35	0.01	0.04	-	-
plus 0.0001 ml α-glycerophosphate dehydrogenase					
Reagent	-	0.015	0.06	-	-
#4	0.61	0.02	0.08*	-	-
E. coli	0.87	0.03	0.12	12**	13.7

* not significantly higher than blank
** corrected for reagent



A. without added enzyme
X—X #1,#2, #4, reagent and endogenous
Δ—Δ E. coli

B. with 0.01 ml triose phosphate isomerase
Δ—Δ endogenous
X—X #4 and reagent
O—O E. coli

Figure 8. α-glycerophosphate dehydrogenase activity of extracts of B. pertussis (strains 1,2 and 4) and Escherichia coli.

Table VIII. α-glycerophosphate dehydrogenase activities of extracts of Bordetella pertussis (strains 1, 2 and 4) and Escherichia coli.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
		without added enzyme			
Reagent	-	0	0	-	-
E. coli	0.87	0.005	0.02	4	4.5*
#1	1.75	0	0	-	-
#2	1.53	0	0	-	-
#4	1.31	0	0	-	-
#4	0.61	+0.01	+0.04	-	-
E.coli-End	0.87	0	0	-	-
#1-Endog	1.75	+0.02	+0.08	-	-
		plus 0.01 ml triose phosphate isomerase			
Reagent	-	0.015	0.06	-	-
#4	0.61	0.015	0.06	-	-
E. coli	0.87	0.045	0.18	24**	27.5
E.coli-End	0.87	0	0	-	-

* coupled system requires both triose phosphate isomerase and α-glycerophosphate dehydrogenase

** corrected for reagent

dehydrogenase activity was demonstrable in B. pertussis either with or without the addition of purified triose phosphate isomerase. The high reagent blanks obtained in the presence of added enzyme would seem to indicate that the triose phosphate isomerase was contaminated with α-glycerophosphate dehydrogenase.

The extracts of E. coli exhibited enzyme activity in both systems.

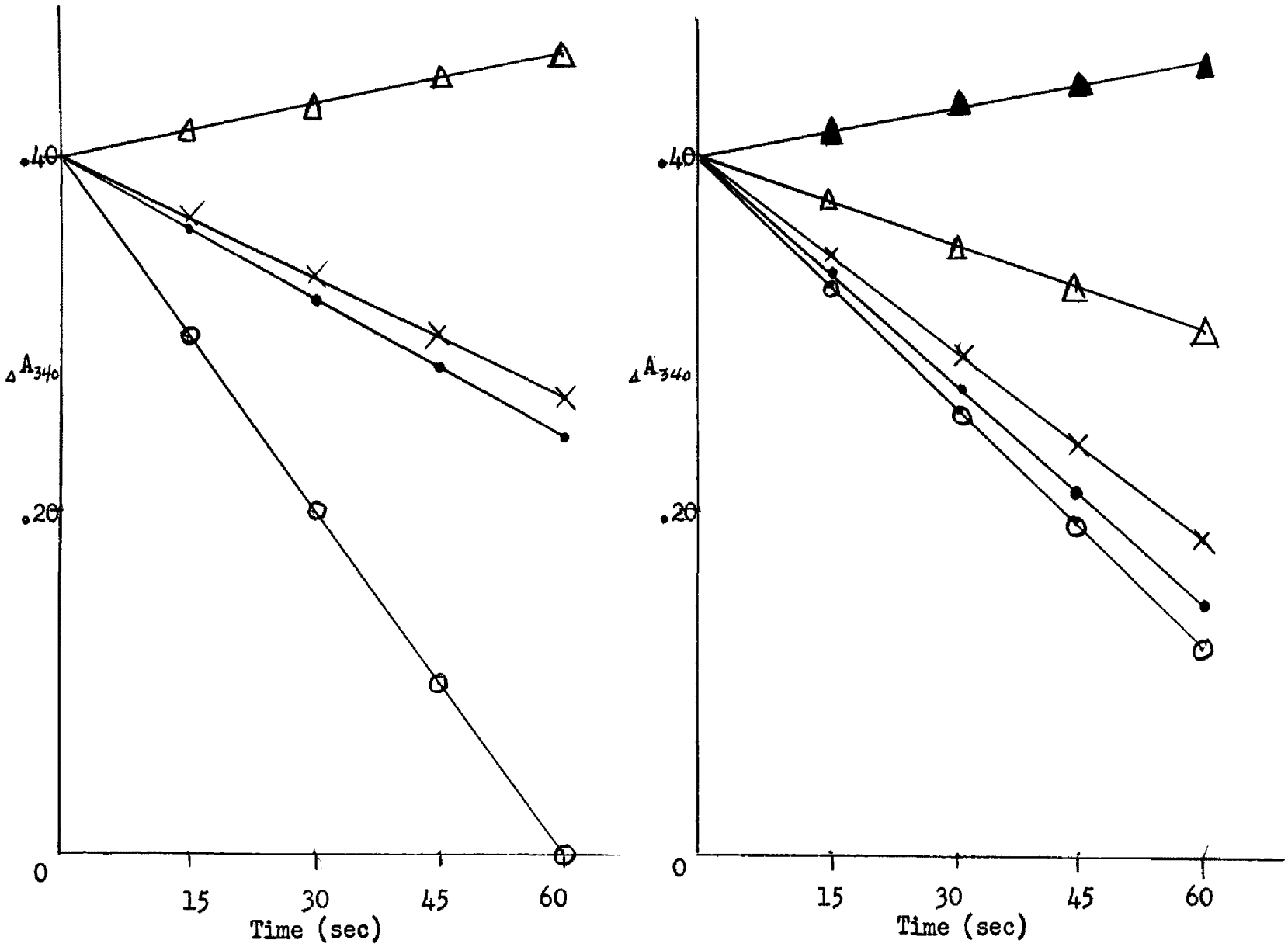
(8) Phosphoglycerokinase:

Either 0.2 or 0.02 ml aliquots of purified GA-3-P dehydrogenase were added to the reaction mixtures. The differences in activity caused by these amounts were very slight (Fig. 9). In both cases, strain #2 exhibited the greatest enzyme activity; however, when #2 was corrected for its unusually high endogenous reaction, the resulting specific activity was more nearly equal that of the other 2 strains (Table IX).

(9) Glyceraldehyde-3-phosphate dehydrogenase:

Because of the slow rate at which this reaction proceeds, a slight variation was employed in the method of representation. The ΔA_{340} for the 15-60 second interval was taken as the initial rate of change, and multiplied by 5 to give a 5-minute span to the graph (Fig. 10). As in the above case, strain #2 exhibited the highest GA-3-P dehydrogenase activity (Table X).

In spite of the different method of representation, a unit of



A. plus 0.2 ml added enzyme
Δ—Δ Reagent and endogenous
x—x #1
•—• #4 and #2-endogenous
o—o #2

B. plus 0.02 ml added enzyme
▲—▲ Reagent and #1- and #4-endogenous
△—△ #2-endogenous
x—x #4
•—• #1
o—o #2

Figure 9. Phosphoglycerokinase activity of extracts of Bordetella pertussis.

Table IX. Phosphoglycerokinase activity of Bordetella pertussis extracts.

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
plus 0.2 ml glyceraldehyde-3-phosphate dehydrogenase					
#1	0.67	0.035	0.14	28	41.7
#2	1.34	0.10	0.40	48*	35.8
#4	1.03	0.04	0.16	32	31.1
plus 0.02 ml glyceraldehyde-3-phosphate dehydrogenase					
#1	0.84	0.063	0.26	52	61.9
#2	0.77	0.07	0.28	36*	46.7
#4	0.80	0.055	0.22	44	55.0

* corrected for endogenous

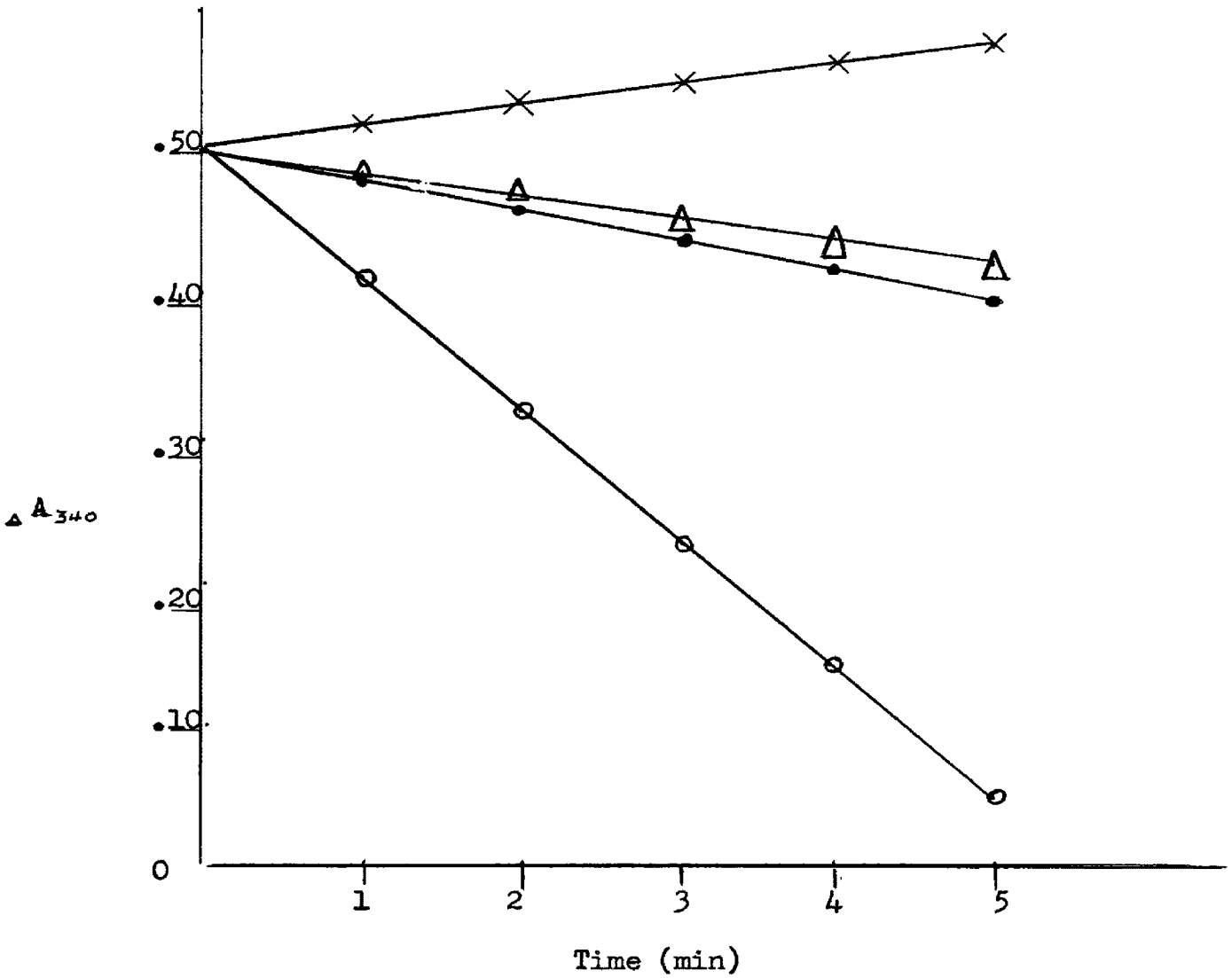


Figure 10. Glyceraldehyde-3-phosphate dehydrogenase activity in extracts of Bordetella pertussis, strains 1,2 and 4.

X—X Reagent and endogenous
△—△ #4
○—○ #2
●—● #1

Table X. Glyceraldehyde-3-phosphate dehydrogenase activity of *Bordetella pertussis* extracts.

Strain	mg N/ml extract	ΔA_{340} 15-60"	ΔA_{340}/5 min	Units/ml	Spec. Act. U/mg N
#1	0.82	0.015	0.075	3	3.7
#1	0.84	0.02	0.10	4	4.8
#2	1.34	0.09	0.45	18	13.4
#2	0.77	0.10	0.50	20	25.2
#4	1.03	0.01	0.05	2	1.9
#4	0.80	0.015	0.075	3	3.7

enzyme activity was still defined as previously.

(10) Pyruvate kinase:

For this and the following ennzyme determinations (enolase and mutase), the μmoles of pyruvic acid formed from the substrate by the enzyme during incubation were determined from a standard curve pictured in Figure 11.

Figure 12 and Table XI show the relative pyruvic kinase activities of strains #1, 2 and 4 of B. pertussis.

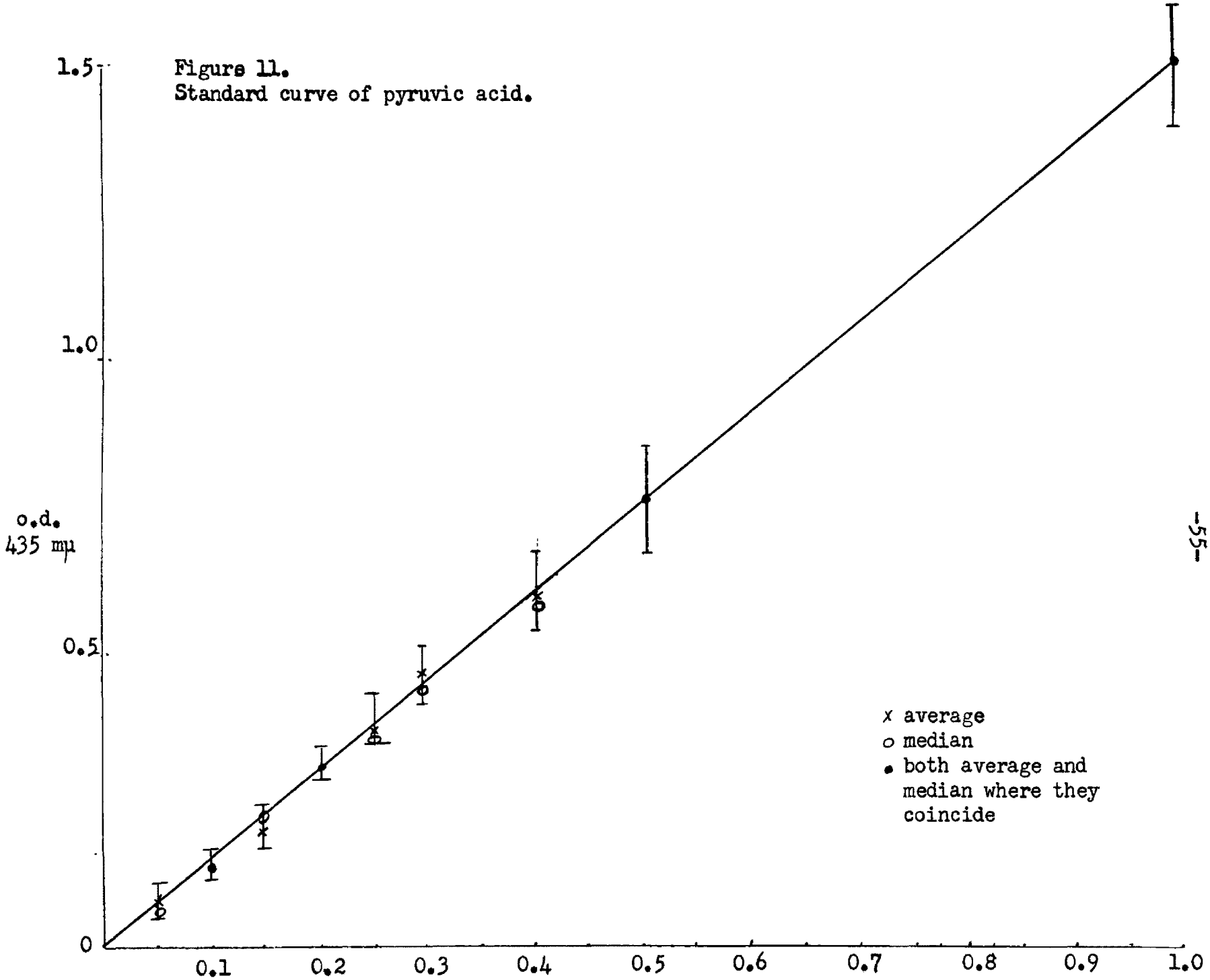
(11) Enolase and mutase:

The extracts of all three strains of B. pertussis catalyzed the formation of pyruvic acid from 3-phosphoglyceric acid, thereby indicating the presence of the enzymes enolase and mutase in these extracts. The results of these experiments are given in Figure 13 and Table XII.

(12) TPN-linked malic dehydrogenase:

Evidence was also presented for the existence of a TPN-linked malic dehydrogenase in cell-free extracts of B. pertussis (Fig. 14 and Table XIII).

Figure 11.
Standard curve of pyruvic acid.



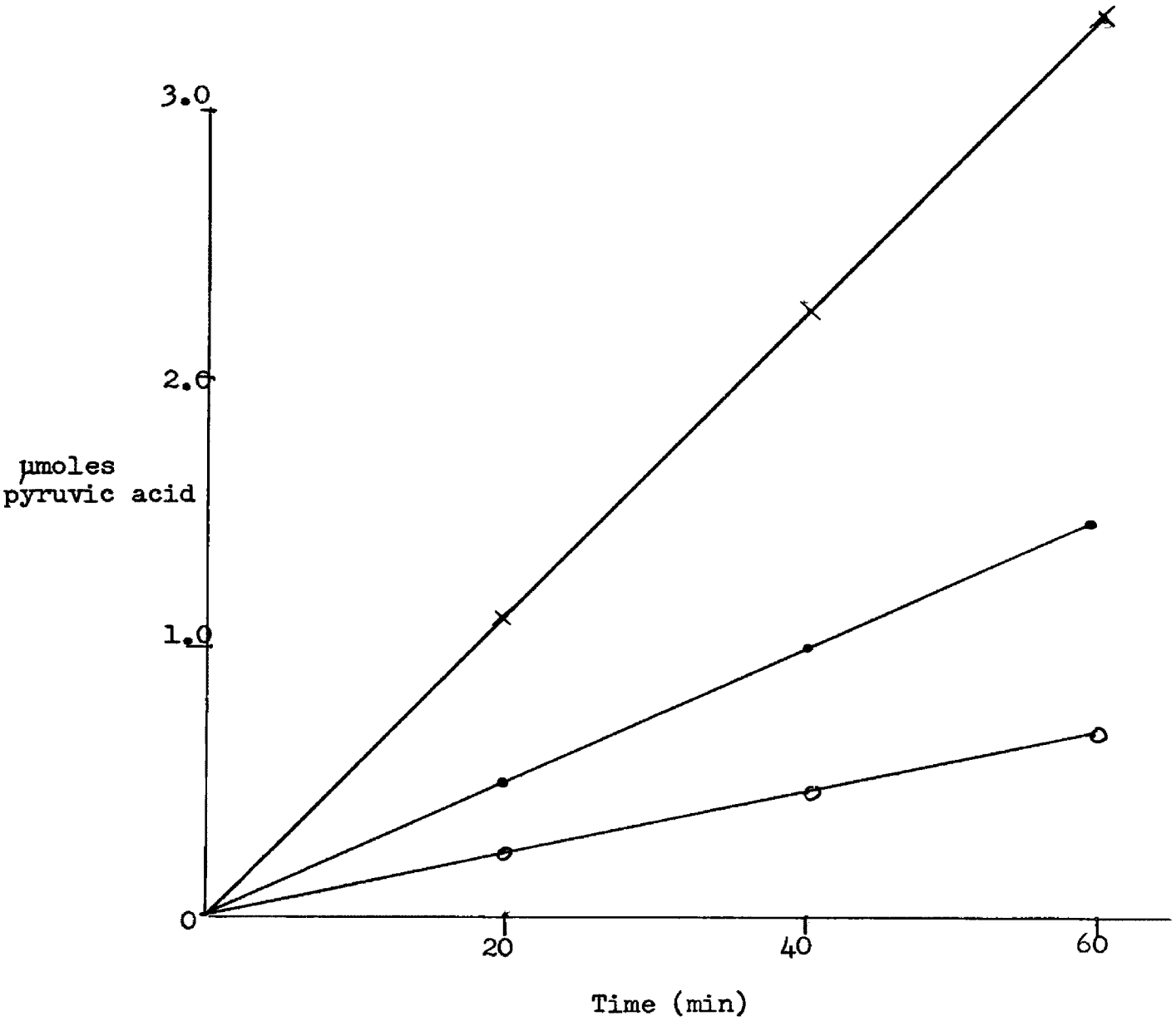


Figure 12. Pyruvic kinase activity of extracts of Bordetella pertussis strains.

×——× #1
•——• #4
○——○ #2

Table XI. Pyruvic kinase activity in extracts of Bordetella pertussis.

Strain	mg N/ml* extract	µmol pyruvic** per 20 min	µmol pyruvic per hour	Units/ml	Spec.Act. U/mg N
#1	0.79	1.125	3.375	33.8	41.9
#1	0.17	0.023	0.069	0.7	4.1
#1	0.31	0.79	0.237	2.4	7.7
#1	0.64	0.078	0.234	2.3	3.6
#2	0.22	0.023	0.069	0.7	3.2
#2	0.40	0.003	0.009	0.1	0.25
#4	0.66	0.37	1.11	11.1	16.8
#4	0.26	0.076	0.228	2.3	8.8
#4	0.56	0.488	1.464	14.6	26.1

* corrected for Tris buffer content
** corrected for endogenous and reagent blanks

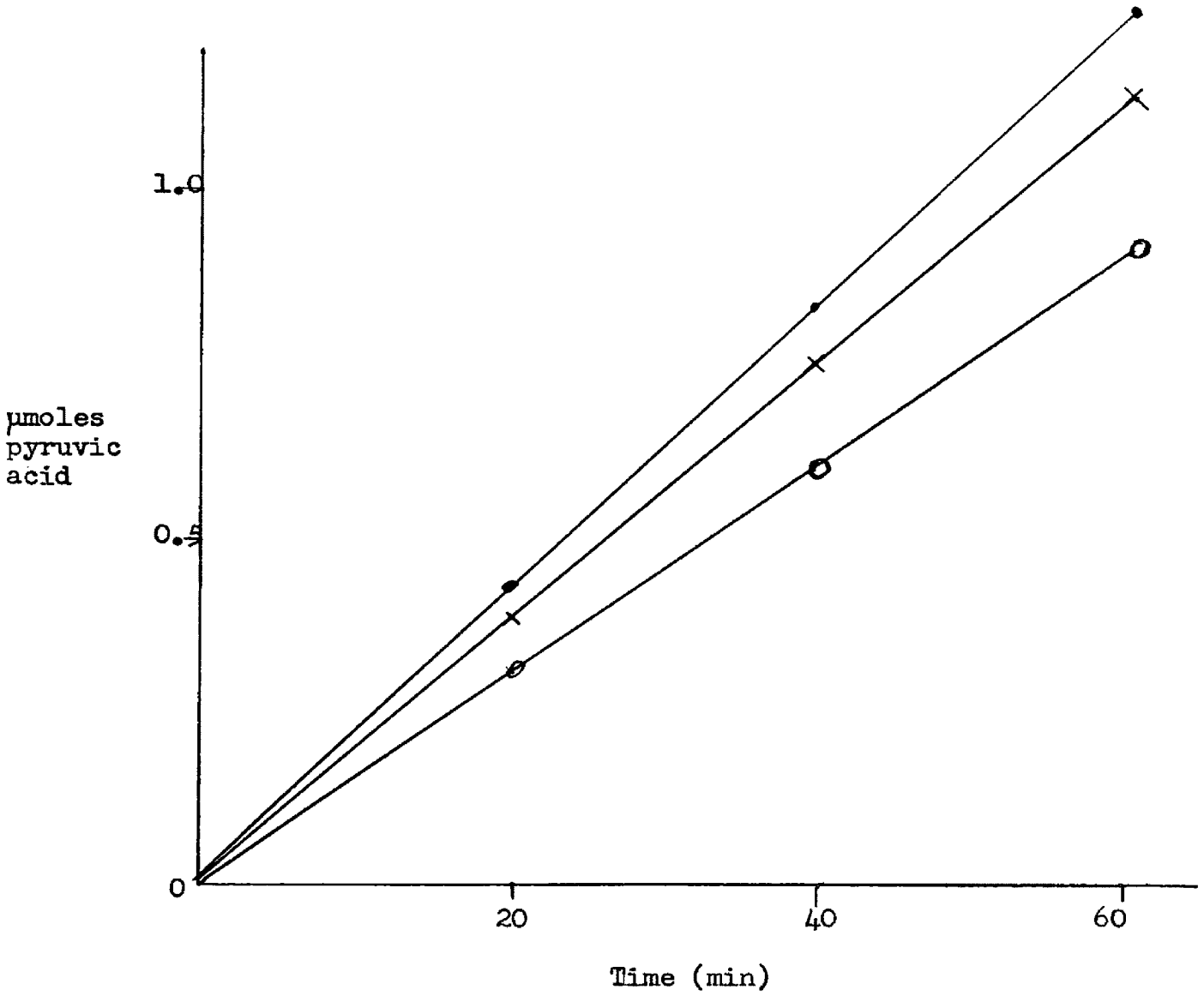


Figure 13. Enolase and mutase activities of extracts of Bordetella pertussis, strains 1, 2 and 4.

●—● #2
x—x #4
o—o #1

Table XII. Enolase and mutase activities of Bordetella pertussis extracts.

Strain	mg N/ml* extract	µmol pyruvic** per 20 min	µmol pyruvic per hour	Units/ml	Spec. Act. U/mg N
#1	0.28	0.31	0.93	9.3	33.2
#1	0.46	0.16	0.48	4.8	10.4
#1	1.17	0.21	0.63	6.3	5.4
#1	0.31	0.21	0.63	6.3	20.3
#2	1.23	0.42	1.27	12.7	10.3
#2	0.77	0.24	0.74	7.4	9.6
#2	0.21	0.17	0.51	5.1	24.3
#4	1.35	0.39	1.16	11.6	8.6
#4	0.82	0.07	0.23	2.3	2.8

* corrected for Tris buffer content
** corrected for endogenous and reagent blanks

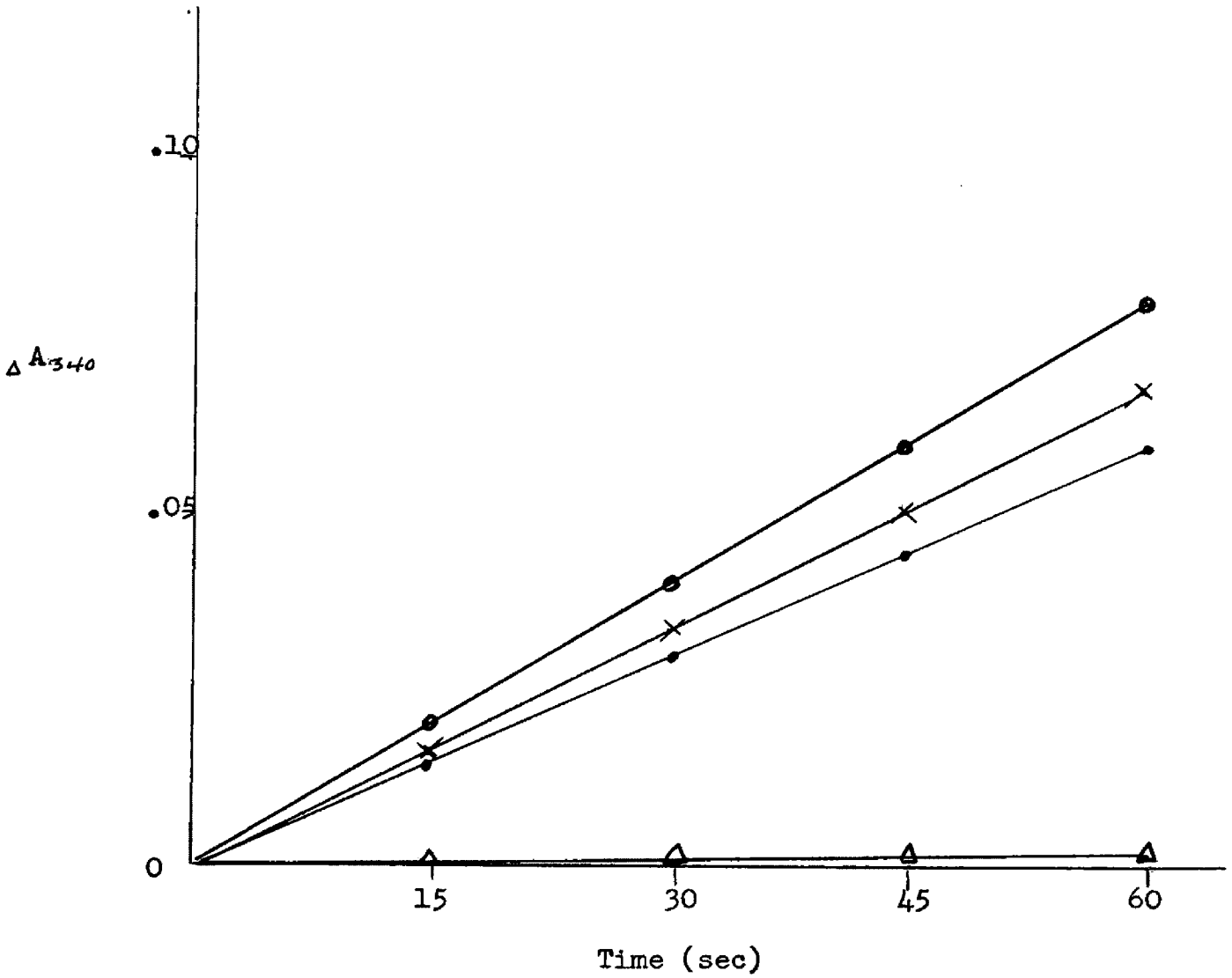


Figure 14. TPN-linked malic dehydrogenase activity in extracts of Bordetella pertussis, strains 1, 2 and 4.

o—o #2
x—x #1
•—• #4
△—△ Reagent and endogenous

Table XIII. TPN-linked malic dehydrogenase activity of Bordetella pertussis extracts

Strain	mg N/ml extract	ΔA_{340} 15-30"	ΔA_{340}/min	Units/ml	Spec. Act. U/mg N
#1	1.75	0.017	0.068	13.6	7.7
#2	1.53	0.02	0.08	16.0	10.5
#4	1.35	0.015	0.06	12.0	8.8

CHAPTER V

DISCUSSION

Enzyme activity is influenced by many factors, such as pH, temperature, concentration of the reaction products, concentration of the substrate, activators and inhibitors. Slight variations in any of these factors can affect the exact duplication of results (Harrow et al., 1960). This could account for the occasionally large discrepancies in the specific activities of an extract for any given enzyme. However, the main purpose of this work was not to characterize a particular enzyme, but rather, to demonstrate which, if any, of the glycolytic enzymes are present in extracts of Bordetella pertussis.

Glycolysis can be defined as the sequence of reactions which results in the conversion of free monosaccharides to lactic acid or pyruvic acid, (Bueding and Farber, 1960), and, as such, Abe (1953) felt that this pathway could not exist in B. pertussis because of the inability of this organism to oxidize sugars. On the other hand, a reversion of glycolysis is considered to be one of the mechanisms for the synthesis of monosaccharides within the cell (Leloir, Cardini and Cabib, 1960) and it has been postulated that the glycolytic pathway is at least partially operative in B. pertussis for the production of polysaccharides (Kobayashi and Fukimi, 1954). In this "inverted" glycolytic pathway, pyruvate may be considered as a key metabolite since the conversion of fats and amino acids to carbohydrates takes

place through the intermediary formation of pyruvic acid (Leloir, Cardini and Cabib, 1960).

It seems to be a fairly well established fact that glutamic acid oxidation is the primary energy source for B. pertussis and that new substances are introduced into the TCA cycle via α-ketoglutaric acid (Jebb and Tomlinson,1951; Rowatt, 1955).

Within the TCA cycle there is a DPN-linked malic dehydrogenase which converts malate to oxaloacetate. There also exists a TPN-linked malic dehydrogenase which converts malate to pyruvate. In pigeon liver and wheat germ extracts this latter "malic" enzyme requires manganese ions for activation (Ochoa, 1955). This activator is apparently not necessary in extracts of B. pertussis since they reduced TPN in the presence of malic acid and buffer without the addition of Mn^{++}. TPN-malic dehydrogenase is probably not the only enzyme capable of producing pyruvate from TCA intermediates, but it is still important as a source of the starting material for reverse glycolysis.

Pyruvic acid formation by B. pertussis extracts from 3-PGA and PEPA demonstrated the presence of pyruvic kinase, enolase and mutase in these extracts. The specific activities for enolase and mutase were higher than those for the kinase, however, 4 times the amount of substrate was used in the demonstrations of the first 2 enzymes than in the third. Enolase is specific for 2-PGA and PEPA (Axelrod, 1960(a)); therefore, the production of pyruvate from 3-PGA would necessarily imply the presence of the mutase which converts 3-PGA to 2-PGA.

Pyruvic kinase catalyzes a transfer of phosphate from PEPA to ADP. This reaction has been shown to be completely reversible in the presence of Mg, ATP and K or NH_4 ions, (Axelrod, 1960(a)), and is the first step in the synthesis of sugars from pyruvate.

Both phosphoglycerokinase and GA-3-P dehydrogenase activities were demonstrated in extracts of B. pertussis. Apparently the dehydrogenase is the limiting factor in this system in B. pertussis as it is in Neisseria meningitidis (Jyssum, Borchgrevink and Jyssum, 1961). An unusually high endogenous blank was consistently noted in the phosphoglycerokinase activity of strain #2. The cells of this strain showed a far greater tendency toward clumping during the washing procedure than did the other strains used, perhaps resulting in an incomplete removal of substrate which might account for the high endogenous activity.

Contrary to what might be expected, neither triose phosphate isomerase nor α-glycerophosphate dehydrogenase activity was demonstrated in B. pertussis extracts. E. coli, however, showed a low but definite activity for both of these enzymes. In the other experiments which utilized this particular dehydrogenation system purified triose phosphate isomerase/α-glycerophosphate dehydrogenase was always added.

The lack of isomerase activity in B. pertussis extracts may be due to the absence of the enzyme in B. pertussis or to the inability of the method used to detect small amounts of the enzyme. However, in regard to the latter postulate, the spectrophotometric method is felt

to be one of the most sensitive for the determination of enzyme activity, and this enzyme was detectable in extracts of E. coli. Nutrient utilization of the glycerol in B-G medium (Dawson et al., 1951; Magasanik, Brooke and Karabian, 1953) would seem to be ruled out by the lack of α-glycerophosphate dehydrogenase activity in B. pertussis extracts.

High activities for both aldolase and phosphofructokinase were demonstrated in extracts of B. pertussis. The specific activity for the kinase appeared to be somewhat higher than that for aldolase, but the strain variation remained about the same for both enzymes with strain #4 exhibiting the highest amount of activity and strain #2 the lowest amount. The presence of aldolase in B. pertussis extracts agrees with the findings of Kobayashi and Fukimi (1954).

Also in agreement with these workers was an inability to demonstrate the presence of hexokinase activity in this organism. The method they used was based upon the formation of acid-labile phosphate and inorganic phosphate, and the slight activity which occurred they attributed to an ATPase present in the extracts. Hexokinase activity is present in all cells capable of utilizing free sugars (Axelrod, 1960(a)). Some hexokinases, such as brain (Bueding and Farber, 1960) and yeast (Berger et al., 1946), exhibit broad substrate specificity, while others have a high specificity for a single substrate (Palleroni et al., 1956; Doudoroff, Palleroni et al., 1956). In certain organisms, Clostridium botulinum (Simmons and Costilow, 1962) and Pseudomonas

saccharophila (Doudoroff, Palleroni et al., 1956), hexokinase activity has been found to be inducible.

No work has yet been reported in which induction of hexokinase activity in B. pertussis has been attempted. From the evidence presented here it appears very probable that B. pertussis lacks a glucokinase, and from its inability to oxidize or ferment free sugars, it would appear equally probable that this organism lacks any kinase capable of phosphorylating unsubstituted monosaccharides.

By neither of the methods used was any hexosephosphate isomerase detected (i.e., specifically phosphoglucoisomerase; G-6-P$\rightleftharpoons$F-6-P). When F-6-P was used as the substrate, extracts suspended in phosphate buffer consistently showed lower activity than the reagent blank. However, when the extracts of these strains of B. pertussis were suspended in barbital buffer their activities equalled that of the reagent blank. It was felt that this might be due to the utilization of the substrate by an enzyme in the extract which required only F-6-P and inorganic phosphate for activity, perhaps a fructose-6-phosphate phosphoketolase such as was found in Acetobacter xylinum by Racker (1962) which catalyzes the reaction:

$$\text{F-6-P} + P_i \rightarrow \text{Erythrose-4-P} + \text{Acetyl-P} + H_2O$$

No evidence was presented for either TPN- or DPN-linked G-6-P dehydrogenase activity in B. pertussis. This would seem to rule out the use of the hexosemonophosphate pathway for glucose catabolism in B. pertussis, but it would not necessarily invalidate the partial

operation of this pathway for the synthesis of pentoses and other carbohydrates in this organism.

In certain species of Pseudomonas and Acetobacter another pathway for glucose catabolism, the Entner-Doudoroff pathway, has been described (Bueding and Farber, 1960). This pathway involves the formation of a phosphorylated intermediate, 2-keto-3-deoxy-6-phosphogluconic acid and its subsequent cleavage to pyruvic acid and glyceraldehyde-3-phosphate. The first means by which this intermediate is formed requires the activity of G-6-P dehydrogenase, which is absent in B. pertussis extracts. The second involves the direct oxidation of glucose to gluconic acid followed by phosphorylation of the latter substance by a specific gluconokinase. Kobayashi and Fukimi (1954) were unable to demonstrate gluconokinase activity in B. pertussis, thereby apparently eliminating the Entner-Doudoroff pathway as a possible means of glucose catabolism in this organism. It is conceivable, however, that the intermediates of this pathway could be formed, beginning with the aldolase-catalyzed condensation of pyruvic acid and glyceraldehyde-3-phosphate to 2-keto-3-deoxy-6-phosphogluconolactone (Leloir, Cardini and Cabib, 1960).

Kobayashi and Fukimi (1954) hypothesized that the inability of B. pertussis to utilize free sugars was due to the lack of hexokinase rather than the lack of permeability, and that sugars could be and probably were synthesized and metabolized within the bacterial cell. Overend et al. (1951) found that DNA preparations of B. pertussis

contained a polysaccharide component, probably in combination with the nucleic acid, and Akiya et al. (1951) found galactose, mannose and fructose to be present in B. pertussis cells, the galactose and mannose probably occurring as polysaccharide components.

The hexosemonophosphates formed from lower molecular weight compounds within the cell can be modified by a series of interconversion reactions, some of which lead to the formation of glycosyl derivatives which may then react with acceptors and lead to the synthesis of polysaccharides (Leloir, Cardini and Cabib, 1960). One of the mechanisms for the synthesis of complex polysaccharides, transglycosylation or transfructosylation, occurs according to the general reaction (Hassid, 1955):

$R'OR''$	+	$R'''OH$	$\longrightarrow$	$R'OR'''$	+	$R''OH$
Glycosyl donor		Acceptor		Product		By-product
hexose-P; sugar nucleotide; di- to polysaccharides.		alcohols; sugar-P; mono- to polysaccharides; H_2PO_4.		hexose-P; glycosides; di- to polysaccharides.		mono- to polysaccharides; H_2PO_4; UDP.

Since fructose-6-phosphate, fructose diphosphate, glyceraldehyde-3-phosphate and phosphoglyceric acid are all metabolized by B. pertussis, it can be assumed that they are also available for interconversion reactions. Mannose-6-phosphate could be formed in the cell by the isomerization of fructose-6-phosphate, providing that B. pertussis contains one phosphohexoisomerase while lacking the other.

Galactose is not directly oxidized by extracts of B. pertussis

(Abe, 1953; Jebb and Tomlinson, 1951). Doudoroff, DeLey et al. (1956) postulated a pathway in P. saccharophila for cell-free extracts of that organism grown on galactose, which involved the direct phosphorylation of 2-keto-3-deoxy-galactonic acid to 2-keto-3-deoxy-6-phosphogalactonic acid followed by cleavage to pyruvic acid and glyceraldehyde-3-phosphate. Kalckar (1957) found this mechanism to be apparently irreversible.

Since glucose-6-phosphate is not formed from fructose-6-phosphate and glucose is not directly phosphorylated it seems unlikely that galactose is formed in B. pertussis by the usual route involving uridine diphosphate (UDP).

Some glucose intermediate must be formed within B. pertussis since the conversion of other monosaccharides to galactose apparently requires the intermediary formation of glucose (Axelrod, 1960(b); Leloir, Cardini and Cabib, 1960). One means by which galactose may be synthesized in B. pertussis is through hexosamine metabolism. It has been shown that fructose-6-phosphate and glutamine (Axelrod, 1960(b)) or ammonia (Leloir, Cardini and Cabib, 1960), in the presence of the necessary enzyme, will produce glucosamine-6-phosphate and glutamic acid or water. Glucosamine-6-phosphate can then react with acetyl coenzyme A to produce N-acetylglucosamine-6-phosphate and coenzyme A. The hexose sugar may be converted by a specific mutase to N-acetylglucosamine-1-phosphate, which, in turn, can react with UTP to form UDP-acetylglucosamine and inorganic pyrophosphate.

Finally, an epimerization reaction may occur transforming UDP-acetylglucosamine into UDP-galactosamine which may serve as a sugar donor in the synthesis of polysaccharides (Axelrod, 1960(b); Leloir, Cardini and Cabib, 1960).

As yet, this pathway has not been studied in B. pertussis. Some evidence exists, however, which might indicate that this mechanism is operative in B. pertussis. Of all the sugars tested as substrate for B. pertussis, a slight oxygen consumption was noticed only with glucosamine (Abe, 1953). The low QO_2(26) which was observed could have been due to the fact that this substrate is not readily metabolized by B. pertussis, or to the fact that glucosamine-6-phosphate is being produced within the cell and competing for the available enzyme.

Gerhardt et al. (1950), studying the utilization of single amino acids for growth by Brucellae, hypothesized that amino acids may serve as organic sources of ammonia, which in turn serves as the primary nitrogen source for synthetic reactions. During the oxidation of glutamic acid by B. pertussis it was noted that the actual yield of ammonia was less than the expected theoretical yield, leaving some 20-30% of the ammonia unaccounted for (Jebb and Tomlinson, 1951; Baddily, Rowatt and Standfast, 1952; Rowatt, 1955). Jebb and Tomlinson (1951) postulated that the missing nitrogen was incorporated into cell substance. It is very possible that this "cell substance" is glucosamine-6-phosphate.

From the data presented it seems probable that B. pertussis

is incapable of catabolizing unsubstituted monosaccharides, primarily because of the absence of hexokinase. The glycolytic enzymes which were demonstrated in extracts of this organism were those leading to the formation of substrates which could easily be converted into a variety of sugars within the cell. The glycolytic cycle is only partially operative in B. pertussis, and its principal function in this organism is probably the anabolism of carbohydrates rather than their catabolism.

CHAPTER VI

SUMMARY

1. A survey was made to determine which glycolytic enzymes were present in extracts of strains 2927 (#1). 5375L45 (#2) and 5373L-2 (#4) of Bordetella pertussis.
2. Activities corresponding to phosphofructokinase, aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerokinase, phosphoglyceromutase, enolase, pyruvic kinase and TPN-linked malic dehydrogenase were demonstrated in these extracts.
3. No activities were demonstrated corresponding to hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, triose phosphate isomerase and α-glycerophosphate dehydrogenase in these extracts.
4. The possibility of the presence of a fructose-6-phosphate phosphoketolase was mentioned.
5. The inability of B. pertussis to catabolize sugars via the glycolytic, hexose monophosphate or Entner-Doudoroff pathways was discussed, together with the probability that those glycolytic enzymes which are present in B. pertussis are primarily involved in the synthesis of poly saccharides.
6. The possible formation of intracellular galactose in B. pertussis through a hexosamine metabolism was also discussed.

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