ASPECTS OF THE BIOCHEMISTRY OF THE THERMOPHILIC MICROORGANISM BACILLUS STEAROTHERMOPHILUS

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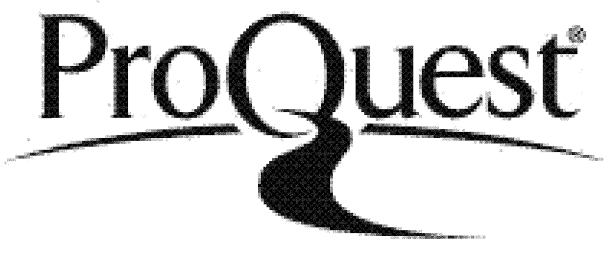

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C O N T E N T S

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1. PRELIMINARY MATTERS.

1.1. General Introduction.

The growth and survival of microorganisms at elevated temperatures has attracted the attention of biologists since the middle years of the nineteenth century. The initial discovery of a thermophilic bacterium is usually attributed to Miquel (1888), who isolated a microorganism from the waters of the river Seine which was capable of growth at 73°. However Gaughran (1947) pointed out that earlier descriptions of the flora of thermal pools by James (1823), Hooker (1854), and Brewer (1866) include species which would now be described as thermophilic bacteria. The extensive reviews by Gaughran (1947) and Allen (1953) provide detailed accounts of the early work on the diversity and occurrence of thermophilic microorganisms and these aspects of the biology of thermophilic microorganisms will not be discussed further. However it should be stressed that while thermophilic microorganisms are usually associated with hot environments such as hot springs or manure heaps they have been isolated from virtually every natural habitat in which they have been sought. For example, although there is no suggestion that the thermophile was able to proliferate in such an inhospitable environment Rabinowitsch (1895) was able to isolate thermophilic microorganisms from freshly fallen snow.

Discussion of the physiology of thermophilic microorganisms has been hampered in the past by the lack of concise and generally accepted definitions of thermophily. The first attempt at a concise definition was made by Morrison and Tanner (1924) who suggested division into (1) Strict Thermophiles, with optimum temperatures for growth above 55°,* (2) Facultative Thermophiles, with optimum temperatures for growth between 50° and 55°, and (3) Thermotolerant Bacteria with optimum temperatures between 40° and 50°.

Frequently encountered in the literature are the terms Stenothermal and Eurithermal proposed by Imsencki and Solnezeva (1945). They refer to organisms which grow at 60° but show no growth (stenothermal) or some growth (eurithermal) at 28-30°.

A quite different basis for distinction between thermophilic, mesophilic and psychrophilic microorganisms has been proposed by Ingraham (1958). He reported values for the Arrhenius constant for growth (or μ, the temperature coefficient) of 9050 cal $mole^{-1}$ and 14200 cal $mole^{-1}$ for a psychrophilic Pseudomonad and the mesophile Escherichia coli respectively and suggested that the value of μ might be a basis for classification. However Hanus and Morita (1968) pointed out that Ingraham's results were miscalculated and also assembled data from a number of sources to show that a) there is

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* Temperature is quoted in degrees Centigrade throughout this thesis.

no relationship between optimum growth temperature and the value of μ and that b) the value of u for a single organism may vary widely according to the experimental conditions employed in different laboratories.

Results obtained by other workers make it clear that the comments of Hanus and Morita (1968) apply equally well to the distinction between mesophiles and thermophiles. Thus Bubela and Holdsworth cite a μ value of 23000 cal $mole^{-1}$ for a strain of Bacillus stearothermophilus while Epstein and Grossowicz (1969a) report for another thermophilic Bacillus (also B. stearothermophilus) a value of 13500-15000 cal $mole^{-1}$. In recent years the tendency has been to ignore precise definitions and Ingraham's suggestion (1962) that the terms psychrophile, mesophile and thermophile need only indicate that an organism grows best at low, intermediate or high temperatures, has now been widely adopted.

Thermophilic members of the genus Bacillus pose two fundamental questions. The first is quite simple, at least to ask. How do these organisms, otherwise closely akin both physiologically and biochemically to mesophilic members of the same genus, survive and proliferate at temperatures far in excess of those usually considered suitable for life? The second question is the converse of the first - why do thermophilic Bacilli not grow at more moderate temperatures?

Early speculations on the question of survival at high temperatures were hampered by the lack of data rather than by any lack of imagination. For instance, von Esmarch (1888) proposed that an insulating shell would afford protection against the heat. Oprescu (1898) was the first worker to report a thermostable enzyme from a thermophilic micro-organism - an extracellular amylase which was still active after being heated for 30 minutes at 85°. In recent years a number of enzymes from thermophiles have been examined and, as Table 1.1. shows, lability to heat is rarely found.

The few enzymes to have been shown to be quite unstable at the growth temperature of the source organism include the pyruvic oxidase studied by Militzer and Burns (1952), the aspartokinase studied by Muramitsu (1970) and the pyruvate carboxylase studied by Cazzulo and others in this laboratory (Cazzulo et al., 1970). Militzer and Burns did not report any effective stabilizing agents but noted that the enzyme was quite stable when subjected to heat treatment prior to extraction from the cells. Pyruvate carboxylase, on the other hand, was shown to be considerably stabilized by its allosteric effectors acetyl- coenzyme A and L-aspartate. Only 2% of its activity was lost after 30 minutes incubation at 55° in a typical reaction mixture lacking only bicarbonate. The rapid inactivation of aspartokinase at 70° reported by Kuramitsu was almost completely prevented by low concentrations

Table 1.1. Thermostability of enzymes from thermophilic bacteria.

Enzyme	EC No.	Source	Treatment °C	Treatment Mins.	%Activity remaining	Reference
Alcohol dehydrogenase	1.1.1.2	Bacillus stearothermophilus	70	10	92	Amelunxen & Lins (1968)
Malate dehydrogenase	1.1.1.37	" "	65	120	60	Militzer et al. (1949)
Isocitrate dehydrogenase	1.1.1.42	" "	63	180	70	Howard & Becker (1970)
Phosphogluconate dehydrogenase	1.1.1.43	Clostridium thermosaccharolyticum	55	30	108	Howell et al. (1969)
Glucose-6-phosphate dehydrogenase	1.1.1.49	B. stearothermophilus	70	10	50	Amelunxen & Lins (1968)
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	" "	70	10	137	" "
" "	"	Cl. thermosaccharolyticum	55	30	44	Howell et al. (1969)
Succinate dehydrogenase	1.3.99.1	B. stearothermophilus	70	10	58	Amelunxen & Lins (1968)
Cytochrome Oxidase	1.9.3.1	" "	65	50	50	Militzer et al. (1950)

Table 1.1. Thermostability of enzymes from thermophilic bacteria.

Enzyme	EC No.	Source	Treatment °C	Treatment Mins	%Activity remaining	Reference
Pyruvate oxidase	-	B.stearothermophilus	65	60	0	Militzer & Burns (1957)
NADH oxidase	-	" "	70	10	48	Amelunxen & Lins (1968)
Hydrogenase	1.98.1.1	Desulfotomaculum nigrificans	65	90	100	Akagi & Campbell (1961)
Aspartate amino-terferase	2.6.1.1	B.stearothermophilus	70	10	48	Amelunxen & Lins (1968)
Hexokinase	2.7.1.1	" "	70	10	42	Amelunxen & Lins (1968)
" "	" "	Cl. thermosaccharolyticum	55	30	87	Howell et al. (1969)
Phosphofructo-kinase	2.7.1.11	" "	70	30	85	" "
" "	" "	Flavobacterium thermophilum	90	60	90	Yoshida et al (1971)
Phosphoglycerate kinase	2.7.2.3	Cl. thermosaccharolyticum	50	30	90	Howell et al (1969)

Table 1.1. Thermostability of enzymes from thermophilic bacteria.

Enzyme	EC No.	Source	Treatment °C	Treatment Mins	%Activity remaining	Reference
Pyruvate kinase	2.7.1.40	Cl.thermosaccharolyticum	70	30	83	Howell et al (1969)
	" "	B.stearothermophilus	60	10	88	Amelunxen & Lins (1968)
Aspartokinase	2.7.2.4	" "	70	5	0	Kuramitsu (1970
Phosphoglucomutase	2.7.5.1	F. thermophilum	85	30	40	Yoshizaki et al. (1971)
Phosphoglyceromutase	2.7.5.3	Cl. thermosaccharolyticum	55	30	100	Howell et al. (1969)
Sulphate adenylyl transferase	2.7.7.4	D. nigrificans	65	120	100	Akagi & Campbell (1962)
DNA dependant RNA polymerase	2.7.7.6	B. stearothermophilus	65	10	50	Remold-O'Donnel & Zillig (1969)
Alkaline phosphatase	3.1.3.1	" "	60	10	88	Amelunxen & Lins (1968)
Fructose diphosphatase	3.1.3.11	F. thermophilum	80	35	50	Yoshida & Oshima (1971)
L-Amylase	3.2.1.1	B. stearothermophilus	60	45	50	Pfueller & Elliot (1969)

Table 1.1. Thermostability of enzymes from thermophilic bacteria

Enzyme	EC No.	Source	Treatment		%Activity remaining	Reference
			°C	Mins		
α-Amylase	3.2.1.1	B. stearothermophilus	90	15	50	Ogasahara et al (1970)
Aminopeptidase	3.4.1.2	" "	80	60	100	Roncari & Zuber (1970)
Protease	-	B. thermoproteolyticus	80	60	100	Ohta (1967)
" "	-	B. stearothermophilus	55	225	100	O'Brien & Campbell (1957)
Asparagine deamidase	3.5.1.1	" "	55	40	71	Manning & Campbell (1957)
" "	" "	B. coagulans	55	40	27	" "
Glutaminase	3.5.1.2	B. stearothermophilus	70	10	124	Amelunxen & Lins (1968)
Inorganic pyrophosphatase	3.6.1.1	" "	80	50	50	Marsh & Militzer (1956)
ATPase	3.6.1.4	" "	65	60	100	Hachimori et al (1970)
" "	" "	" "	65	120	100	Militzer & Tuttle (1951)

Table 1.1. Thermostability of enzymes from thermophilic bacteria.

Enzyme	EC No.	Source	Treatment		%Activity remaining	Reference
			°C	Mins		
Fructose diphosphate aldolase	4.1.2	B. stearothermophilus	70	90	60	Sugimoto & Nosoh (1971)
" "	" "	" "	70	60	95	Thompson & Thompson (1962)
" "	" "	Cl. thermosaccharolyticum	55	30	77	Howell et al. (1969)
Isocitrate lyase	4.1.3.1	B. stearothermophilus	55	34	50	Daron (1967)
" "	" "	" "	55	30	65	Griffiths & Sundaram (personal communication)
Enolase	4.2.1.11	Cl. thermosaccharolyticum	70	30	108	Howell et al (1969)
" "	" "	B. stearothermophilus	70	10	88	Amelunxen & Lins (1968)
L-threonine deaminase	4.2.1.16	" "	70	30	20	Thomas & Kuramitsu (1971)

Table 1.1. Thermostability of enzymes from thermophilic bacteria.

Enzyme	EC No.	Source	Treatment		%Activity remaining	Reference
			°C	Mins.		
Triosephosphate isomerase	5.3.1.1	Cl.thermosaccharolyticum	55	30	102	Howell et al. (1969)
Glucosephosphate isomerase	5.3.1.9	" "	63	30	105	" "
Pyruvate carboxylase	6.4.1.1	B.stearothermophilus	55	4	5	Cazzulo et al (1970)

of the allosteric inhibitors lysine and threonine. High concentrations of aspartate and ATP together also afforded protection.

The case for the general thermostability of enzymes from thermophilic microorganisms would appear on the basis of the data in Table 1.1., fairly convincing, but the objection remains that in spite of its length Table 1.1. represents only a small fraction of the total number of different enzymes within the cell. Nevertheless it should be borne in mind that if we assume that the enzymes listed in Table 1.1. represent a random selection with respect to thermostability the probability would appear to be very small that a newly studied enzyme from a thermophile would prove to be thermolabile. Koffler and co-workers (1957) and more recently Amelunxen and Lins (1968) have demonstrated that the greater thermostability of individual proteins from thermophilic Bacilli is reflected in the smaller amount of total protein precipitation when crude, cell free extracts of these organisms are heated.

Inspection of Table 1.1. shows that the bulk of the data has accumulated since Allen completed her review in 1953. At that time the generality of enzyme thermostability seemed much less likely, particularly in the light of Allen's (1950) own conclusion that the catalase (EC 1.11.1.16) from a thermophilic strain of Bacillus subtilis was rapidly

inactivated at 55°. (The observation that the substrate, hydrogen peroxide, rapidly inactivated the catalase of an unidentified thermophile (Nakamura, 1960) at high temperatures may account for Allen's results.) Allen (1953) therefore lent her support to the suggestion originally made by Gaughran (1947a) that thermophiles have the facility for rapid resynthesis of heat damaged proteins. As descriptions of thermostable enzymes have accumulated this theory has attracted less support and the experiments of Koffler and co-workers (1957) on the coagulability of total cell proteins produced results that led to the rapid repair theory being generally discarded. The possibility that rapid repair has a role in the physiology of thermophilic microorganisms was revived recently by Bubela and Holdsworth (1966) who reported very rapid protein turnover in B. stearothermophilus. Under their particular conditions isotopically labelled proteins had a half life of only 4 minutes at 63°. While they were not prepared to commit themselves to the absolute physiological validity of their results Bubela and Holdsworth were convinced that very rapid protein synthesis did occur. These results called for a re-examination of the question of protein turnover and its relationship to the thermostability of enzyme proteins in thermophiles. Experiments to be described in this thesis show that a measurable rate of protein turnover does occur during the growth of the strain of B. stearothermophilus used in these studies but that the rate of turnover does not remotely approach that claimed by Bubela and Holdsworth, or

that required by the "rapid repair" theory. It has proved possible in this study to correlate the rate of turnover with the rate of inactivation in vivo of a number of enzymes that were examined. When Allen developed the theory of rapid repair or resynthesis it was assumed to apply only to proteins, the most obvious potential victims of high temperatures. However the thermal denaturation of other macromolecular components of the cell remains a possibility and may conceivably be a significant element in the physiology of thermophiles. Recent work has focussed attention on the thermostability of these components. Marmur (1960) demonstrated that the DNA of B. stearothermophilus has a similar "melting temperature" (Tm) to that of E. coli but the temperature at which denaturation sets in in both instances is well above the optimum growth temperature of the thermophile. DNA therefore is unlikely to be subjected to any significant damage at the high temperatures at which thermophiles grow.

The messenger RNA of B. stearothermophilus has been isolated (Saunders and Campbell, 1966) but no attempt was made to determine its Tm value. Bubela and Holdsworth (1966) and Grinstead and McQuillen (1970) have shown that the messenger RNA of B. stearothermophilus is turned over at similar rates to that of E. coli and B. subtilis (Levinthal et al., 1962) but in all cases the half lives of the RNA were sufficiently short (less than 5 minutes) for the question of thermostability to be largely irrelevant.

The ribosomes of thermophilic microorganisms have received considerable attention in recent years. The general features of the ribosomes of B. stearothermophilus are similar to those of mesophilic microorganisms, although there is evidence that the binding between the protein and RNA components is stronger in this thermophile than in E. coli (Mangiantini et al., 1965). Friedman and Weinstein (1966) found the ribosomes of B. stearothermophilus to contain 59% RNA and 41% protein, proportions similar to those reported for E. coli ribosomes (Tissieres et al., 1959). In contrast Mangiantini and co-workers (1965) found an average value of only 46% RNA in a different strain of B. stearothermophilus. Stenesch and Yang (1967) reported a similar low value for the RNA content of ribosomes from three strains of B. stearothermophilus and from three mesophilic species of the same genus. The ribosomes of the extreme thermophile Thermus Aquaticus (Brock and Freeze 1969) have been shown to contain 41% RNA (Zeikus et al., 1970). It would appear therefore that such differences as do occur in overall composition may have no particular significance for thermophily.

The thermal stability of ribosomes, as measured by their Tm value, has been studied by a number of groups. Comparisons between B. stearothermophilus ribosomes and those from E. coli (Saunders and Campbell, 1966; Friedman et al., 1967; and Mangiantini et al., 1965) have shown that, while the experimental conditions, particularly the concen-

tration of Mg^{2+} ions or spermidine, have a marked effect on the values obtained, the ribosomes from the thermophile give values for Tm consistently some 10^{o} to 20^{o} higher. Zeikus and co-workers (1970), for instance, reported Tm values of 86^{o}, 78^{o} and 71^{o} for the ribosomes from T. aquaticus, B. stearothermophilus and E. coli respectively. The relationship between ribosomal Tm value and maximum growth temperature has received particular attention from Pace and Campbell (1967). They demonstrated a very strong correlation between these parameters for a range of psychrophilic, mesophilic and thermophilic microorganisms. The increased stability of the ribosomes of thermophilic microorganisms has also been observed in studies of their biological activity. Algranati and Lengyel (1966) showed that whereas E. coli ribosomes lost 90% of their activity (measured by their ability to support poly A and poly U dependant incorporation of lysine and phenylalanine into polypeptide) after treatment at 65^{o} for 5 minutes the ribosomes of B. stearothermophilus were actually more active after such treatment. Similar results have been obtained by Friedman and Weinstein (1966) and Friedman and co-workers (1967).

The separate contributions of the protein and RNA to the thermostability of the ribosomes of B. stearothermophilus has been examined by a number of workers. Ribosomal RNA (rRNA) from thermophilic bacteria has been shown to have a higher guanine plus cytosine content and correspondingly higher Tm value than that from mesophilic

bacteria (Friedman et al., 1967; Mangiantini et al., 1965; Saunders and Campbell, 1966; Stenesh and Holazo 1967). The gross amino acid composition of B. stearothermophilus ribosomes reported by Saunders and Campbell (1966) does not differ significantly from that of E. coli ribosomes (Spahr, 1962) although polyacrylamide gel electrophoresis shows the individual proteins to be quite different (Nomura et al., 1968). (It should be borne in mind however that the differences found by Nomura's group were no greater than those between unrelated mesophiles.)

Nomura and co-workers (1968) have also prepared hybrid 30S ribosomal particles with protein and 16S rRNA from the ribosomes of E.coli and B. stearothermophilus. Their results indicate that the thermostability of B. stearothermophilus ribosomes is dependent upon properties of both the RNA and the proteins specific to the thermophile and that neither component alone is able to confer complete thermostability on a hybrid particle although the hybrid of E. coli RNA and B. stearothermophilus protein was much more stable than the native E. coli 30S particle.

The ribosomes of three strains of B. stearothermophilus were also more resistant than those of three mesophilic members of the same genus to the ribonuclease (EC 2.7.7.16) mediated release of soluble nucleotides which follows treatment with EDTA (Stenesch and Yang, 1967).

The other non protein element of the protein synthesizing machinery, the soluble or transfer RNA

(tRNA), has also been examined in thermophilic microorganisms. The thermal denaturation profiles of the tRNA from thermophilic bacilli have been found to be virtually identical to those obtained with the tRNA from mesophilic microorganisms (Friedman and Weinstein, 1966; Mangiantini et al., 1965; and Saunders and Campbell, 1966). The tRNA from T. aquaticus however did give a Tm of 86°, compared with a value of 80° for the tRNA of both E. coli and B. stearothermophilus determined under the same conditions (Zeikus et al., 1970). It would appear that, as with DNA, the thermal stability of tRNA is sufficiently great for the stability to acquire physiological significance only in microorganisms living, as does T. aquaticus, at temperatures approaching or greater than 80°.

The accumulated data on the protein synthesizing machinery of thermophiles summarized above leaves little doubt that these organisms have no special requirement for the rapid resynthesis of nucleic acids or ribsomes. The thermostability of cell walls and membranes is less well established however. The cell walls of B. stearothermophilus and B. coagulans have been examined and shown to contain a glyceroteichoic acid with some interesting features (Wicken, 1966; Forrester and Wicken 1966a and b) but the possible significance of the cell wall structure in relation to the thermophilic habit was not discussed. Chaloupka and co-workers (1962 and 1964) have shown that the diaminopimelate moiety of the cell wall mucopeptide of the mesophile B. megaterium KM turns over at a rate of 10-15% per generation.

The question of "rapid resynthesis" of the cell wall macromolecules of thermophilic macromolecules therefore remains open, although, in the light of the relatively high turnover rate seen in B. megaterium any turnover of components will have to be very carefully assessed in order to establish whether it is a special consequence of thermal degradation.

The remaining fraction of the cell to which Allen's rapid resynthesis hypothesis could apply is the cell membrane. The data on the thermostability of the adenosine triphosphatase and cytochrome oxidase (Table 1.1.) suggest that membrane bound enzymes from thermophilic microorganisms may not be any less resistant to thermal inactivation than the soluble enzymes. However when considering membrane function the question of organisation becomes important. A membrane bound enzyme must, besides its activity, retain a correct spatial relationship to other members of a metabolic pathway and remain accessible to its substrate(s). Recent work has left little doubt that the lipid constituents of membranes play a major role in the structure and function of membranes. Although phospholipids are not normally thought of as substances vunerable to thermal denaturation at moderate temperatures the possibility of phospholipid turnover, well established in animal tissues (for review see Dawson, 1966), must be considered. There is no general consensus of opinion on the extent and significance of phospholipid turnover in micro-organisms. Studies with Mycoplasma laidlawii B. (McElhaney and Tourtellotte, 1970) demonstrated

the absence of turnover, during growth, of the fatty acid, phosphorus or glucose moieties of the membrane phospholipids. Kanfer and Kennedy (1963) and Kanemasa and co-workers (1967) studied the turnover of 32-P in the phospholipids of E. coli. Both groups found that the phosphate of phosphatidyl ethanolamine was essentially stable but that of phosphatidyl glycerol was relatively labile. Kanemasa's group found a half life of about one hour for the 32-P-phosphatidyl glycerol and also found that the phosphate of cardiolipin was quite rapidly exchanged ($t_{\frac{1}{2}}$ approx. 3 hours). The only recorded study on a member of the genus Bacillus is that of Morrison and Morowitz (1970) who found no substantial turnover of the fatty acid moieties of the phospholipids of B. megaterium KM. As with cell walls the question of phospholipid turnover remains open and again any turnover that does occur may not be primarily a direct consequence of high temperature.

Any exceptional turnover of macromolecules that occurs will inevitably require the expenditure of energy. The expenditure of this "maintenance energy" has received considerable attention from microbiologists. Early experiments using batch cultures indicated that very low levels of energy expenditure could be expected. For example the data of Mallette (1963) suggest that a culture of E. coli would consume about 0.3 mMoles glucose per hour per gram (dry weight) of cells for maintenance. Continuous culture techniques have permitted more precise determinations but in general aerobic cultures of microorganisms

have yielded similar values. The accurate determination of maintenance energy requirement usually involves a study of the dependence of the molar growth yeild, Y, on the growth rate of the organism when controlled by the supply of a limiting nutrient (Pirt, 1956). However the value of the molar growth yield itself may provide, by comparison with the value obtained with other organisms under similar conditions, considerable insight into the energy requirements during growth of the organism in question. The massive energy wastage that the theory of rapid resynthesis would entail should be revealed by measurements of molar growth yield for thermophiles and comparison of the results with those obtained for closely related mesophiles. Farrell and Rose (1967) have pointed out the possible value of such measurements. This thesis contains a detailed analysis of the molar growth yield of B. stearothermophilus and the effects of temperature on the energy metabolism of the organism. As far as is known no study of this aspect of the biochemistry of thermophiles has previously been reported.

Some aspects of the failure of many thermophilic microorganisms to grow at "mesophile temperatures" have also been the subject of experiments to be described in this thesis. Foter and Rahn (1936) were the first to reason that since the growth of a microorganism may be regarded as being dependent on chemical reactions, growth rate should decrease with fall in temperature according to the Arrhenius

equation, at least until the medium freezes solid. However it is now well established (Farrell and Rose, 1967) that the temperature dependence of growth rate in thermophiles and mesophiles only obeys the Arrhenius relationship over a limited (20^{o} to 30^{o}) temperature range. Outside this range the growth rate falls off rapidly to zero. The lower temperature of growth of psychrophilic microorganisms may well result in the Arrhenius relationship being maintained in some of these cases to the point at which the medium freezes.

Farrell and Rose considered in their review (1967) a number of possible explanations for this deviation from the Arrhenius relationship. Cold lability, now well known as a property of many allosteric or membrane bound enzymes (Stadtman, 1966), was suggested by Farrell and Rose (1967) as a possible explanation of this phenomenon. However cold lability is associated most particularly with relatively pure enzyme fractions and under conditions which resemble or mimic those to be expected in their normal intracellular environment such as in crude cell extracts or in the prescence of high concentrations of polyhydric alcohols cold lability is not found (e.g. see Penefsky and Warner, 1965 and Dennis and Coultate, 1967). It seems unlikely therefore that cold lability is of significance in this context.

A more attractive hypothesis discussed by Farrell and Rose is that the function of cell membranes is dependent on the physical state of the membrane lipids, which is in turn dependent on temperature.

Heilbrun (1924) and Bělehrádek (1931) both suggested that the melting of lipids at high temperatures was the cause of thermal death. The frequent observation that the lipids of poikilothermic organisms contain higher proportions of unsaturated fatty acids or more highly unsaturated fatty acids if they are grown at low temperatures (for examples see Marr and Ingraham, 1962) led Gaughran (1947) to the opposite conclusion; that is, the membrane lipids must be in a molten state for the membrane to be biologically active.

More recently the availability of sophisticated physical techniques, in particular differential thermal analysis (D.T.A., also known as differential scanning calorimetry) has permitted a direct examination of this question.

Chapman and Collins (1965) showed that the significant conformational changes take place in pure phospholipids at temperatures far below their capillary melting point. The temperature at which these transitions take place is highly dependent on the nature of both the polar and non polar parts of the molecule. Corresponding thermal transitions have now been demonstrated in the whole cells and isolated membranes of Mycoplasma laidlawii (Steim et al., 1969) and Micrococcus lysodeikticus (Ashe and Steim, 1971). In the light of these results, others obtained by X-ray diffraction (Engelman, 1970 and 1971; Wilkins et al., 1971) and their own spin labelling studies Jost and co-workers (1971) have concluded that the biologically relevant structure of membrane lipid bilayers is that of "melted" lipid chains, showing

a considerable degree of flexing and not the close packed paracrystalline array one finds at low temperatures.

Steim (1969) and Tourtellotte and McElhaney (1969 have taken advantage of the extent to which the fatty acid composition of the membrane lipids of M.laidlawii may be modified by the supply of fatty acids in the growth medium to demonstrate the biological significance of these transition temperatures. Drastic effects on the cells, including changes in morphology and leakage of cell components, resulted when the incubation temperature fell below the manipulated transition temperature.

Studies of other microorganisms have shown that the degree of apparent "meltedness" (as judged from the fatty acid composition) may be more significant for some species than for others. Thus Esfahani and co-workers (1968) found that the extent of incorporation of exogenous fatty acids by an unsaturated fatty acid auxotroph of E. coli was primarily dependent on the melting properties of the fatty acid so that the apparent "meltedness" of the membrane lipids remained essentially constant. Sifieriz and co-workers (1973) have recently demonstrated that the Hill coefficient of the inhibition by Na^+ ions of the membrane-bound (Ca^{2+})-adenosine triphosphatase of E. coli was dependent on the degree of unsaturation of the membrane lipids. In contrast Weekes and Wakil (1970) found that the proportion of unsaturated fatty acids in the membrane lipids of Lactobacillus plantarum could be varied between 3 and 61% without noticeable

effects on the growth of the organism.

To date investigations of the membranes of Bacilli have been largely restricted to the analysis of their component lipids. With the exception of those of some psychrophilic species the membrane lipids of Bacilli contain only small proportions of unsaturated fatty acids (Kaneda, 1971a) but frequently large proportions of branched chain saturated fatty acids of both the iso- and anteiso- series (Shen et al., 1970; Kaneda, 1967 and Bulla et al., 1970). The effects of growth temperature on the average melting points of the fatty acids of Bacilli is remarkably slight. For example a comparison of the fatty acid compositions of the lipids from three mesophilic Bacillus species with those of three strains of B. stearothermophilus (Shen et al., 1970) revealed a somewhat lower proportion of anteiso fatty acids in the thermophile lipids. Although the anteiso fatty acids have melting points some 20° lower than the corresponding members of the unbranched and iso series the average melting point of the thermophile fatty acids was only 5° higher than that of the mesophiles. Fatty acid analyses of Thermus aquaticus (Ray et al., 1971; Heinen et al., 1970) and other unidentified hot spring microorganisms (Baumann and Simmonds, 1969) have not indicated a preponderance of high melting point fatty acids in these organisms.

Kaneda (1963 and 1971b) has shown that since the branched chain fatty acids are derived from the keto acid derivatives of branched chain amino acids

it is possible to modify the fatty acid composition of the lipids of Bacilli by the supply of such amino acids, or their derivatives, in the growth medium. Changes in fatty acid composition obtained in this way were not accompanied by obvious changes in morphology or growth rate. Daron (1970) and Yao and co-workers (1970) both demonstrated that the fatty acid composition of B. stearothermophilus lipids varied with the growth temperature. However the variations observed were no greater than those also obtained by Daron when the carbon source for growth was changed from acetate to glucose. It may well be therefore that in the Bacilli temperature dependent variations in fatty acid composition are merely secondary manifestations of changes in the pattern of amino acid metabolism.

The apparent similarity of physical properties between the membrane lipids of mesophilic and thermophilic Bacilli makes it unlikely that conformational changes in membrane lipids are responsible for the deviation from linearity of the Arrhenius plots of growth of thermophilic Bacilli at temperature at which mesophilic Bacilli grow readily. This question has also been studied by examination of temperature effects on membrane function in the intact organism. Bubela and Holdsworth (1966) attempted to measure the rate of transport of leucine and uracil into the cells of B. stearothermophilus as a function of temperature. However their technique also measured incorporation of these compounds into protein and nucleic acid respectively

rather than transport alone. Studies to be described in this thesis show that the respiratory capacity of the membrane of B. stearothermophilus shows no anomolous effects of temperature even at temperatures some 20° below the minimum for growth.

A promising approach to the problem of the non linearity at low temperatures in Arrhenius plots of growth rate is suggested by the work of Ingraham's group. Their studies with E. coli have shown that low temperatures have a drastic effect on biochemical regulatory mechanisms. In particular Ingraham's group have studied the effects of temperature on the control of enzyme synthesis. For example Ng (1963) showed that tryptophan failed to induce the synthesis of tryptophanase below 15° even though it was shown (a) that tryptophanase activity is stable in cells growing at low temperature and (b) that tryptophan does enter the cell at low temperatures. The same group have also demonstrated (Ng et al., 1962) that the extent of glucose repression of β - galactosidase (EC 3.2.1.23) synthesis is reduced at temperatures below 20° and is virtually abolished at 10°. These observations and others discussed by Ingraham and Marr (1965) suggest that derangement of the regulation of protein synthesis is a major factor in establishing the lower temperature limits for bacterial growth. Ingraham has suggested (see Farrell and Rose, 1967) that the most probable explanation of these effects is a temperature dependent change in the affinity of the inducer for the repressor protein.

Such changes in the affinity of small molecules for proteins could be expected to extend to the interactions of enzymes with substrates, inhibitors and allosteric effectors. Most of the early studies of temperature effects have been directed towards an understanding of the thermodynamics of enzymic catalysis rather than the effect of temperature on the physiology of the organism (e.g. see Dixon and Webb 1964). Recently Hochachka's group have made a particular study of the kinetic and regulatory parameters of a number of enzymes from fish muscle and related the effects of temperature on these parameters to the water temperature naturally experienced by the fish (Somero and Hochachka, 1968; Behrisch and Hochachka, 1969; Behrisch, 1969; Hochachka and Lewis, 1971; Somero and Johnson, 1970). Of less physiological significance but providing striking illustration of the magnitude of the changes in regulatory parameters that may accompany change in temperature are the results of Taketa and Pogell (1965) with rat liver fructose diphosphatase and Llorente and co-workers (1970) with pyruvate kinase from the same source. Taketa and Pogell found that the concentration of AMP for half maximal inhibition ($I_{0.5}$) of fructose diphosphatase fell from about 0.5mM at 46° to 0.005mM at 2°. In contrast the concentration of phosphoenolpyruvate (PEP) for half maximal activity ($S_{0.5}$) of pyruvic kinase increased with temperature so that at low PEP concentrations there was an inverse relationship between temperature and activity.

The effect of temperature on the allosteric transitions of rabbit muscle phosphorylase b (EC 2.4.1.1) has been the subject of detailed analysis by Kastenschmidt and co-workers (1968) who found that the values of the constants L^1 and L, representing the relative proportions of the R and T forms of the enzyme (as defined by Monod et al., 1965) in the prescence and absence of the substrate, glucose-1-phosphate, fall by factors of approximately 1000 and 600 respectively as the temperature was lowered from 29° to 4°.

At the present time there have been few observations of temperature effects on the regulatory parameters of enzymes from microorganisms. The deoxythymidine kinase (EC 2.7.1.) of E. coli has been subjected to a similar analysis to that of phosphorylase (Iwatsuki and Okazaki, 1967). At low temperatures the sigmoidicity of the plot of the response of reaction velocity to increasing ATP concentration was abolished and the affinity of the enzyme for the substrates increased so that an inverse effect of temperature on velocity at low substrate concentrations, first noted with rat liver pyruvate kinase (Llorente et al., 1969), was found. Iwatsuki and Okazaki did not consider any physiological implications of their results. O'Donovan and Ingraham (1965) have described a cold sensitive mutant of E. coli which required histidine for growth only at low temperature (20°). The first enzyme of histidine biosynthesis, phosphoribosyl ATP pyrophosphorylase (EC 2.7.6.1) was found to be

1000 times more sensitive to feedback inhibition by histidine in the mutant than in the parent strain. The enzymes from both mutant and parent were also found to have their sensitivity to histidine inhibition increased about five times as the temperature was lowered from 37° to 20°. In the case of the mutant this temperature effect was sufficient to cause a cessation of growth at the lower temperature in the absence of exogenous histidine.

A similar increase in sensitivity to allosteric inhibition with lowering of temperature was observed by Kuramitsu (1970) with the threonine and lysine inhibition of the aspartokinase from B. stearothermophilus. At 23° 1 mM threonine almost completely inhibited the enzyme but at 55° only 50% inhibition was obtained at saturating levels of threonine. Another example is the allosteric regulation of the pyruvate carboxylase of B. stearothermophilus an enzyme required by the organism for the anaplerotic fixation of carbon dioxide during growth on glucose (Sundaram et al., 1969). Like the enzyme from some other sources it requires acetyl CoA as a positive effector and is inhibited by L-aspartate (Cazzulo et al., 1970). The concentration of acetyl CoA required for half-maximal activation was virtually unaffected by temperature but the inhibition by L-aspartate, as indicated by its antagonism of acetyl CoA activation doubled when the temperature was raised from 30° to 51.5°.

The effects on the metabolism of the entire cell that changes in temperature may cause through

changes in the patterns of enzyme activity and regulation have only recently become the subject of detailed examination. In a recent review Rose (1969) has shown that such effects may be relatively minor but major effects of temperature alterations on the composition of microbial cells are also found. For example the production of the red pigments of both Serratia marcescens (Williams et al., 1965) and B. cereus var. alesti (Uffen and Canale-Parola, 1966) is enhanced at temperatures below the optimum for growth. More significant effects may be illustrated by those on the fatty acid composition of the lipids of some microorganisms mentioned earlier in this introduction and changes in the carbohydrate content of the cells of Candida utilis (Brown and Rose 1969) grown under conditions of NH_4^+ limitation in a thermostat.

The observation of Campbell and Williams (1953) that many strains of thermophilic Bacilli have nutritional requirements that vary with the growth temperature indicates that the patterns of metabolic activities of thermophiles as well as mesophiles and psychrophiles may be modified by changes in temperature. However, while the supply of a particular nutrient may sometimes permit growth at a somewhat lower temperature the anamolous form of Arrhenius plots of growth rate remains.

The final part of this thesis presents the results of a brief examination of the effects of temperature on the regulation of an enzyme with a central role in cell metabolism pyruvate kinase.

The study of regulation, as affected by temperature, is of potential significance in another context. The molar growth yield studies to be described in this thesis reveal that the temperature optimum for rate of growth and that for most efficient utilization of substrate need not be identical. At higher temperatures (where growth is fastest) it will be shown that more of the carbon of the substrate is wasted and that this phenomenon may well be the consequence of a derangement of metabolic regulation at these temperatures.

1.2. Generally applicable methods.

1.2.1. Materials. All laboratory chemicals were the purest grades normally available, usually the "Analytical Reagent" grades of British Drug Houses Ltd. or Fisons Scientific Apparatus Ltd. Biochemical reagents, including enzyme preparations, were the products of either the Sigma Chemical Company Ltd. or the Boehringer Corporation Ltd. Glass distilled water prepared in the laboratory was employed throughout this work.

1.2.2. Organisms. With the exception of one experiment in which the mesophile Bacillus licheniformis was used all the experiments described in this thesis were conducted with a single strain of the thermophile B. stearothermophilus. This strain was a generous gift to this laboratory from Professor N. Grossowicz of the Hebrew University-Hadassah Medical School, Jerusalem, Israel. This organism was originally described as B. coagulans (Sundaram and Kornberg 1969; Cazzulo, Sundaram and Kornberg 1969; Sundaram et al. 1969) but has since been characterized as an amylase-

negative variant of B. stearothermophilus (Epstein and Grossowicz 1969a). A closely similar strain of B. stearothermophilus has been described by Daron (1967).

The growth requirements of this thermophile are remarkably simple; rapid growth is obtained in minimal salts media containing only a single carbon source. As discussed elsewhere this facility for prototrophic growth (rare amongst thermophiles) makes it ideally suited to studies of the energy requirements for growth.

Cultures of the thermophile were maintained on nutrient agar slopes incubated overnight at 55° and then stored, tightly sealed to prevent drying out, at 4° for 1-2 months. Cells taken from solid to liquid media were always first grown in nutrient broth; this procedure shortens considerably the long lag period that is observed when cells are transferred directly from nutrient agar slopes to liquid, minimal salts media.

This strain of B. stearothermophilus lacks the ability to reduce nitrates (Epstein and Grossowicz 1969a) and must therefore be vigorously aerated during growth on all liquid media. For most purposes in this work (and except where indicated in the text) a gyrotary shaking incubator (New Brunswick Scientific Co., Model G25), maintained at a temperature of 55°, was employed for the incubation of liquid cultures. Normal Erlenmeyer flasks with liquid medium occupying about 20% of flask's capacity were used except in certain experiments in which a water bath shaker having a reciprocal shaking action was used (see Section 3.2.2.).

1.2.3. Media. A number of different media were employed for the culture of microorganisms in this work.

Solid media. Stock cultures of microorganisms were maintained on slopes of Oxoid Nutrient Agar (No.1) prepared according to the manufacturers directions.

Liquid media. As demanded by the requirements of particular experiments the following media were employed.

a) Nutrient broth. Oxoid Nutrient Broth (No.1) prepared according to the manufacturers directions.

b) Nutrient broth/succinate. Nutrient broth as described above supplemented with sodium succinate to give a final concentration of 10 mM. This supplementation was necessary to ensure that a cell density of at least 0.5 mg dry weight per ml. was reached in cultures of the thermophile.

c) Minimal salts medium. The salts medium employed was essentially that described by Epstein and Grossowicz (1969a) except that their procedure for the preparation of the trace elements solution (described by Baker and co-workers(1960)) was not adopted. The medium contained, per litre: Sodium Chloride, 1g; potassium chloride 1g; ammonium nitrate, 1g; dipotassium hydrogen phosphate, 5.02g; potassium dihydrogen phosphate, 1.52g; disodium ehtylenediaminetetracetate ($EDTANa_2$), 80 mg; trace element solution, 2 ml and D-biotin 50 µg. The trace element solution was prepared by dissolving the following reagents in a mixture of

concentrated hydrochloric acid (100 ml) and water (100 ml): $MgCl_2$, 4g; $Fe_2(NH_4)_2(SO_4)_3.6H_2O$, 2.5g; $MnSO_4$, 0.2g; $CoSO_4.7H_2O$, 20 mg; $ZnSO_4$, 0.2g; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1g and $CaCl_2.6H_2O$, 4g. Although the strain og B. stearothermophilus used in this work will grow adequately in the absence of biotin work in this laboratory (Sundaram et al. 1969; Sundaram 1973) showed that with certain carbon sources the presence of biotin in the culture medium considerably enhanced the rate of growth.

The carbon sources used (and their concentrations) varied with the requirements of particular experiments and with the exception of glucose were added to the medium prior to sterilization. All media were sterilized by autoclaving at a pressure of 15 lbs per square inch for 20 minutes. Where applicable glucose (as a sterile solution) was incorporated after autoclaving.

Abbreviations. The following abbreviations are employed in this thesis for the identification of liquid media:

Nutrient broth	- NB
Nutrient broth/succinate	- NB/S
Minimal salts	- MS

Except where otherwise indicated the carbon sources included with minimal salts medium were at a concentration of 50 mM, and indicated, when at that concentration, thus:

MS plus acetate	- MS/A
MS plus glucose	- MS/G
MS plus succinate	- MS/S

2. PROTEIN STABILITY

2.1. Introduction

Studies of mesophilic microorganisms have established that significant levels of protein turnover only occur in these organisms in the absence of growth. (Mandelstam 1960 and 1963, Halvorson 1962). Turnover appeared to occur in response to a requirement for the synthesis of new enzymes under conditions where little net synthesis of protein could occur such as during starvation of essential amino acids (Mandelstam 1968), sporulation (Mandelstam and Waites, 1968) and the intermediate lag phase of diauxic growth (Halvorson 1962). The dependence of protein synthesis, under conditions of nitrogen starvation, on the release of amino acids by protein turnover has been confirmed recently by Goldberg (1971). He showed that the isopropyl thiogalactoside induced synthesis of β-galactosidase in nitrogen starved *E. coli* cells could be prevented by specific inhibitors of serine proteases. The inhibitors (phenyl methane sulphonyl fluoride and p-toluene sulphonyl fluoride) had no effect on growing cells but inhibited the degradation of proteins in non-growing cells.

This role of protein turnover in the nitrogen economy of mesophilic organisms is quite different from that suggested for protein turnover in thermophiles. The "rapid repair" theory of Allen (1953) requires a facility for exceedingly rapid turnover of protein in order to reuse the amino acids of the thermally inactivated enzymes in thermophiles growing

at high temperatures. This theory has been generally discarded in view of the relatively great thermostability of virtually all thermophile proteins examined. However, the report by Bubela and Holdsworth (1966) of very rapid protein turnover in B. stearothermophilus appeared to lend fresh support for the theory. The techniques adopted by Bubela and Holdsworth unfortunately did not meet the criteria laid down by Tarver (1954) and Mandelstam (1960) for obtaining meaningful results from turnover experiments and it was felt that a further examination of the turnover question was warranted. During the course of this work Epstein and Grossowicz (1969b) published details of their study of protein turnover in a prototrophic strain of B. stearothermophilus. They found that under non-growing conditions (nitrogen - starvation) the rates of protein turnover were similar to those established in mesophilic microorganisms. During growth they were unable to detect any protein turnover over a period of 1 hour (equivalent to about one mean generation time).

The experiments to be described here show that a measurable level of protein turnover does occur during growth of B. stearothermophilus at 55° but it is in no way compatible with the results of Bubela and Holdsworth or the "rapid repair" theory of Allen.

The usual experimental procedure in protein turnover experiments, and the one adopted in most of the turnover experiments to be described here is as follows. Growing cells are exposed for several generations to an isotopically labelled amino acid

in the culture medium which is thus incorporated into the proteins of the cell. It is assumed that this procedure will ensure uniform labelling of all the cell's proteins with the radiactive isotope. The cells are withdrawn from the labelling medium, washed and incubated under appropriate experimental conditions in the presence of a high concentration of the non-radioactive form of the same amino acid. The loss of label from the cell protein may then be followed with only a small possibility of error from reincorporation.

An alternative method of preventing reincorporation of labelled amino acid is to prevent protein synthesis altogether with an inhibitor such as chloramphenicol. This treatment will, of course, prevent further growth of the cells and render the system somewhat unphysiological. However there is little reason to suppose protein stability within the cell will be significantly altered under these conditions, at least during the first few hours. This supposition proved substantially correct and left the way open for experiments designed to test the stability of particular enzymes in the intact cell after chloramphenicol treatment. This approach has recently been adopted by Sirotrak (1971) in a study of the dihydrofolate reductase (EC 1.5.1.4) of Diplococcus pneumoniae. The observation that synthesis of the glyoxalate cycle enzyme, isocitrate lyase is repressed during growth of B. stearothermophilus on glucose as sole carbon source facilitated confirmation with this particular enzyme of the

results obtained with chloramphenicol.

2.2. Methods

2.2.1. Protein turnover during growth.

The method for the study of turnover was essentially that of Mandelstam (1960). The organism was grown for several generations in the appropriate medium supplemented with L-[1-^{14}C] leucine in order to ensure general labelling of the cellular proteins with the radioactive amino acid. The culture was harvested under aseptic conditions while still in the exponential phase of growth by centrifugation at 25°. The cells were washed with medium containing unlabelled L-leucine (1mgml^{-1}), inoculated into a fresh medium also supplemented with unlabelled L-leucine (1 mg ml^{-1}) and incubation was continued. Except where otherwise indicated this medium was the same as that used in the labelling phase of the experiment. The amount of radioactive protein remaining undegraded in the growing cells was determined at appropriate times as follows. Portions (1ml) of the culture, usually in triplicate, were taken into 1 ml of 10% (w/v) trichloracetic acid at 0°. After standing for at least 30 minutes the precipitate was collected on glass fibre filters (Whatman GF/C diameter 2.5 cm) and washed several times with cold 5% (w/v) trichloracetic acid. The last traces of water and trichloracetic acid were removed by washing the filter with acetone. After drying, the radioactivity of the precipitate was measured with a Packard 4000 Scintillation Spectrometer using a toluene based scintillation fluid

(0.5% (w/v), 2,5 diphenyloxazole and 0.03% (w/v) 1,4-bis [2(4-methyl-5-phenyl-oxazolyl)] benzene in toluene).

2.2.2. Protein degradation in cells treated with chloramphenicol.

The organism was grown for several generations in the presence of L-[I-^{14}C] leucine until a cell density of 0.25 - 0.35 mg dry weight per ml was reached when growth and further incorporation of radioactive leucine into protein were stopped by the addition of chloramphenicol (250 μg ml^{-1}) (Cazzulo et al., 1969). The radioactive protein remaining undegraded during continued incubation was measured at suitable times as described in Section 2.2.1.

2.2.3. Stability of enzyme systems in intact cells treated with chloramphenicol.

The organism was grown on MS/A until a cell density of 0.30 - 0.40 ug dry weight per ml was reached. Chloramphenicol (250 μg ml^{-1}) was then added to stop further enzyme synthesis and growth and incubation was continued. At intervals thereafter samples (50-100 mls) were removed and cell extracts prepared as follows. The cells from accurately determined volumes of samples were harvested by centrifugation at 5°, washed once with 50mM tris (hydroxymethyl)-amino methane hydrochloride (tris-HCl) buffer (pH 7.6) containing 0.15 M potassium chloride and 1 mM EDTA and each lot of cells was suspended in the same buffer to a final volume of 4 ml. Lysozyme (EC 3.2.1.17) (750 μg) was added and the mixture was incubated at 37° for 30 minutes. 1mM EDTA was added

to give a final volume of 8 ml and the mexture was centrifuged at 25000X g for 30 minutes at 5°. Enzyme activities in the supernatent fraction (cell-free extract) were determined spectrophotometrically at 30° using a Unicam SP800 spectrophotometer. The following assay systems were used.

Pyruvate carboxylase was assayed by the method of Cazzulo and co-workers (1970). The reaction mixture (contained in glass cuvettes of 1 cm path length) consisted of 300 μ moles of tris-HCl, pH 8.0; μmoles of magnesium chloride; 300 μmoles of potassium bicarbonate; 10 μmoles of sodium pyruvate; 0.65 μmoles of NADH; 0.2 umoles of acetyl coenzyme A; 5 umoles of ATP; 1 unit of pure malate dehydrogenase; 0.1 ml of cell-free extract and water to a final volume of 3 ml. The reaction was started by the addition of the ATP and the oxidation of NADH observed at 340 nm.

Isocitrate lyase was assayed by the method of Kornberg (1963). The reaction mixture (contained in quartz cuvettes of 1 cm pathlength) consisted of 25 μmoles of imidazole-HCl, pH 6.8; 5 μmoles of magnesium chloride; 1 μmole of EDTA; 4 μmoles phenylhydrazine-hydrochloride; 0.1 ml of cell-free extract and water to 1.00 ml. The reaction, which was initiated by the addition of isocitrate, was followed by monitoring the change in absorbance at 324 nm (against a blank containing all the ingredients except isocitrate) due to the formation of the phenylhydrazone of glyoxalate.

Isocitrate dehydrogenase was assayed by the method of Self and Weitzman (1970). The reaction mixture contained (in glass cuvettes of 1 cm path

length) 20 µmoles tris-HCl pH 8.0; 10 µmoles of magnesium chloride; 1 µmole of EDTA; 0.2 µmoles of NADP; 2 µmoles sodium DL isocitrate; 0.05 ml of cell-free extract and water to a final volume of 1 ml. The reaction was started by the addition of the cell-free extract and was followed by the change in absorbance at 340 nm due to the reduction of NADP.

Pyruvate kinase was assayed by a procedure based on that of Bucher and Pfleiderer (1955). The reaction mixture (contained in glass cuvettes of 1 cm path length) 250 µmoles of tris-HCl pH 8.0; 12.5 µmoles each of phosphoenol pyruvate, ADP and magnesium chloride; 125 µmoles of potassium chloride; 0.167 µmoles of NADH; 100 µg of lactate dehydrogenase and water to a volume of 2.4 ml. The reaction was started by the addition of 0.1 ml of cell-free extract and followed by the fall in absorbance at 340 nm as the NADH was oxidised by the pyruvate produced by the action of the pyruvate kinase.

Malate dehydrogenase was assayed by the method of Ochoa (1955). The reaction mixture contained (in glass cuvettes of 1 cm pathlength) 25 µmoles of tris-HCl pH 7.4; 0.165 µmoles of NADH; 0.25 µmoles of oxaloacetate and water to a volume of 0.98 ml. The reaction was started by the addition of 0.02 mls cell-free extract and was followed by the fall in absorbance at 340 nm due to the oxidation of NADPH.

Oxygen uptake: The rate of respiration of intact cells following treatment with chloramphenicol was examined in similar experiments to those described

above. Suitable volumes of the cultures were withdrawn at intervals following chloramphenicol (250 μg^{ml-1}) addition and diluted with fresh medium which had been heated to the growth temperature (55^{o}). The rate of oxygen uptake of the diluted cultures was then measured at 55^{o} with an oxygen electrode as described later in this thesis.

2.2.4. Stability of isocitrate lyase in growing cells.

The organism was grown on MS/A for several generations and harvested by centrifugation (at 25^{o}) while still in the exponential phase of growth. They were then resuspended in MS/G and incubation continued. At this point and at appropriate intervals thereafter samples of the culture (80 ml) were withdrawn and the total isocitrate lyase activity was determined by the methods already presented in Section 2.2.3.

2.3. Results.

2.3.1. Protein degradation during growth. The rates of protein degradation during growth were examined during growth at 55^{o} on both minimal salts medium with 50 mM glucose as carbon source and also nutrient broth supplemented with 10 mM sodium succinate and are shown in Figure 2.1. It will be seen that there is a striking difference between the rate of breakdown on the two media. During growth on the salts medium the labelled proteins were degraded slowly, a half life ($t_{\frac{1}{2}}$) of 17.5 hr was calculated from this plot. In contrast proteins labelled during growth on nutrient broth were degraded to a significant extent during continued growth on the same medium.

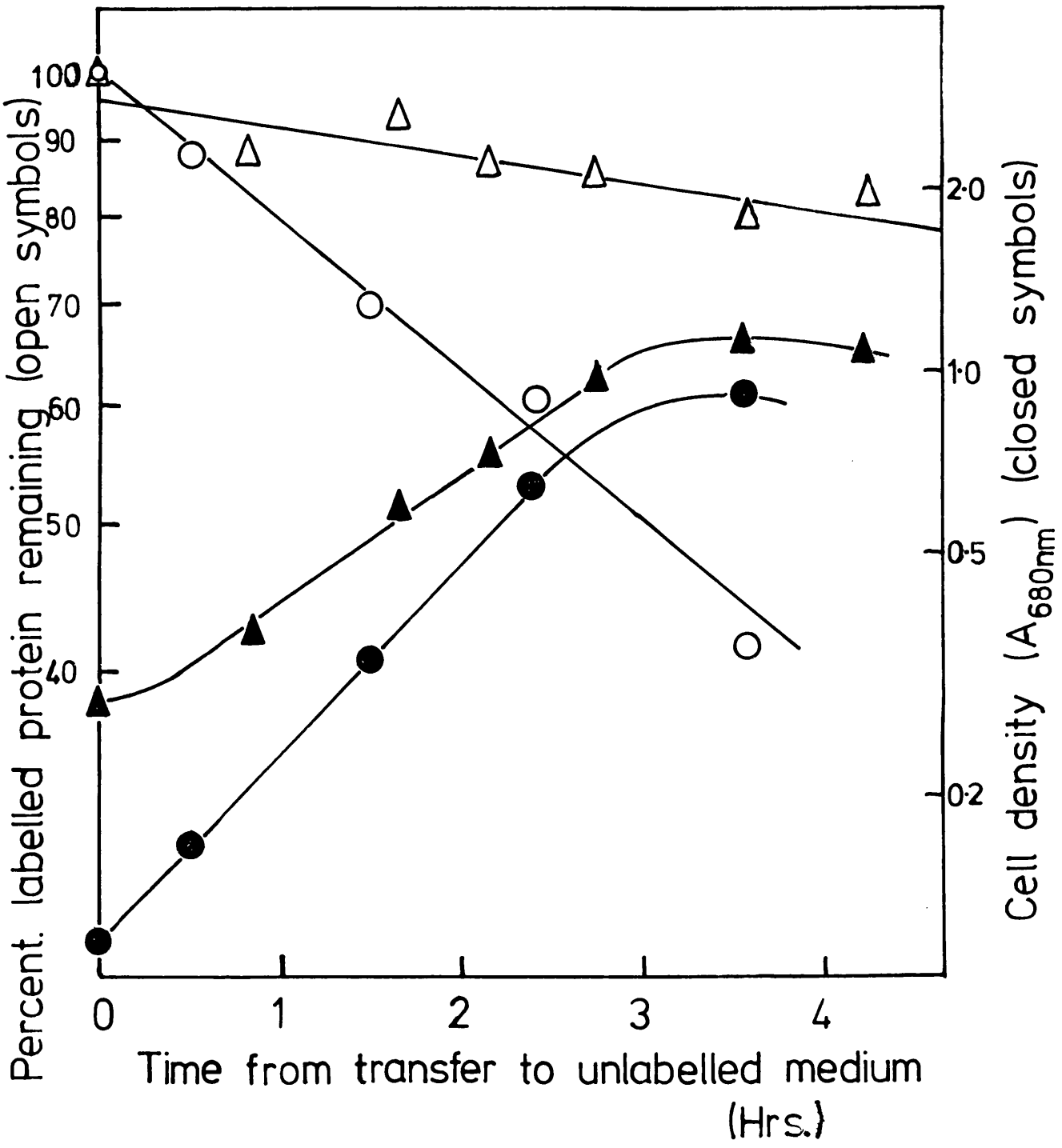


Figure 2.1. The stability of labelled protein during growth on a complex medium (NB/S) (circles) and on a minimal medium (MS/G) (triangles).

In this case the value of $t_{\frac{1}{2}}$ was 3 hr. Taking into account the different growth rates on the two media one can calculate that, in the exponential phase of growth, there is 5% degradation per generation (1.3 hr) on the salts medium and 19.5% degradation per generation (1 hr) during growth on the nutrient broth. Under virtually identical conditions and with the same strain of B. stearothermophilus Epstein and Grossowicz (1969b) were unable to detect any loss of labelled protein during growth on a minimal salts medium after 1 hr. The low rate of turnover on this medium found in the experiment reported here would probably have gone undetected over a period of only 1 hr. In the present study turnover was examined over 4 hours during which time the cell mass increased 4 fold (Fig. 2.1).

These results show clearly that under growing conditions the rate of protein turnover is not nearly high enough to support Allen's hypothesis (1953). Even during growth on nutrient broth only one fifth of the cell protein is degraded while the cell mass is doubled.

The marked difference in the degradation rates on the two media was rather unexpected although in studies with E. coli Podolsky (1953) found that the addition of casein hydrolysate or broth to a minimal salts medium raised the rate of protein turnover during growth over a 30 hr. period from about 8% to about 40% (i.e. 0.27%/hr. to 1.3%/hr.).

Three possible explanations, not necessarily mutually exclusive, may be considered. The first is

that the protein pool of cells grown on the minimal medium contains a higher proportion of heat stable enzymes; for instance enzymes required for the synthesis of amino acids and other metabolites supplied by the nutrient broth may be more stable. This seems improbable and the data of Table 1.1 provide no support for this suggestion. The second possibility, also not very probable, is that during growth on a minimal salts medium the chemical or physical properties of the cytoplasm are less conducive to the thermal inactivation of enzymes. The third and most plausible hypothesis is that the activity of proteolytic enzymes, essential if thermal inactivation is to be manifested as degradation, is dependent on the nature of the growth medium.

A number of experiments were performed in an effort to clarify this point. The first step was to establish ~~the~~ with greater precision which media components induced a high rate of turnover. Labelled cells were prepared by growth of the organism on MS/S supplemented with L- [1-14C] -leucine. After harvesting and washing in unlabelled medium the rate of protein degradation was followed during growth on batches of MS medium with the same carbon source but modified by the inclusion of separate components of nutrient broth as indicated in Figure 2.2. The presence of peptone as nitrogen source, or the addition of beef or yeast extracts did not induce any more protein degradation than that found during growth on unmodified MS medium. In this experiment the labelled proteins had a half life of about 27 hr. In another

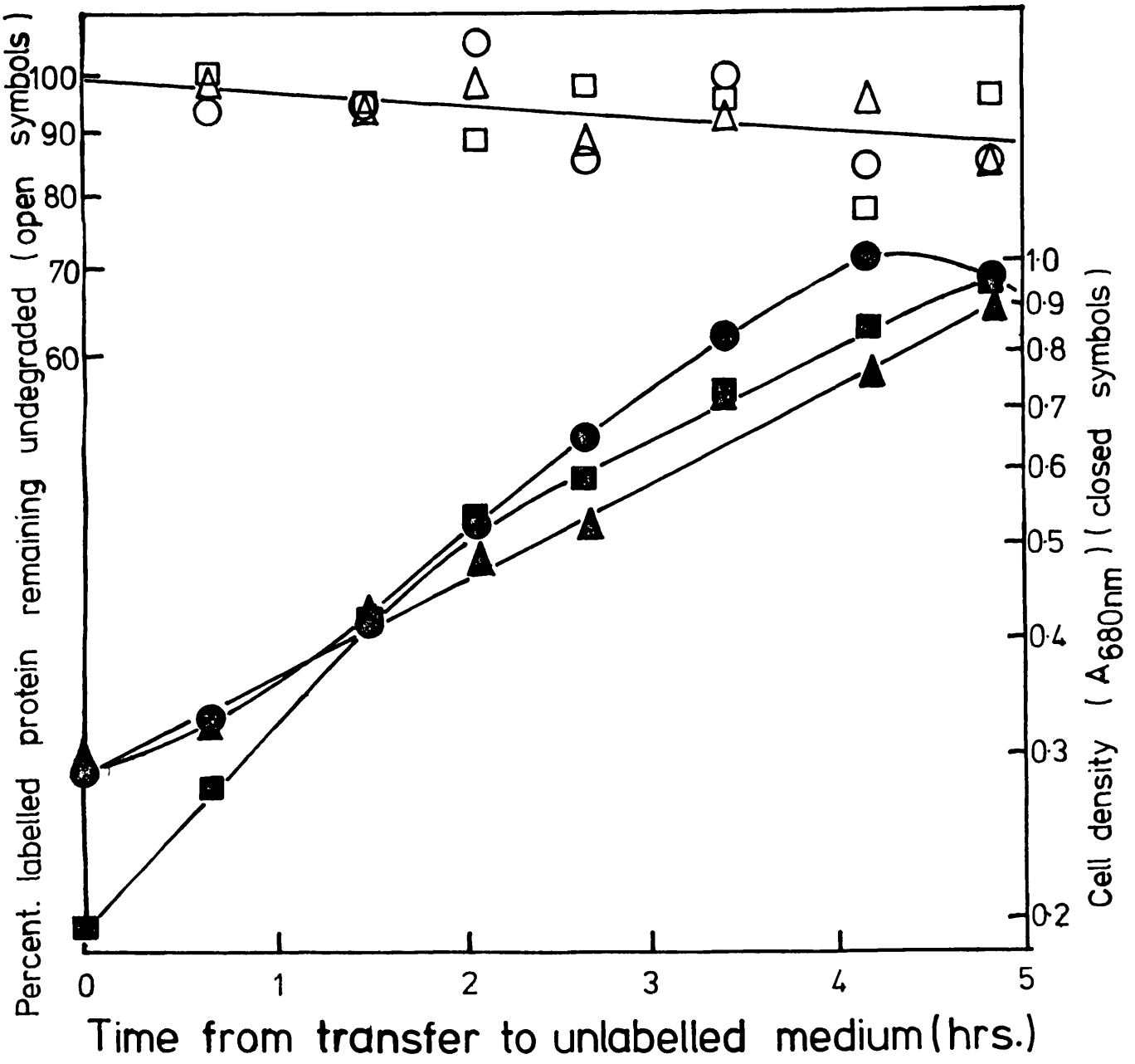


Figure 2.2. The stability of labelled protein during growth after transfer from minimal medium (MS/S) to supplemented minimal media: MS/S plus 2% peptone but lacking NH_4NO_3 (circles); MS/S plus 0.1% beef extract (triangles); MS/S plus 0.2% yeast extract (squares).

experiment cells were labelled as before with L- [1-14C] -leucine during growth on MS medium, harvested and allowed to grow in NBS medium. Protein turnover was followed over a period of 6 hours, the extended period made possible by an intermediate 10-fold dilution with fresh NBS medium. As shown in Figure 2.3 the rate of turnover observed was similar to that characteristic of growth in minimal salts medium rather than the richer nutrient broth (see Fig. 2.1). This apparent anomaly is probably explained by the somewhat different procedures followed in the two experiments. In the experiment presented in Figure 2.1 cells from a nutrient agar slope were directly inoculated into nutrient broth and exposed to radioactive leucine in this medium after a brief interval to allow for exponential growth to be established. In the experiments presented in Figures 2.2 and 2.3 cells from a slope were grown up in nutrient broth and a small portion of this culture served as the inoculum for the culture grown on minimal salts medium in which the labelling took place. Thus the history of the cells that were used in these experiments appeared to have a bearing on the rate of turnover observed.

The results of a further experiment, which are presented in Figure 2.4, are consistent with this idea. In one case a culture that had been established in minimal salts medium served as the inoculum for a nutrient broth culture containing radioactive leucine; the labelled cells were collected and transferred into fresh, non-radioactive NB/S where protein degradation

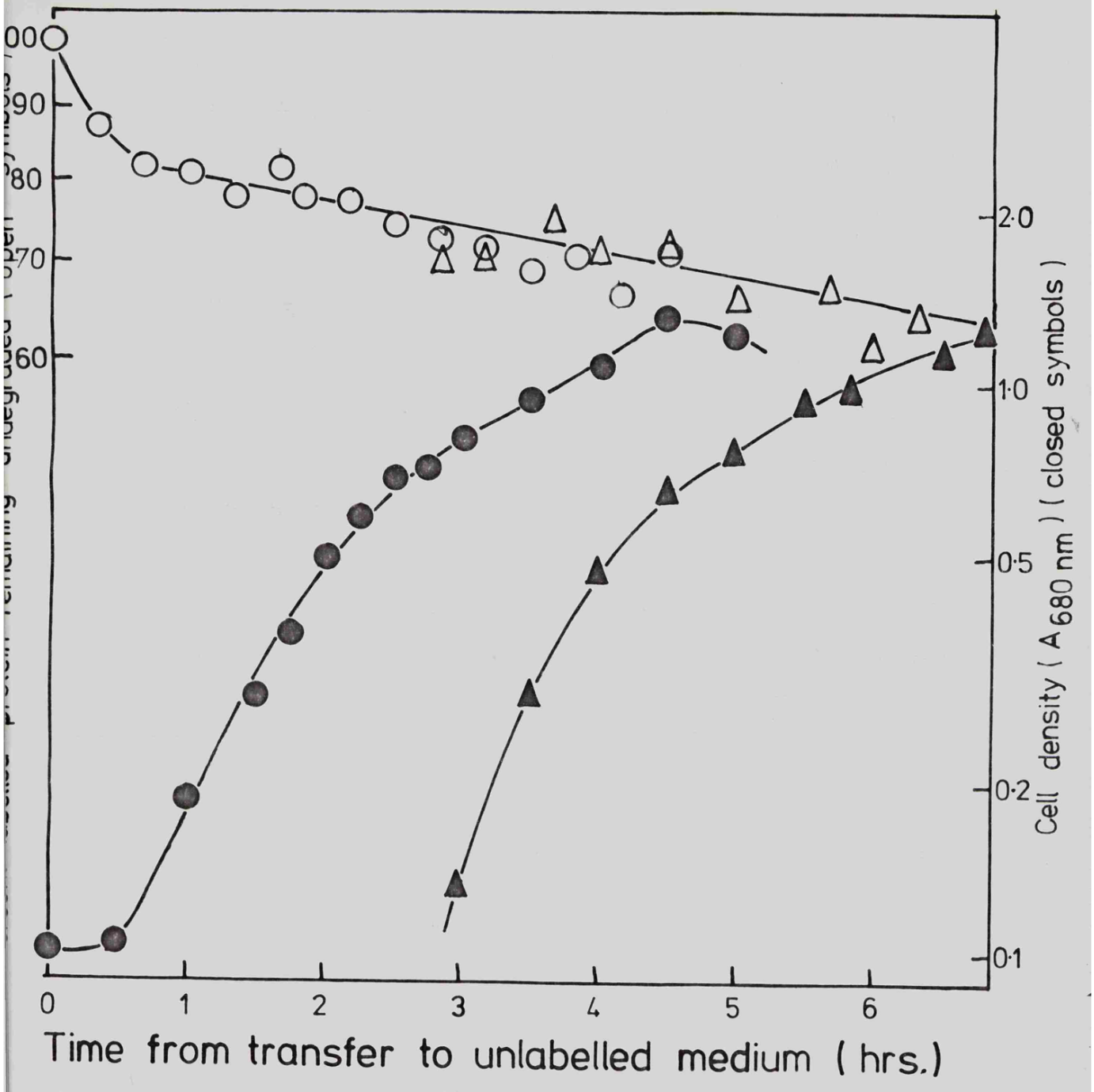


Figure 2.3. Stability of protein labelled during growth on minimal medium (MS/S) after transfer to complex medium (NB/S) (circles) and after further dilution (10x) with fresh NB/S.

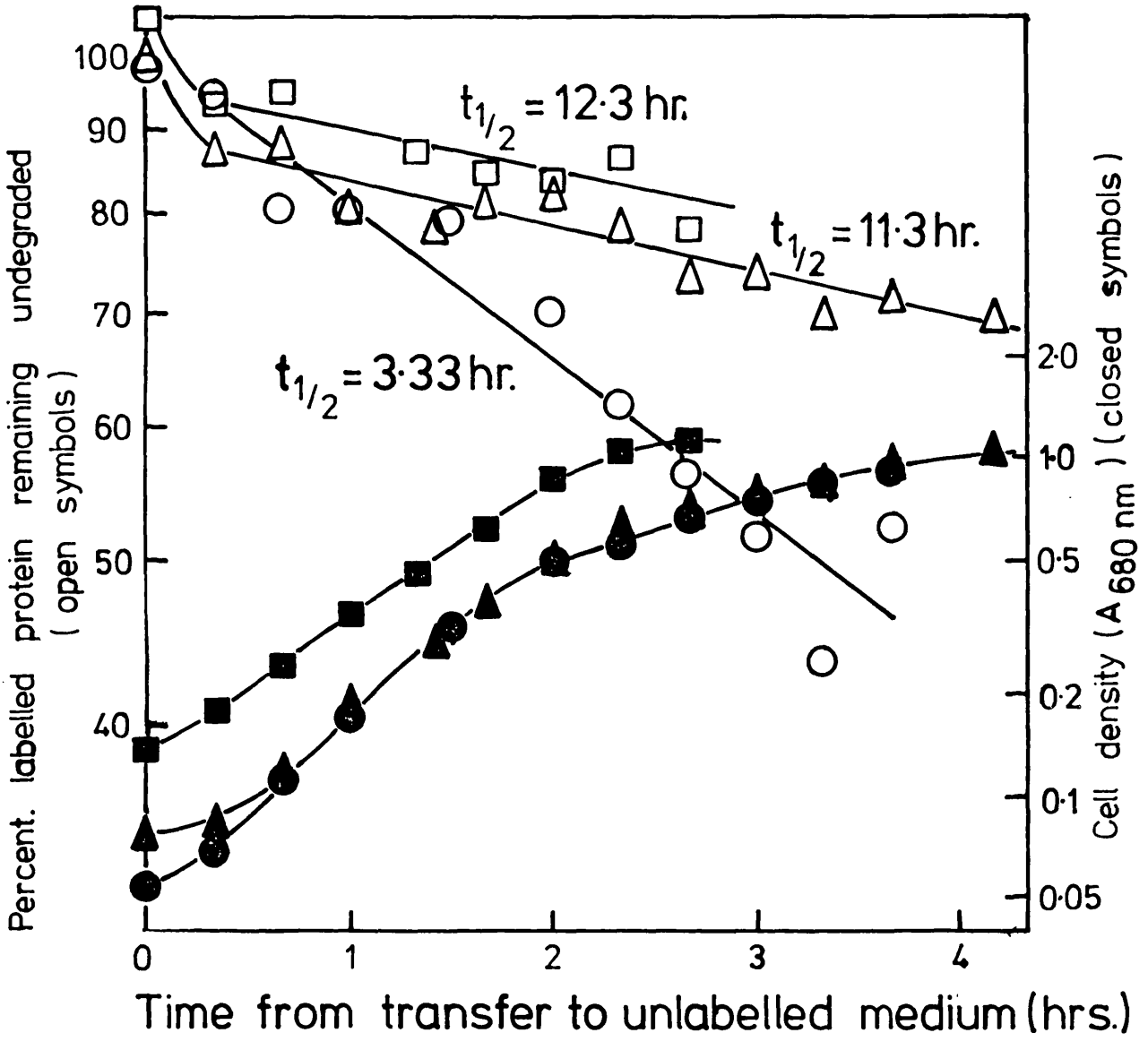


Figure 2.4. The stability of protein labelled during growth of cells (obtained directly or indirectly from stock nutrient agar slopes) on complex and minimal media. The sequences of different media and the stages at which the proteins were labelled and their degradation subsequently followed were as follows:

(A) Slope - NB/S* - NB/S (circles)

(B) Slope - NB/S - MS/S* - NB/S (triangles)

(C) Slope - NB/S - MS/S - NB/S* - NB/S (squares)

The labelling stage is indicated with an asterisk, the stage during which degradation was monitored is underlined.

was followed (curve A). In a second case cells labelled in minimal salts medium were transferred to NB/S where the course of protein degradation was followed (curve B). In a third case cells from a nutrient agar slope were inoculated directly into NB/S containing radioactive leucine; the labelled cells were taken into fresh NB/S where protein degradation was again monitored (curve C). Only in the last case was the high turnover rate observed. Thus an uninterrupted passage from slope through the rich medium of nutrient broth appears to be necessary for the high rate of protein degradation to be manifested.

A possible explanation is that proteolytic activity associated with the cells on the solid medium, perhaps arising during the formation or germination of spores, is maintained during subsequent growth in nutrient broth and is involved in the protein turnover observed. An experiment in which cells from the slope were labelled in nutrient broth and then transferred to minimal salts medium for the measurement of protein degradation might have yielded useful information on this point. However the long lag phase that follows such a transfer prevents the meaningful interpretation of any result that may be obtained.

Attempts to demonstrate an elevated level of proteolytic activity in cells which exhibited the high rate of protein degradation were unsuccessful. No protease activity could be detected in any cell extracts by the <u>in vitro</u> assays described by Mandelstam

and Waites (1968) using casein as substrate and Lin and co-workers (1969) using N,N dimethyl casein as substrate. This failure may be attributed to two possible causes. The maximum turnover rate observed (about 20% per hour) would be equivalent, in an *in vitro* assay, to an enzyme activity of only 0.2 mg of protein hydrolysed per hour per mg of protein in the cell extract. If the level of proteolytic activity in the cell did approach such a low, limiting value the figures just quoted indicate that it would be very difficult to detect by these assays. It may be significant that in their study of *B. cereus* Aronson and co-workers (1971) found that the protein turnover associated with sporulation in this organism (a rate of about 6% per hour) was unchanged in mutants having greatly reduced levels of proteolytic activity.

Secondly the proteolytic activity may be similar to that described in *B. licheniformis* by Bernlohr and Clark (1971) in being highly specific for denatured proteins so that it would go undetected in assays using native proteins as substrates.

It must be said that these results do not define the precise origin of the higher rate of protein turnover in nutrient broth grown cells. However further investigation of this comparatively minor point appeared fraught with technical complexities was not therefore undertaken.

2.3.2. Protein degradation in non-growing cells.

Protein degradation in non-growing cells was investigated by the use of chloramphenicol (CAP). That CAP at the concentration used (250 $\mu g\ ml^{-1}$) would

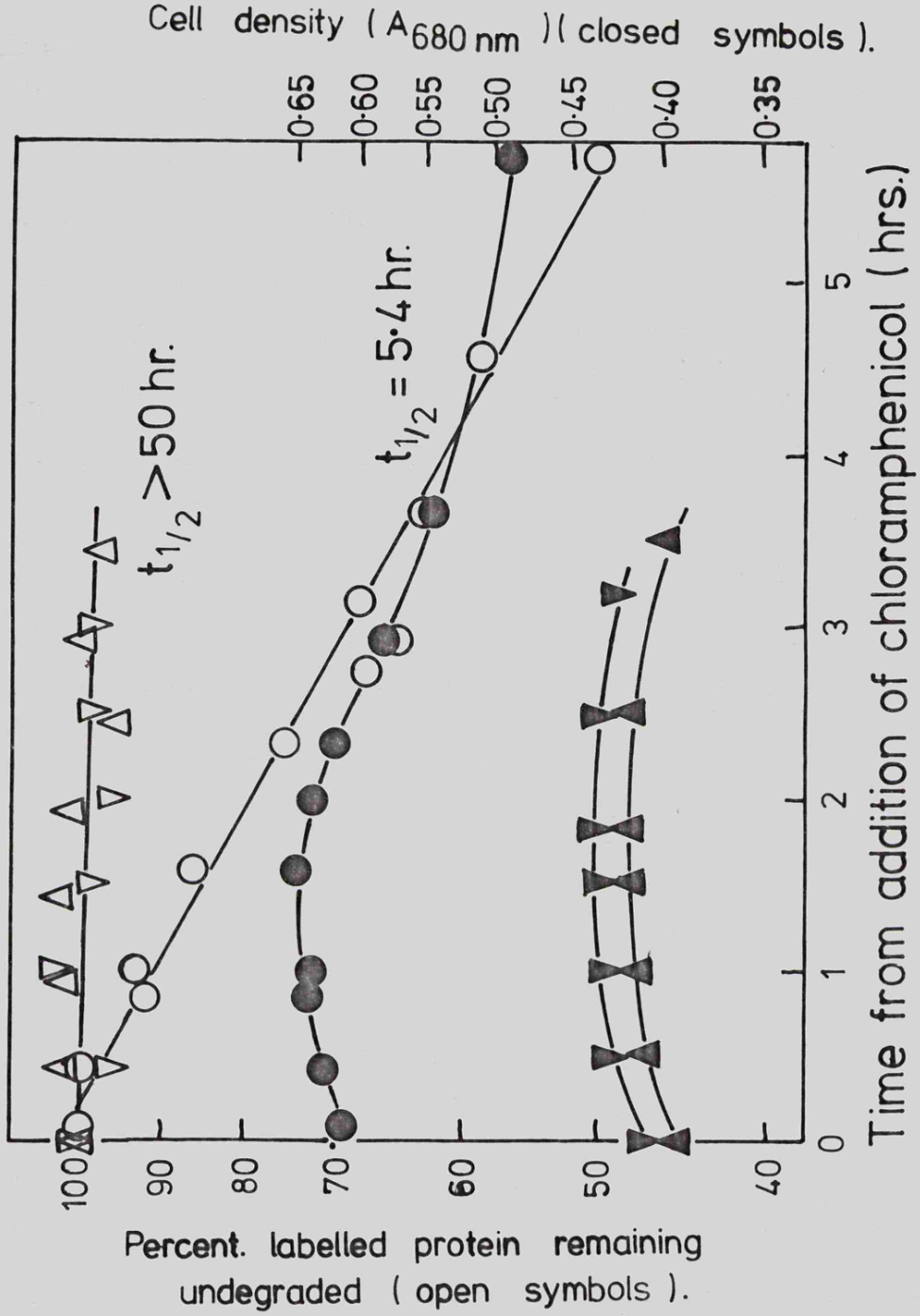


Figure 2.5. Degradation of labelled protein following the addition of chloramphenicol (250 μg ml^{-1}) to cultures growing on complex (NB/S - circles) and minimal (MS/S - triangles; MS/A - inverted triangles) media. The radioactivity of the protein at the time of chloramphenicol addition was taken as 100%.

prevent any reincorporation of amino acids liberated from labelled proteins was confirmed as follows. CAP was added to a rapidly growing culture followed 5 minutes later by L- [1-C14] -leucine (1 nCi ml^{-1}).

Samples were withdrawn 10 and 85 minutes later and the radioactivity of their TCA insoluble fractions was determined. Mean values of 11.6, at 10 min., and 9.7, at 85 min., counts per minute per ml of culture were obtained indicating a negligibly small degree of incorporation of leucine into protein after treatment with this antibiotic.

The extent of protein degradation in cultures grown on NB/S, MS/A and MS/S media was measured after treatment of the culture with CAP. The results, presented in Figure 2.5 show that in non-growing cells protein is degraded significantly faster in cells grown in nutrient broth than in cells grown in minimal salts media, a pattern identical to that observed in growing cells (Fig. 2.1). The slight rise in apparent cell density after the addition of CAP was presumably caused by a brief continuation of synthesis of non-protein cell material. Since, after this initial increase the absorbance of the culture remained stationary for about 2½ hours it may be assumed that the integrity of the cell was maintained fairly well during this period.

2.3.3. Enzyme stability in the intact cell. The close similarity between the rates of protein degradation in growing and CAP treated cells suggested that the rates of enzyme inactivation in intact, CAP treated, cells would be similar to those prevailing

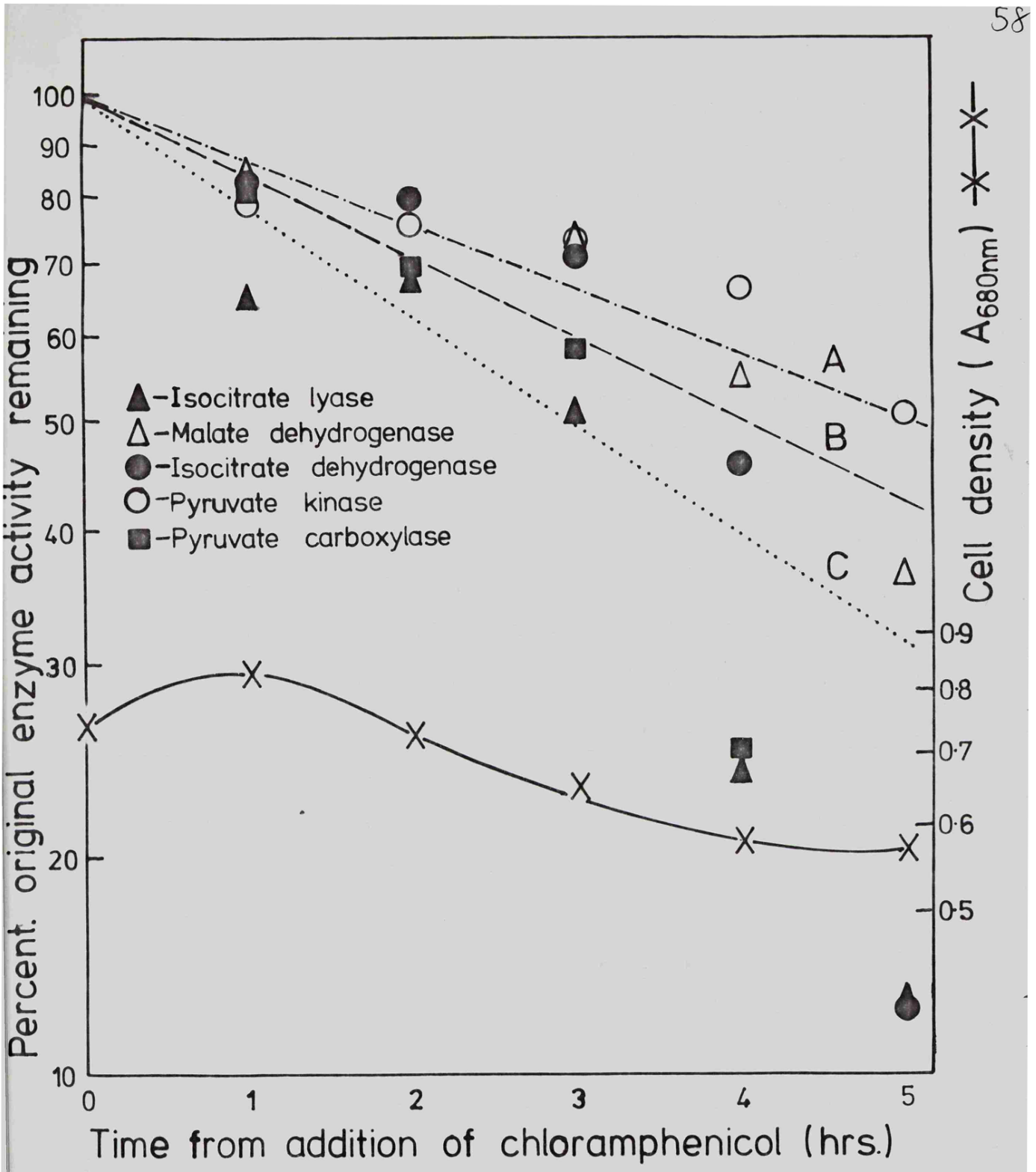


Figure 2.6. The decay of enzyme activities in intact cells following treatment with chloramphenicol. The total activity of each enzyme (units per ml of culture) at the time of chloramphenicol (250 μg ml^{-1}) addition was taken as 100%. For clarity individual curves have not been drawn; instead the theoretical lines (A, B and C) that would be given by exponential decay with half-lives of 5, 4 and 3 hours respectively have been drawn for comparison with the experimental points.

in growing cells. A number of enzymes were assayed in cell free extracts prepared from cells incubated in the prescence of CAP under conditions of temperature, medium and aeration normally employed for growth of the organism. The plots in Fig. 2.6 show the course of decay, following CAP addition, of the five enzymes that were examined. In Table 2.1 are presented the initial activities and approximate half-lives of these enzymes during a 3 hour period of CAP treatment.

The data of Figure 2.6 show that for at least three hours the enzyme activities studied decay, apparently exponentially, at rather similar rates. This experiment was conducted using a New Brunswick Gyrotary Shaker in which, as was realised subsequently, accurate temperature control is not obtained. A limited repeat of this experiment was therefore made when a New Brunswick Gyrotary Water Bath Shaker became available. The results shown in Figure 2.7 essentially confirm the results obtained in the first experiment.

The measurement of respiration affords a convenient test of the sensitivity to inactivation of a membrane bound, multienzyme system. Cultures growing on MS/A or MS/G were treated with chloramphenicol as before and their respiratory activity (after suitable dilution in fresh medium) measured at suitable intervals using an oxygen electrode. The ability of cells to oxidise these carbon sources is shown by the results in Figure 2.8 to decay exponentially and somewhat more rapidly than activities of the single enzymes examined. Parallel experiments conducted with this strain of B. stearothermophilus

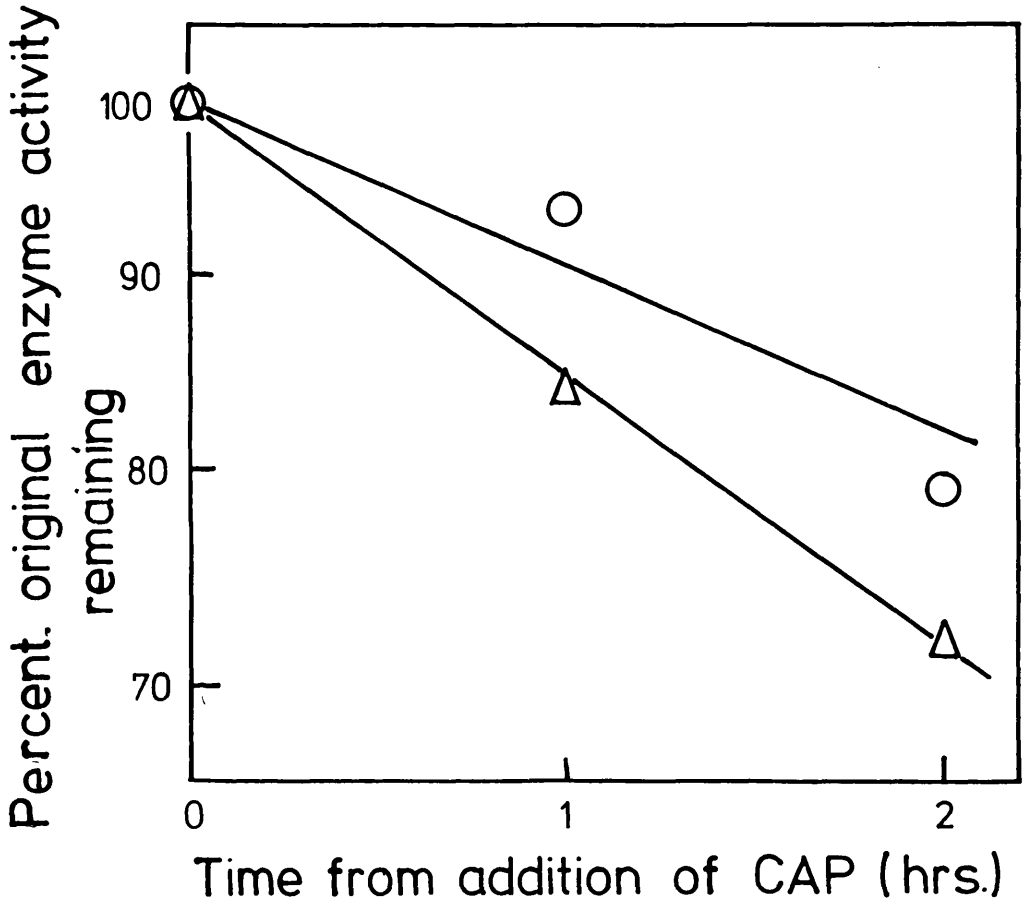


Figure 2.7. The decay of isocitrate lyase (triangles) and pyruvate kinase (circles) activities in intact cells after treatment with chloramphenicol.

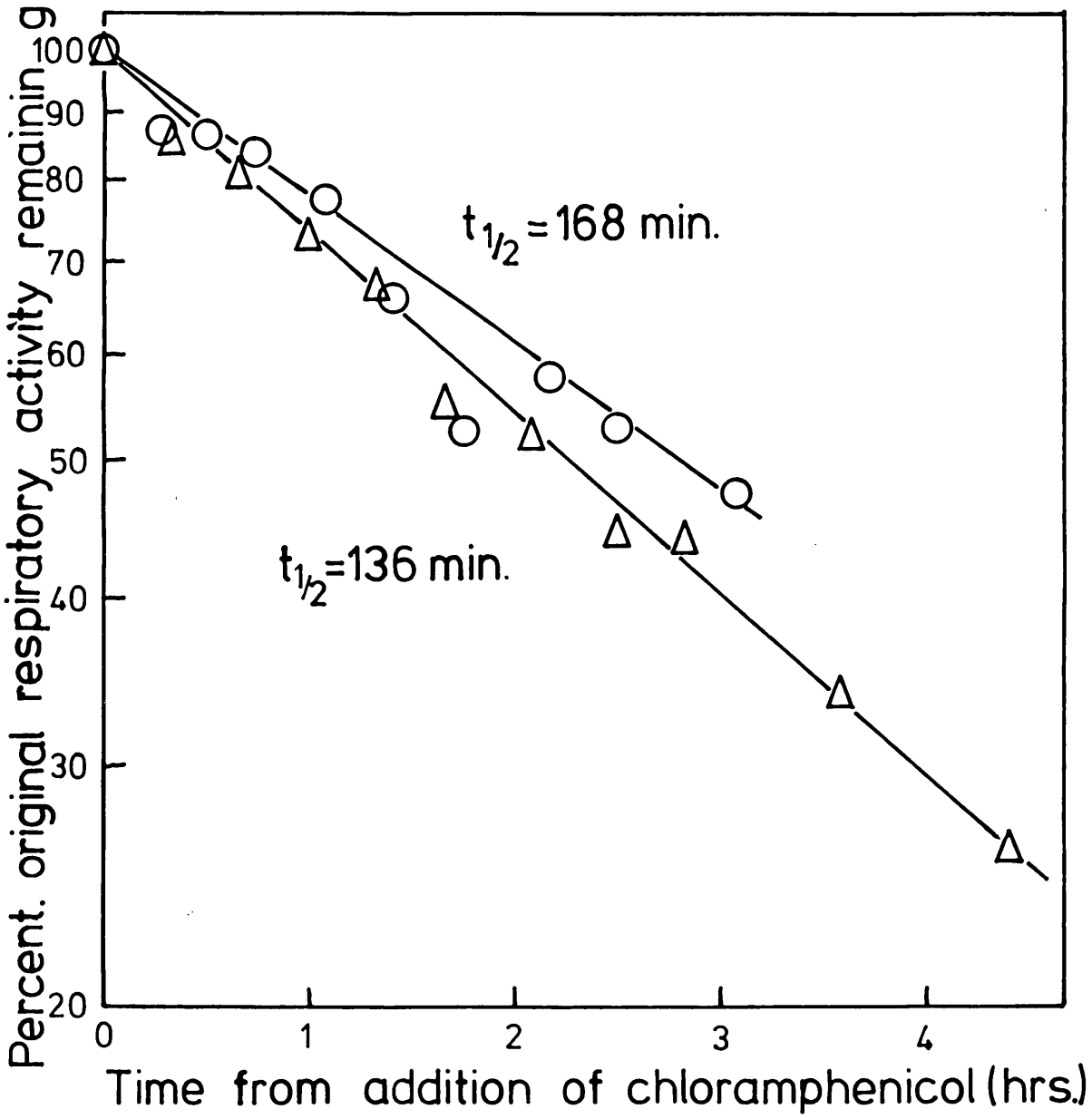


Figure 2.8. The decay of respiratory activity with acetate (circles) and glucose (triangles) as carbon source following treatment of cultures growing on MS/A and MS/G respectively with chloramphenicol. The respiratory activity of each culture (expressed as μ moles O_2 consumed per minute per ml) at the time of chloramphenicol (250 μg ml^{-1}) addition was taken as 100%.

in this laboratory by P. Harris showed that the facility for active uptake of glucose also decayed exponentially following treatment with CAP; the half life of this system was about 80 minutes.

Table 2.1 The stabilities of enzymes in intact cells during exposure to chloramphenicol for 3 hours.

The approximate rate of decay (expressed as the half life) was obtained from the data of Figure 2.6. The initial activity of the cell-free extracts, expressed in terms of the original culture, is also indicated.

Enzyme activity	Half life (hr.)	Initial activity (nmol $min^{-1}ml^{-1}$)
Malate dehydrogenase	5.55	54.8
Isocitrate dehydrogenase	6.22	14.2
Pyruvate carboxylase	3.70	17.4
Pyruvate kinase	5.75	19.2
Isocitrate lyase	3.00	11.9

2.3.4 Stability of isocitrate lyase in growing cells.

During growth in the prescence of glucose synthesis of isocitrate lyase by this organism is repressed; the specific activity in glucose grown cells is less than 2% of the value in acetate grown cells (T. K. Sundaram, personal communication). It was therefore possible to examine the stability of this enzyme in growing, intact cells after their transfer from acetate as carbon source to glucose. The results presented in Figure 2.9 show that although synthesis of the isocitrate lyase

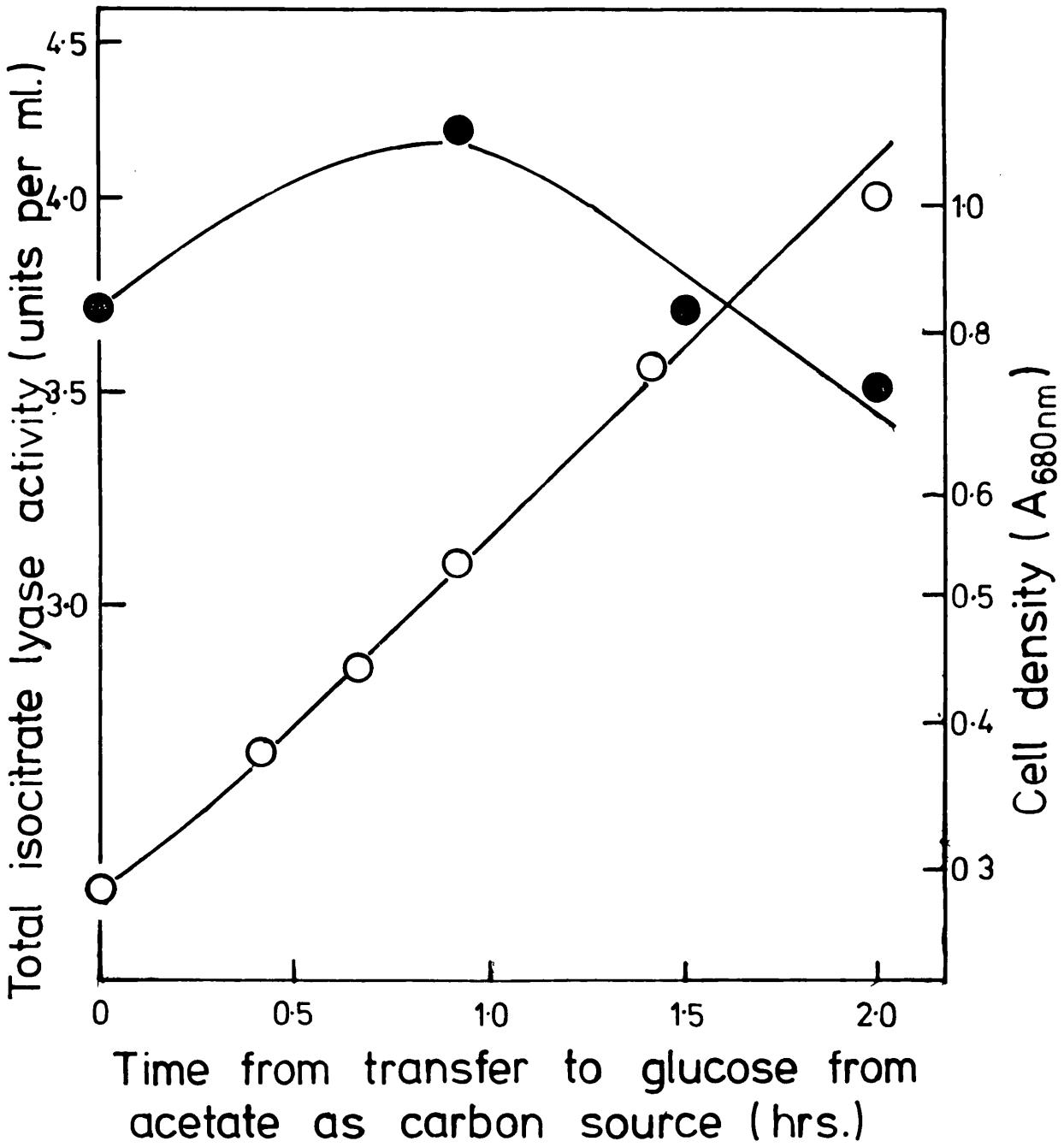


Figure 2.9. The decay of isocitrate lyase activity in cells transferred from MS/A to MS/G. One unit of isocitrate lyase catalyses the cleavage of 1 μ mole of isocitrate per minute.

did not cease quite as abruptly after transfer to MS/G as had been expected activity is subsequently lost at a rate similar to that found after chloramphenicol treatment.

2.4 Discussion

The results obtained in this study clearly rule out the possibility of validity for Allen's "rapid repair" hypothesis that was apparently supported by the results of Bubela and Holdsworth (1966). There is a definite and significant level of protein degradation during the growth of this strain of B. stearothermophilus but it is not sufficient to present a special strain on the overall physiology of the organism. If one assumes that the rate of accumulation of active protein is a factor limiting the growth rate, even the maximum degree of protein degradation found in the experiments reported here would only reduce the rate of growth by about 20%.

The exact reason for the extraordinarily high apparent turnover rates found by Bubela and Holdsworth (1966) is not at all clear. Their results with E. coli (labelled proteins had a half life of 17 minutes) were so different from those reported by other workers (Mandelstam (1958), Borek and co-workers (1958), Chaloupka (1960) and Willets (1967)) all found protein turnover rates of 2 to 5% per hour) that it seems possible Bubela and Holdsworth's experiments only involved a distinct labile protein fraction such as that whose existence in E. coli has been established by Pine (1970) and also Nath and Koch (1970). The very high cell densities employed by Bubela and

Holdsworth in their studies of protein turnover make it unlikely that cell growth, and therefore normal protein synthesis, was taking place during the labelling phase of their experiments.

The similarity between the rates of intracellular enzyme inactivation after chloramphenicol treatment (half lives 3-6 hr. for single enzyme systems) and the fastest rates of protein degradation suggests that in this thermophile at least protein degradation occurs in response to a need of the growing cell to dispose of redundant, thermally inactivated, enzyme molecules rather than to provide amino acids for reincorporation into new protein. The increase in the rate of protein degradation during growth on media providing ample supplies of amino acids would not be expected if this latter function was significant. A role for protein degradation in the reutilisation of amino acids under conditions of nitrogen starvation is not of course ruled out by these results. Epstein and Grossowicz (1969b) studied protein turnover in the same strain of B. stearothermophilus during nitrogen starvation following growth on minimal media and found degradation rates of 5-6% per hour.

Brock (1967) has suggested that the enzymes of thermophilic microorganisms may represent a compromise between the structural simplification possibly required for thermostability and the structural complexity required not only for catalysis but also for the fine control of metabolism mediated by allosteric interactions. The results of studies reported later in

this thesis on pyruvate kinase and the work of others with pyruvate carboxylase (Cazzulo et al., 1970), isocitrate lyase (Sundaram and Kornberg, 1969), threonine deaminase (Thomas and Kuramitsu, 1971), fructose 1,6-diphosphatase (Yoshida and Oshima, 1971), phosphofructokinase (Yoshida et al., 1971) and aspartokinase (Kuramitsu, 1970) leave no doubt that thermophile enzymes are susceptable to allosteric regulation. It is not possible to say in the absence of detailed comparisons whether the regulation of thermophile enzymes is as extensive as that of their mesophilic counterparts.

The significant degree of *in vivo* protein instability indicated by the experiments reported here may imply that in this particular strain of *B. stearothermophilus* adaptation to the thermophilic habit may be incomplete. However unpublished experiments of T. K. Sundaram in this laboratory show that at least one other more typical strain of *B. stearothermophilus* (NCIB 8928; ATCC 12980) exhibits a significant degree of protein turnover during growth at its optimum temperature.

The existance of a correlation between environmental temperature and enzyme stability demonstrated by Ushakov (1967) in poikilothermic animals living at relatively low temperatures (2-15^{o}) suggests that even at low temperatures many enzymes acquire no more than a minimum degree of thermostability. If this is in fact the case then the compromise position reached by a thermophile may not be unexpected.

The greater lability of the multienzyme systems of respiration is particularly interesting. The most likely explanation is that one or more key enzymes are particularly thermolabile. Membrane bound enzymes must retain the integrity of both their active sites and their membrane binding surfaces. This additional constraint may well reduce the scope for structural adaptations directed towards greater thermostability. An alternative explanation assumes firstly that a multienzyme process, in providing a larger "target" for thermal damage, will have a greater probability of losing activity than a single enzyme process and secondly that there is no transfer of electrons or other intermediates between the components of the discrete multienzyme complexes. White and Sinclair (1971) have recently concluded that such evidence as there is does not indicate the existence in bacterial membranes of the discrete electron transport complexes that this explanation requires.

3. ENERGETICS

3.1. Introduction

The simple nutritional requirements of the strain of B. stearothermophilus used in this laboratory make it ideally suited to the studies of the energetics of growth. Its prototrophic nutrition means that its carbon requirement for growth is readily determined in quantitative terms and may be compared directly with the requirements of mesophiles, including those of the same genus.

An examination of the "maintenance energy requirement" (see the General Introduction, Section 1.1) will provide insight into the need for energy for the repair or replacement of thermally damaged macromolecules and organelles. The determination of the maintenance energy requirement unfortunately demands continuous culture techniques which at high temperature would pose special problems. Therefore no direct measurement of this parameter was attempted. However the energy requirements envisaged by any theory of rapid repair (Allen, 1953) are large and would be expected to be revealed by the study of molar growth yield of this thermophile.

The results obtained in the present study show that the utilization of carbon sources for energy and synthesis of cell material by our strain of B. stearothermophilus occurs with comparable efficiency to that observed in mesophiles. The efficiency was nevertheless found to decrease as the growth temperature was raised and the reduced efficiency at high temperatures to stem largely from incomplete oxidation of substrate

carbon. Studies of the effect of temperature on respiratory rate also reported here provide circumstantial evidence that the uncoupling of respiration from energy production is another factor responsible for the reduced growth yield at high temperatures.

3.2. Methods

3.2.1 The relationship between dry cell weight and the absorbance of cultures. Even at low temperatures (5°) and in the prescence of high concentrations of potassium chloride (0.15 M) a significant proportion of the cells of this organism were found to lyse rapidly in the absence of a carbon source. The preparation of cells for dry weight determination therefore posed particular problems. The method described below was adopted, and found to give values for the dry weight which led to satisfactory agreement between our results and generally accepted values on such points as total carbon content of the cells.

Cells were grown at 43°and 55.6° on MS/G in the usual way. At a suitable time in the exponential phase of growth of each culture the culture flask was removed from the shaker, rapidly cooled and its absorbance at 680 nm recorded using a Unicam SP 600 spectrophotometer. At the same time samples of 10-40 ml were removed and the cells recovered by filtration on tared Millipore filters (pore size 0.45 μ). The adhering medium was washed off with a few d rops of ice cold water. The filters were transferred to a dessicator and dried under vacuum over P_2O_5 at room temperature. In order to provide a correction for changes in the water content of the filters during the drying of the cells three

tared control filters were wetted with fresh medium, and washed and dried as indicated above. After drying to constant weight the change in weight of each filter was plotted against the volume of the sample and the relationship between absorbance at 680 nm and cell density (dry weight per ml) obtained.

3.2.2. The determination of molar growth yield.

The method initially adopted was based on the technique of Ng (1969) and is referred to here as the growth increment method. This technique had a particular drawback, as explained below, and a modified technique, referred to as the terminal cell density method was adopted in later experiments.

The growth increment method. The essential principle of this technique is to grow the organism on a low level of carbon source until growth stops due to exhaustion of nutrient. Small increments of carbon source are then added and the resulting successive increments of growth are measured to provide a linear plot of the weight of cells versus the amount of carbon source from which an average value for the molar growth yield can be obtained. In the present study the procedure was as follows.

An overnight culture on minimal salts medium with 50 mM glucose as carbon source provided the inoculum (2ml) for 50ml of the same medium contained in a 250ml Erlenmeyer flask but having only 10 mM glucose. This flask had 3 ripples formed across the bottom (see Figure 3.1) designed to enhance the aeration during incubation on a reciprocating shaker. This culture was then incubated at 55^{o} in the New Brunswick Gyratory

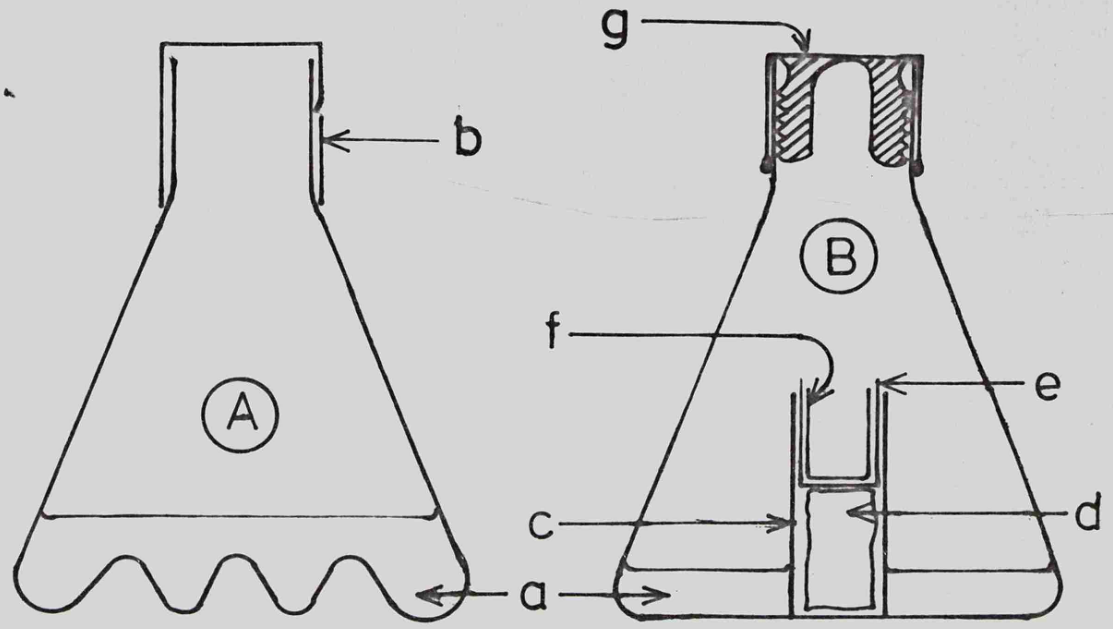


Figure 3.1. Modified 250 ml. Erlenmeyer flasks for use in a reciprocating shaker (A) and for CO_2 collection (B). Key :-(a)culture medium;(b)metal cap;(c)fixed glass well, 15 x 50 mm;(d)polythene tubing support; (e)inner removable metal cap to contain "f" ; (f)glass vial (12 x 15 mm) containing a filter paper or glass fibre paper wick and KoH solution; (g) airtight rubber cap (Suba-seal).

Shaking Incubator until its absorbance at 680 nm was 0.7-0.8. At this point the culture was transferred to a Grant Water Bath Shaker (reciprocal action) maintained at the temperature of the experiment. Incubation then continued until exponential growth at the new temperature was well established. This culture was then used to inoculate a series of prewarmed experimental flasks. These too had rippled bottoms and each contained 45 mls of minimal salts medium without glucose or other carbon source. Each flask received 5 mls of the inoculum culture and, after closure with snugly fitting metal caps, incubation with shaking commenced. Growth in each flask was monitored by the withdrawal of precisely measured 1 ml samples and measurement of their absorbance at 680 nm in semi micro cuvettes using a Unicam SP 500 spectrophotometer. When these measurements indicated, by the cessation of growth, that the glucose carried over in the inoculum was exhausted, an appropriate volume of sterile 0.2M glucose was added together with sufficient carbon free medium to return the volume to precisely 50 ml.

Incubation was then continued until the frequent measurements of the absorbance indicated that growth had again ceased. The course of a typical experiment is shown in Figure 3.2.

The chief difficulty with this procedure is illustrated by Figure 3.2. When growth of the thermophile ceased there was no plateau of steady absorbance as Ng (1969) found with *E. coli* ML 30 but instead an immediate fall, presumably due to cell lysis. This

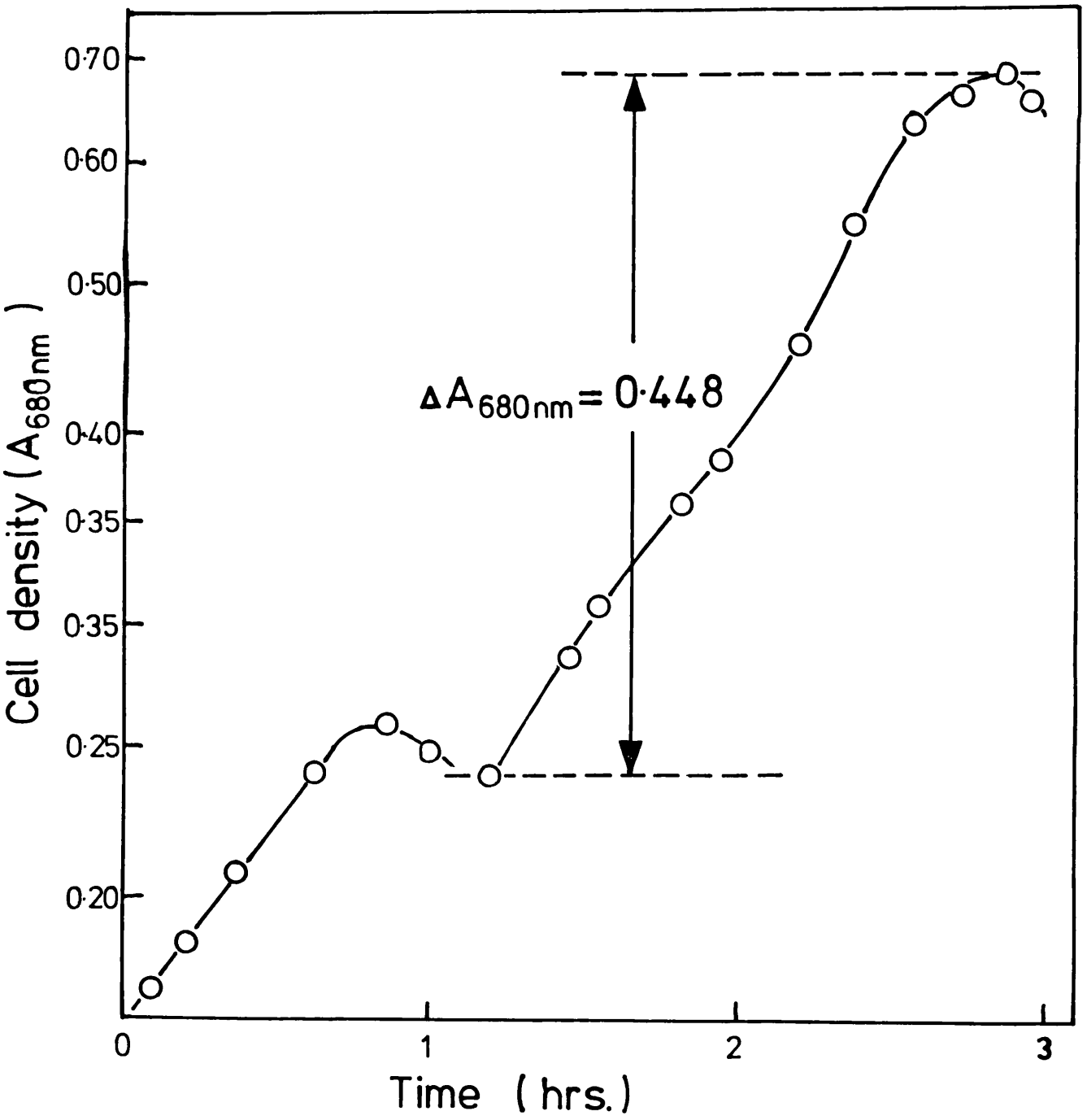


Figure 3.2. The course of a typical molar growth yield determination (at 55°) by the growth increment method. The break in the curve indicates the point at which 0.2 M glucose was added to give, in this particular case, a final concentration of 3mM and the volume corrected to 50 mls by the addition of medium lacking carbon source.

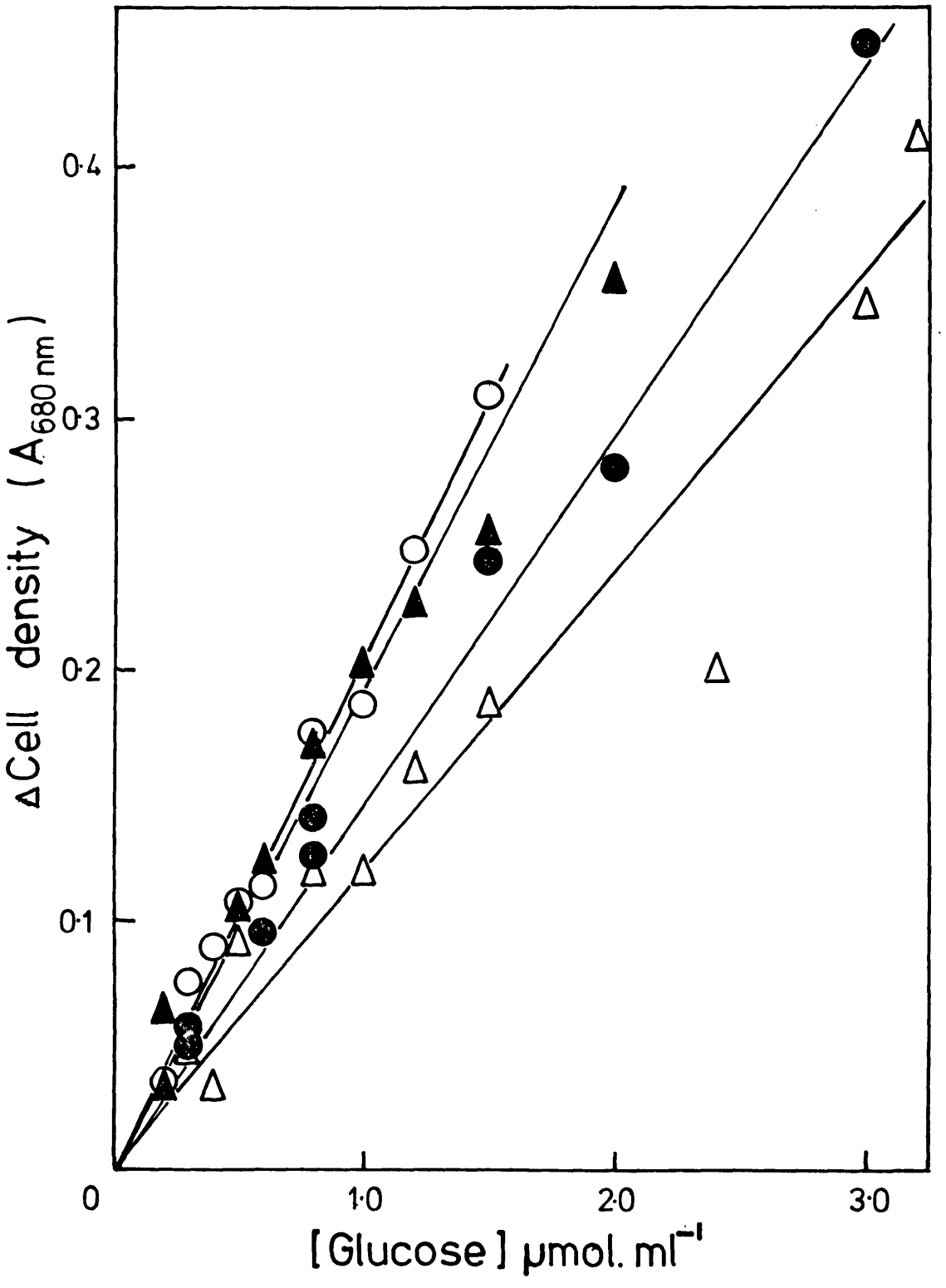
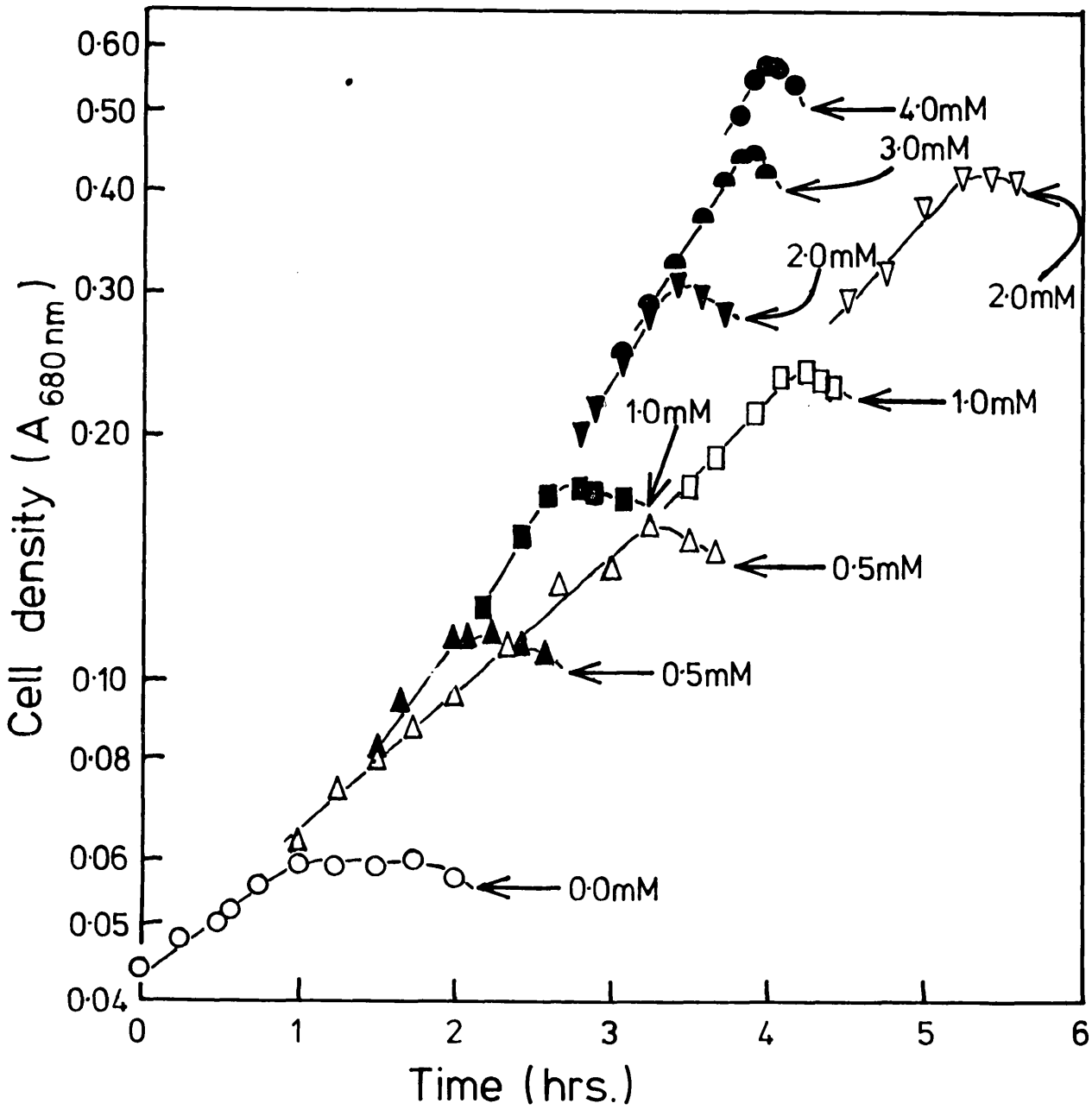


Figure 3.3. The determination of the molar growth yield by the growth increment method at 41.0° (open circles), 45.0° (closed triangles), 55.0° (closed circles) and 60.0° (open triangles). At of 62.0° there was no detectable growth at any of the glucose concentrations used (0.3 - 3.0 μ mole ml^{-1}).

made it difficult to define accurately the base line absorbance when measuring the growth increment, a difficulty reflected in the somewhat scattered points in Figure 3.3.

The terminal cell density method. This was a modification of the growth increment method designed to avoid the difficulty discussed above. It was adopted for the determination of the molar growth yield on succinate and all subsequent experiments. Up to the point at which the inoculum culture was transferred to the experimental temperature, the procedure was as described above. After adaptation of the inoculum culture to the experimental temperature each experimental flask received 5 mls of the inoculum. Each experimental flask contained 45 mls of prewarmed carbon free medium plus quantities of carbon source giving a range of final concentrations (i.e. after inoculation) of 0-4 μmoles ml^{-1} over and above the unknown constant amount carried over in the inoculum. Incubation with shaking then continued and the cell density reached when growth stopped determined as in the first method. The course of a number of typical experiments is shown in Figure 3.4. This method eliminated the necessity for any manipulation of the experimental flasks after inoculation and consequently the scatter of points in plots of final cell density against carbon source concentration was greatly reduced (see Figure 3.5).

When a New Brunswick Gyrotary Shaking Water Bath became available some of the experiments originally performed using the Grant Reciprocal Shaker were



molar growth yield by the

Figure 3.4. The determination of the final cell density method. The curves plotted are the final stages of the growth curves of cultures containing, in addition to glucose carried over in the inoculum, the amounts of glucose shown. Plots shown with open symbols were obtained at 43.5°, those with closed symbols at 57.5°.

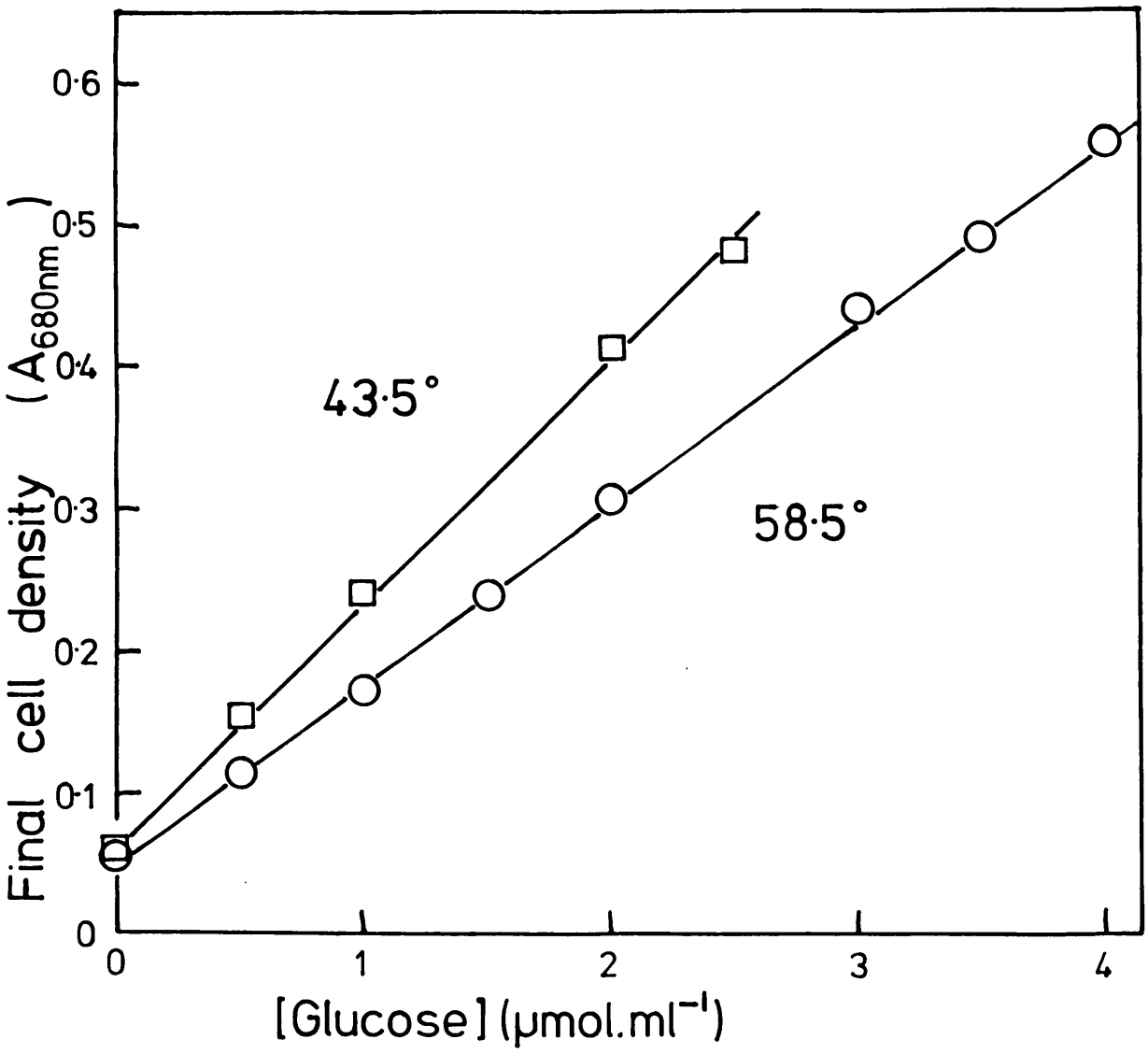


Figure 3.5. The relationship between cell yield and glucose concentration determined by the final cell density method. The data was obtained from the plots in Figure 3.4. and others obtained at the same time but omitted from that Figure for the sake of clarity.

repeated. Since the gyrotary shaking action greatly improved aeration there was no necessity to use the ripple bottomed flasks and ordinary Erlenmeyer flasks (with metal caps) were used.

The differences in the geometry of the optical systems of the various spectophotometers used in the experiments (the Unicam SP500, SP600 and SP1800) raised the possibility that the relationship between absorbance at 680 nm and cell density established with the SP600 may not hold for other instruments. This was checked but no significant differences of absorbance were found between readings taken with these different instruments. The use of semi-micro cuvettes with the SP 500 and SP 1800 had no effect on the absorbance either.

3.2.3 The fate of glucose consumed during growth.

Preliminary experiments indicated that a significant proportion of the glucose that disappeared from the medium during growth was neither oxidised to carbon dioxide nor incorporated into cell material. The precise determination of the proportion of glucose carbon thus "wasted" was performed as follows.

A culture was grown up on minimal salts medium with 10 mM glucose as carbon source. A small amount of this culture was used as inoculum for a similar culture having 10mM U-C14-glucose as a carbon source. (Throughout these experiments the U-C14-glucose had a uniform specific activity of 2.5nCi.μmol^{-1}.) When this culture reached a cell density of about 25 mg.ml^{-1} it was transferred from the New Brunswick Gyrotary Incubator (at 55^{o}) to the New Brunswick Gyrotary Water

Bath Shaker maintained at the temperature of the experiment. In this way any temperature adaptation processes would be completed before the actual experiment commenced. When a cell density of about 0.45 $mg.ml^{-1}$ was reached 5 mls of this culture were used to inoculate 95 mls (contained in a 500 ml Erlenmeyer flask) of the same medium containing 0.2-0.5 µmoles of U-C14-glucose and incubation was started. Immediately, and at appropriate intervals thereafter, 3 ml aliquots of the culture were removed. Approximately 1 ml was set aside for the determination of cell density in semi micro cuvettes (1 cm path length) in a Unicam SP 1800 spectrophotometer. Exactly 2 mls of the sample was filtered through a Millipore filter (diameter 2.5 cm., pore size 0.45 µ). The cell-free filtrate was transferred to an ice bath to await further treatment. Meanwhile the filter was washed briefly with 5 ml of ice cold carbon free salts medium, dried by suction and then transferred to a scintillation vial in which drying was completed after transfer to a 55^{0} air incubator. When dry, 3 mls of dioxane based scintillation fluid (Bray 1960) was added to each vial and the radioactivity of the cells measured in the Packard Tricarb 4000 Scintillation Spectrometer. 1 µmole of U-C14-glucose was added to each of a number of duplicate filters to provide an internal standard of counting efficiency. The number of µmoles of glucose incorporated into the cell material was then calculated by reference to the radioactivity of the internal standards.

The total glucose carbon content of the culture

filtrate was measured as follows. A sample (0.1 ml) of filtrate was placed in a scintillation vial with 10 ml of dioxane based scintillation fluid, mixed and the radioactivity determined as above. Internal standards were provided by the addition of 1 μmole of U-C14-glucose to a number of duplicate 0.1 ml samples. The carbon content of the medium could then be calculated by reference to the radioactivity of the internal standards. The concentration of free glucose remaining in the medium was determined on the cell free filtrate by the glucose oxidase method. One capsule of mixed peroxidase and glucose oxidase (Sigma Ltd., London) was dissolved in 100 ml of water, and 3 ml of this solution was added to 0.2 ml samples of cell free filtrate (where necessitated by high glucose concentrations, 0.2 ml of appropriately diluted samples were used). The addition of the enzyme solution was immediately followed by the addition of 0.05 ml of o-dianisidine (2.5 mg ml^{-1}). The complete reaction mixture was vigorously stirred on a vortex mixer and left at room temperature for 30 minutes before measurement of the brown colour at 420 nm. The glucose concentration was determined by reference to a series of standards of pure D-glucose run at the same time.

3.2.4. <u>The measurement of carbon dioxide liberated during growth on glucose.</u> Eight 250 ml Erlenmeyer flasks fitted with removable wells (see Figure 3.1) containing 1 ml of 20% w/v KOH and a filter paper wick, each contained 50 ml of minimal salts medium were divided into 4 pairs. One flask

in each pair (the pilot flask) contained a suitable amount of 12C-glucose in the range 25-125 μmoles (i.e. 0.5-2.5 $\mu mol.ml^{-1}$) and the other flask (the experimental flast) an identical amount of U-14C-glucose. After equilibriation to the temperature of the experiment each flask was inoculated with 2.5 ml of a culture growing exponentially on minimal salts medium with 10 mM glucose as sole carbon source. This amount of inoculum ensured that the carry over of glucose from inoculum to experimental and pilot flasks was minimal. All the flasks were closed with airtight rubber caps (Suba-Seal) and incubation started in the New Brunswick Gyrotary Shaking Water Bath. Calculations showed that the flasks contained ample amounts of oxygen for the complete oxidation of the amounts of glucose supplied. At intervals the absorbance of the pilot cultures was measured, the experimental flasks remained sealed throughout the experiment. When the pilot absorbance readings indicated that growth had ceased in a particular flask 10 ml of 25% w/v sulphuric acid was added to the cognate experimental culture by syringe in order to prevent further metabolism and release any CO_2 remaining in the medium. The experimental flask was then shaken at room temperature for 1 hour to ensure that trapping of 14-CO_2 in the KOH was complete. The well containing the KOH was then transferred to the body of a second flask of similar design to the experimental flask. The removable glass well of the second flask contained 2 ml of hyamine hydroxide (10% w/v in methanol). After the flask was sealed 20 mls sulphuric acid was added by

syringe to the KOH to liberate the 14-CO_2. After 1 hour for the trapping of the 14-CO_2 in the hyamine to be completed, the hyamine containing well was transferred to a scintillation vial containing 10 mls of scintillation fluid (Bray, 1960) and the radioactivity measured in a Packard 4000 Spectrometer. The number of counts obtained was expressed as umoles of glucose by the use of internal standards. For this purpose 0.1 mls of 50 mM U-C14-glucose was added to each vial after counting was complete and the mixture recounted. Tests in which similar amounts of non-radioactive glucose were added to 14-CO_2 samples showed the counting efficiency to be unchanged by the glucose addition.

3.2.5 The oxidation of 6-C14-glucose during growth

A comparison of the extent of 14-CO_2 production at 57° from 6-C14 and U-C14-glucose was made using a simplified version of the procedure just described.

Two sets of triplicate experimental flasks contained U- or 6-C14-glucose in a final concentration of 1 mM, 1 nCi ml^{-1}. The procedures for their inoculation and incubation were similar to those described above (Section 3.2.4.) except that 2 mls of inoculum and a final volume of 20 mls of culture were used for each flask.

Glass fibre paper wicks (cut from Whatman GF/C filters) were used in the KOH wells ('f' in Figure 3.1) and the radioactivity of the potassium carbonate was measured directly as follows. After trapping of the liberated CO_2 was completed the glass wells were transferred, complete with the metal caps which

contained them ('e' in Figure 3.1) to a hot plate where they were maintained at about 100° for 1 hour. When dry the glass wells and their contents were transferred to scintillation vials containing 10 mls of toluene based scintillation fluid (see Section 2.2.1) and their radioactivity was measured in the scintillation counter as before.

3.2.6 The identification and determination of the products of glucose metabolism remaining in the spent culture medium.

The products of glucose metabolism were sought in the cell free filtrate from a culture grown on minimal salts medium at 55° with 5 mM U-C14-glucose as carbon source.

The concentrations of free glucose and of total glucose carbon in the filtrate were determined by the glucose oxidase method and radioactivity measurements as described previously (Section 3.2.3). The very low levels of waste products expected (no more that 2 mM for a 3-carbon atom compound) and the difficulties of concentration in the presence of high levels of inorganic salts precluded the use of paper chromatography for the identification of the most likely products, viz., pyruvic, lactic and acetic acids.

(a) Pyruvic acid was assayed spectrophotometrically (at 340 nm) by its reduction to lactate in the presence of NADH and lactate dehydrogenase. Cell free filtrate (2 ml) was placed in a 3 ml glass cuvette together with 0.2 mls of 10 mM NADH in 50 mM potassium phosphate buffer (pH 6.8). After

After mixing the absorbance at 340 nm was recorded and then 5 µg of L-lactate dehydrogenase in 0.1 mls of the same buffer added. After allowing 5 minutes for any reaction to be completed the new absorbance was noted and then 0.2 mls of 5 mM sodium pyruvate added as an internal standard. Once again the absorbance at the completion of the reduction of the pyruvate was noted. All absorbance readings were made with a water blank and were corrected for the volume changes involved.

(b) Lactic acid, both the L- and D- isomers, was assayed spectrophotometrically (at 340 nm) by its oxidation to pyruvate in the presence of the appropriate lactate dehydrogenase, NAD and hydrazine to trap the pyruvate (Hohorst, 1965). The assay mixture contained, 0.45 mls of hydrazine-glycine buffer, pH 9.5 (7.5 g glycine, 5.2 g hydrazine sulphate, 0.2 g EDTA, 51 ml 2N NaOH, water to 100 ml), 0.05 ml 50 mM NAD and 0.5 ml of cell free filtrate. After mixing, the absorbance was recorded and 5 µg of L- or 25 µg of D-lactate dehydrogenase added in 0.05 ml of phosphate buffer (50 mM pH 6.8). After allowing 5 minutes for any reaction to occur the absorbance was recorded and then 0.1 ml of 5 mM sodium DL lactate was added as an internal standard and the change in absorbance again recorded. As before all the absorbance readings were made with a water blank and were corrected for volume changes.

(c) Acetic acid was determined in a coupled assay based on the reactions catalysed by acetate kinase (i), pyruvate kinase (ii) and lactate dehydrogenase (iii).

$$\text{Acetate} + \text{ATP} \longrightarrow \text{Acetyl phosphate} + \text{ADP} \quad \text{(i)}$$

$$\text{ADP} + \text{PEP} \longrightarrow \text{ATP} + \text{Pyruvate} \quad \text{(ii)}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \longrightarrow \text{Lactate} + \text{NAD}^+ \quad \text{(iii)}$$

This assay was preferred to the more usual one based on acetate kinase and hydroxylamine (Rose, 1955) since that extinction coefficient of NADH, which is nearly 6 times as high as that of the acetyl hydroxamate $FeCl_3$ complex would provide much greater sensitivity. The assay mixture contained (in each of two cuvettes) 0.1 ml of 50 mM ATP; 0.1 ml of 20 mM PEP; 0.15 ml of 2.5 mM NADH; and 0.1 ml of 100 mM $MgCl_2$ all contained in potassium phosphate (50 mM) EDTA (1 mM) buffer (pH 6.8). 10 µg each of pyruvate kinase and L-lactate dehydrogenase were added followed by 0.02 ml of cell free filtrate and water to give a final volume of 1.0 ml. After mixing the absorbance, referred to a water blank, of both cuvettes was recorded at 340 nm for 5 minutes. During this period a steady fall in absorbance was noted. The test reaction was then started by the addition to one cuvette of 100 µg of acetate kinase; the other cuvette served as a control. The change in absorbance at 340 nm continued to be monitored in both cuvettes using the automatic cell change facility of the spectrophotometer until the rates of decrease in absorbance in the test and control cuvettes were again identical. The difference in absorbance between the two cuvettes at this point was a measure of the acetate present in the sample of cell free filtrate. A series of runs in which the cell free filtrate was replaced by different amounts of pure sodium acetate showed that there was the expected stoichiometric relationship between

NADH oxidation (measured by the difference in absorbance between the test and control cuvettes) and the amount of acetate added.

3.2.7 Respiration. Studies of the effect of temperature on the rate of respiration of whole cells were made using an oxygen electrode. An overnight culture (400 ml) was grown up at the appropriate temperature on a minimal salts medium with the desired carbon source included at a concentration of 50 mM. When the cell density reached 0.35-0.40 mg dry wt. per ml (i.e. towards the end of exponential growth) the culture flask was transferred to an ice bath and the culture stirred until the temperature fell to about 5°. The cells were then harvested by centrifugation at 5° and resuspended in fresh cold medium to a cell density between 5 and 10 mg dry wt per ml. This cell suspension was kept in ice until used for experiments. A small quantity (2-4 ml) of fresh medium was placed in the cell of the oxygen electrode (Rank Bros., Bottisham, Cambs.) and stirred until a steady potentiometer reading from the electrode indicated that saturation with air and temperature equilibriation were complete. An appropriate volume of cell suspension was rapidly brought to the experimental temperature and then added to the medium in the cell. The cell was then closed, stirring started and the rate of uptake of oxygen by the bacterial cells recorded. The oxygen content of air saturated buffers or culture media at temperatures above 40° has only rarely been recorded in the literature. For this reason the rates of oxygen uptake were calculated on

°C	$10^3/°K$	Subtract Difference	
		.1	13
0	3.6609	.2	26
1	3.6475	.3	40
2	3.6343	.4	53
3	3.6211	.5	66
4	3.6080	.6	79
5	3.5950	.7	92
6	3.5821	.8	106
		.9	119
7	3.5695		
8	3.5568	.1	12
9	3.5442	.2	25
		.3	37
10	3.5317	.4	49
11	3.5192	.5	62
12	3.5069	.6	74
13	3.4964	.7	86
14	3.4824	.8	98
15	3.4703	.9	111
16	3.4583		
17	3.4464		
18	3.4345	.1	12
19	3.4228	.2	23
		.3	35
20	3.4111	.4	46
21	3.3985	.5	53
22	3.3879	.6	69
23	3.3765	.7	81
24	3.3651	.8	92
25	3.3538	.9	104
26	3.3426		
27	3.3315		
28	3.3205	.1	11
29	3.3095	.2	22
		.3	32
30	3.2985	.4	43
31	3.2877	.5	54
32	3.2769	.6	65
33	3.2662	.7	76
34	3.2555	.8	86
35	3.2450	.9	97
36	3.2344		
37	3.2242		
38	3.2138	.1	10
39	3.2035	.2	20
		.3	30
40	3.1933	.4	40
41	3.1831	.5	51
42	3.1730	.6	61
43	3.1630	.7	71
44	3.1530	.8	81
45	3.1431	.9	91
46	3.1332		
47	3.1234		
48	3.1137	.1	10
49	3.1040	.2	19
		.3	29
50	3.0944	.4	38
51	3.0848	.5	48
52	3.0753	.6	57
53	3.0659	.7	67
54	3.0565	.8	76
55	3.0472	.9	86
56	3.0379		
57	3.0289		
58	3.0197	.1	9
59	3.0106	.2	18
		.3	27
60	3.0016	.4	36
61	2.9926	.5	45
62	2.9837	.6	53
63	2.9748	.7	62
64	2.9660	.8	71
65	2.9562	.9	80
66	2.9485		
67	2.9399		
68	2.9313	.1	8
69	2.9227	.2	17
		.3	25
70	2.9142	.4	34
71	2.9057	.5	42
72	2.8973	.6	50
73	2.8889	.7	59
74	2.8805	.8	67
75	2.8723	.9	76
76	2.8640		

Figure 3.6. A table for the conversion of degrees centigrade into the reciprocal of the absolute temperature, i.e. °C to $10^3/°K$, for use in the construction of Arrhenius plots.

the basis of the oxygen content of pure water saturated with air for which reliable values are available (Handbook of Chemistry and Physics - Chemical Rubber Publishing Co.). Such data as have been recorded (Egorova and Pozmogova, 1966) indicate that the error resulting from this procedure is small and without significant effect on the conclusions to be drawn from these experiments.

The derivation of Arrhenius plots from the results of these and other experiments was greatly facilitated by the construction of a table for the direct conversion of the temperature in degrees Centigrade to the reciprocal of the absolute temperature (as $1000/^{o}K$). The table was derived from the five-figure reciprocal tables by Castle (1966) and is presented here as Figure 3.6.

3.3 Results

3.3.1 The relationship between dry cell weight and the absorbance of cultures.

The results in Figure 3.7 show that growth temperature causes no significant difference in the dry weight/absorbance relationship of the culture. The relationship between dry weight and absorbance at 680 nm is clearly linear and the figure obtained from these plots (0.45 mg per ml at $A_{680\ nm} = 1.0$) was applied in all the experiments described in this thesis. Experiments conducted in this laboratory by Miss S. N. Dilks showed that the absorbance of cultures and cell suspensions of the thermophile obeys Beer's Law up to a maximum absorbance at 680 nm of 1.0.

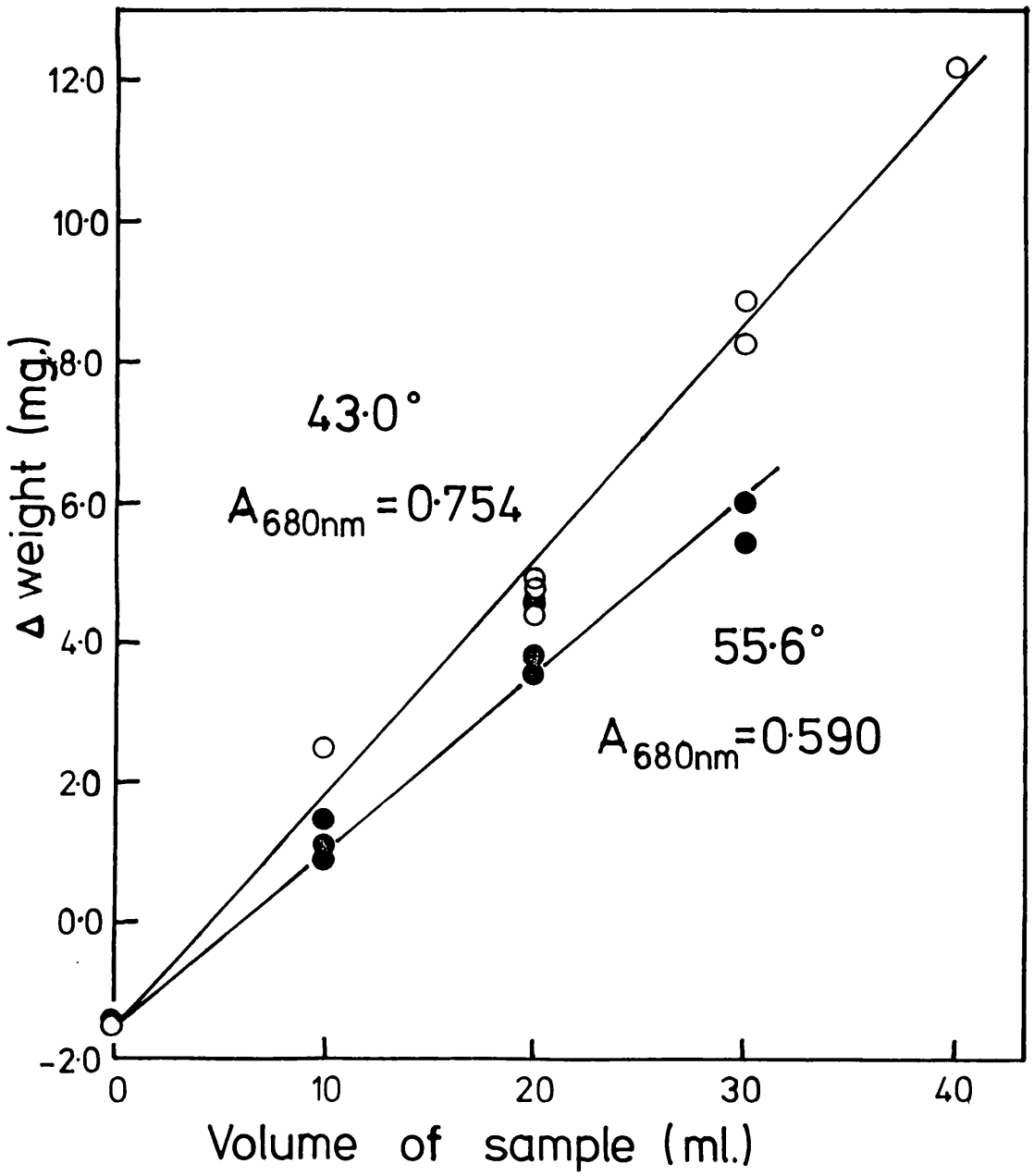


Figure 3.7. The determination of cell dry weight. From the slopes of these plots the dry weight of cells from 1 ml of a culture grown at 43.0° to the absorbance shown was 0.337 mg ; a culture grown at 55.6° similarly gave a figure of 0.267 mg. These figures gave values of 0.447 and 0.453 mg respectively for the weight of cells in 1 ml of culture of A_{680nm} = 1.0.

3.3.2 Molar growth yield. The results giving the molar growth yield on glucose are shown in Figure 3.8. The striking effect of temperature on the values of Y_G (g. (dry wt) of cell material per mole of glucose utilized) was clearly unaffected by the modifications of technique involved in the introduction of the "Terminal cell density method" (see Section 3.2.1.). The value of zero for the yield at 62° was based on the finding that when the inoculum culture, which was growing at 55°, was transferred to this temperature there was an immediate cessation of growth, the absorbance of the culture remaining steady. When in another experiment the inoculum culture was transferred to the higher temperature of 65° there was a rapid fall in absorbance accompanying extensive cell lysis.

The possibility was considered that the aeration obtained with the Grant reciprocal shaker was inadequate and hence the cause, through the low solubility of oxygen at high temperatures, of the observed effect of temperature on yield. However the later use of the New Brunswick Gyrotary Water Bath Shaker resulted in essentially similar results although the aeration of the culture was now greatly improved.

Two other observations supported the view that the degree of aeration obtained in these experiments was adequate. Firstly the affinity of the terminal oxidation systems for oxygen appeared to be very high in the respiration experiments using the oxygen electrode. The rate of oxygen uptake only slowed

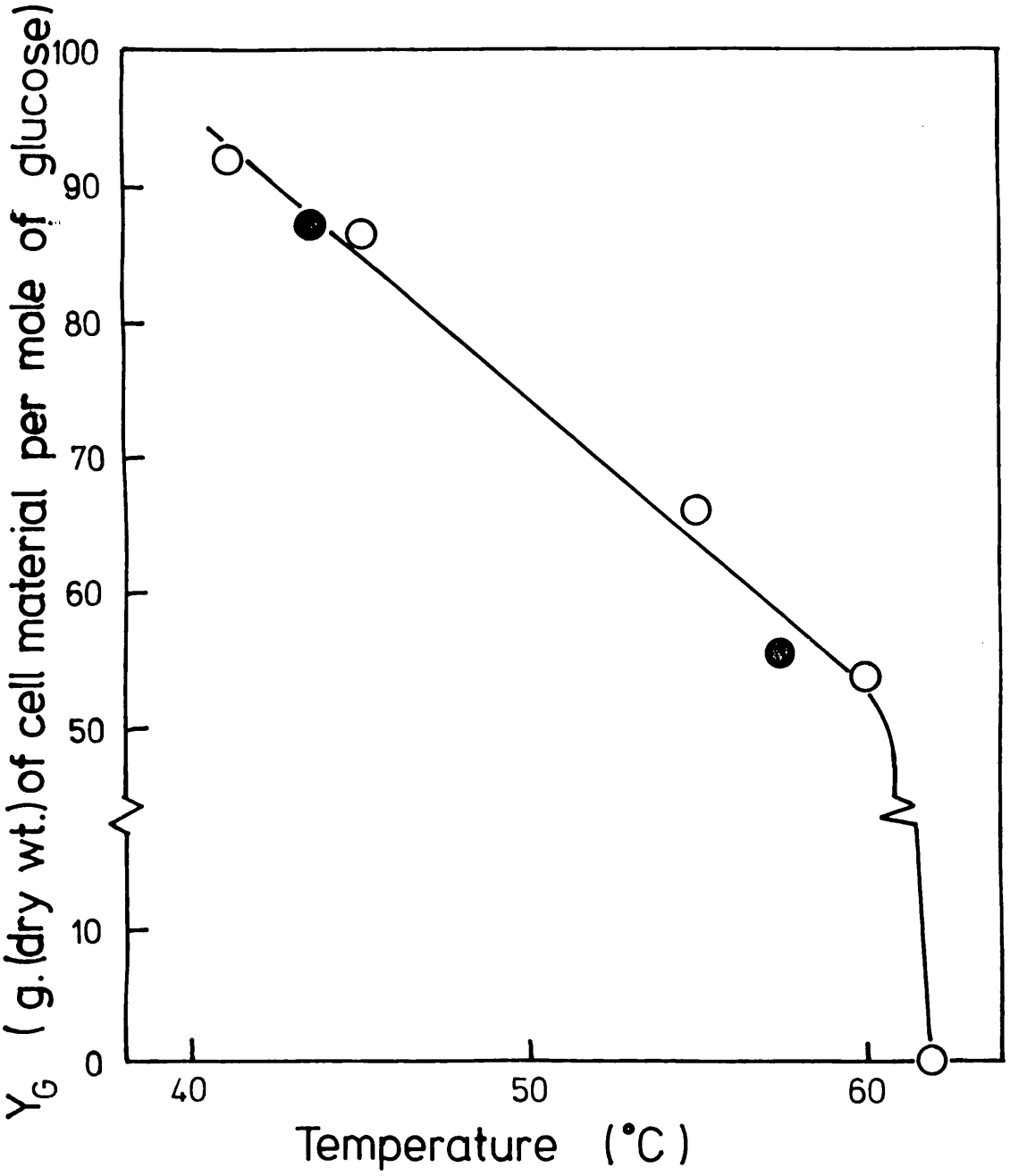


Figure 3.8. The effect of temperature on the molar growth yield with glucose (Y_G) Results were obtained by the growth increment method using a Grant reciprocal shaker (open circles) or by the terminal cell density method using a New Brunswick Gyrotary Shaker (closed circles).

when the oxygen content of the medium fell to 2-3% of the content of the air saturated medium. Secondly the inoculum cultures in all the molar growth yield experiments continued to grow exponentially even though cell densities in them were five fold greater than those encountered in the experimental cultures. There was thus no evidence to support the suggestion that the concentration rate of supply of oxygen limited growth under the conditions of these experiments.

The values for molar growth yield on succinate (Y_{succ}) obtained from the plots in Figure 4.9 are 54.0 and 31.5 gm per mole of succinate at 43.3° and 59.3° respectively. In his review Payne (1971) has suggested that molar growth yields may be meaningfully related to the number of electrons available from the carbon source during prototrophic growth with ammonium ion as nitrogen source, conditions fulfulled in these experiments. Data presented by Payne for a number of mesophilic bacteria indicate that the growth yields per available electron ($Y_{av.e^-}$) generally approximate to 3.14 although values as low as 1.1 and as high as 5 have occasionally been recorded. The values for $Y_{av.e^-}$ calculated from the molar growth yields on glucose and succinate are given in Table 3.1. There is a remarkable degree of agreement between the values obtained for the two carbon sources. Particularly at the lower temperatures the values obtained for the thermophile are closely similar to those reported for mesophilic bacteria.

With the exception of the work of Ng (1969) with

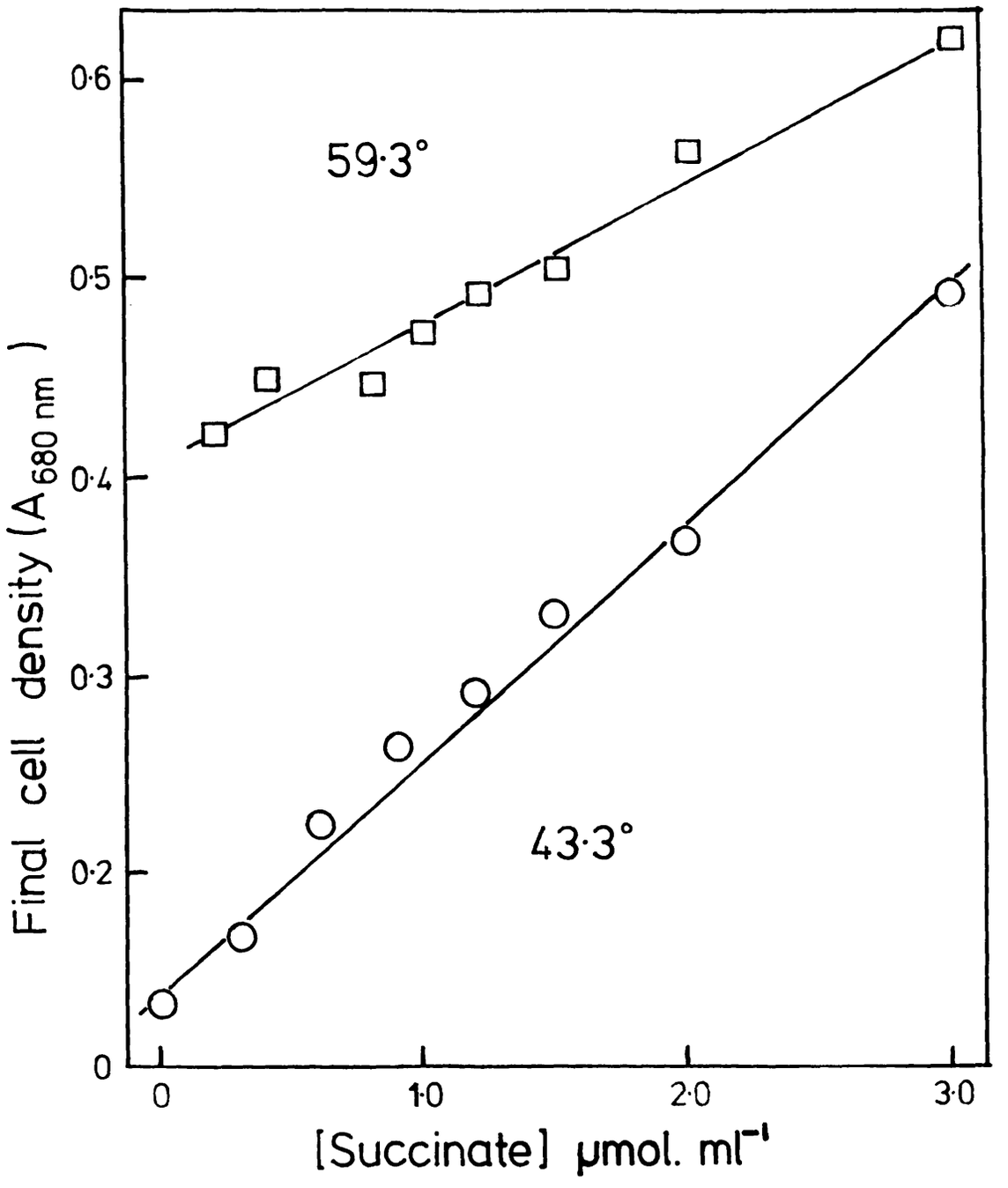


Figure 3.9. The relationship between cell yield and succinate concentration determined by the final cell density method at high and low temperatures.

E. coli ML 30 the few studies of the effect of temperature on molar growth yield have given similar results. Monod (1942) with E. coli, Senez (1962) with Aerobacter aerogenes and Desulfovigrio desulfuricans and Forrest (1969) with Zymomanas mobilis all found that the yield of cells was virtually constant at temperatures between that giving the maximum growth rate and the lowest temperatures of their experiments. Above the optimum temperature for growth rate the yield in each case fell off rapidly to reach zero only a few degrees above the optimum. It would appear that the rapid fall in yield of B. stearothermophilus above approximately 58° (Figure 3.8) corresponds to that noted by these workers. However there is no indication in their results of the type of change in yield at suboptimal temperatures shown by B. stearothermophilus in the experiments reported here. Ng (1969) examined the yield of E. coli cells at temperatures near the minimum for growth. His result was also quite different in character from the results with the thermophile in that he found the yield to fall sharply below 18° but remain constant between 18° and 35°.

Table 3.1 Yields of cells per available electron.

Values of Y_G were obtained from the curve in Figure 3.8.

Substrate	Temp. (°C)	$Y_{substrate}$ ($g.mole^{-1}$)	(av.e-) per mole	$Y_{av.e^-}$ ($g.(av.e^-)^{-1}$)
Glucose	43.3	88.0	24	3.67
Succinate	43.3	54.0	14	3.86
Glucose	59.3	54.0	24	2.25
Succinate	59.3	31.5	14	2.25

Ng, Senez and Forrest all considered that uncoupling between energy production and respiration was the cause of low molar growth yields at extremes of temperature and this appeared at first to be the most plausible explanation of the effect of temperature on the molar growth yield of B. stearothermophilus. The determination of Y_{O_2} values at different temperatures is one possible way of checking this point. Although this was not done in this study the results of experiments on the respiratory capacity of intact cells of the thermophile at different temperatures (results examined in greater depth in the Discussion, Section 3.4) suggest that at the higher temperatures there is some uncoupling of energy production from respiration.

Another possibility was that higher temperatures might have an effect on the pattern of glucose utilization. This has been examined in some detail here by studying the fate of the carbon atoms of radioactive glucose during growth at different temperatures.

3.3.3. The fate of glucose consumed during growth.

The results of experiments at 43.5° and 57.5° are shown in Figures 3.10 and 3.11 respectively. The three plots in each figure, linear until the cessation of growth, relate to the increase in cell mass the amount of glucose remaining in the medium, the amount of glucose incorporated into cell material and the amount of glucose carbon remaining in the medium. These plots yielded the data presented in Table 3.2 for an increment of cell mass during growth of 0.1 mg dry weight per ml. This analysis makes it clear that a major part of the effect of temperature on Y_G stems from the different degrees at "wastage" of the carbon source as unutilized material. Thus at 43.5° 7.82% of the glucose is wasted whereas at 57.5° the wastage figure rises to 29.8%. The values for Y_G obtained in this experiment (87.0 at 43.5° and 55.3 at 43.5°) agree well with those obtained by other methods (see Figure 3.8).

The assumption that the glucose carbon unaccounted for as cell material or waste (VI in Table 3.2) was CO_2 was confirmed by direct determination of CO_2 production during growth (Table 3.3). The results in Table 3.3 further show that the proportion of the glucose supplied that is oxidised to CO_2 is not greatly affected by the growth temperature.

These results suggested that the oxidative and the non-oxidative phases of glucose catabolism are not well co-ordinated at the high temperatures at which growth is most rapid. This question was further explored by examining the utilization and wastage of

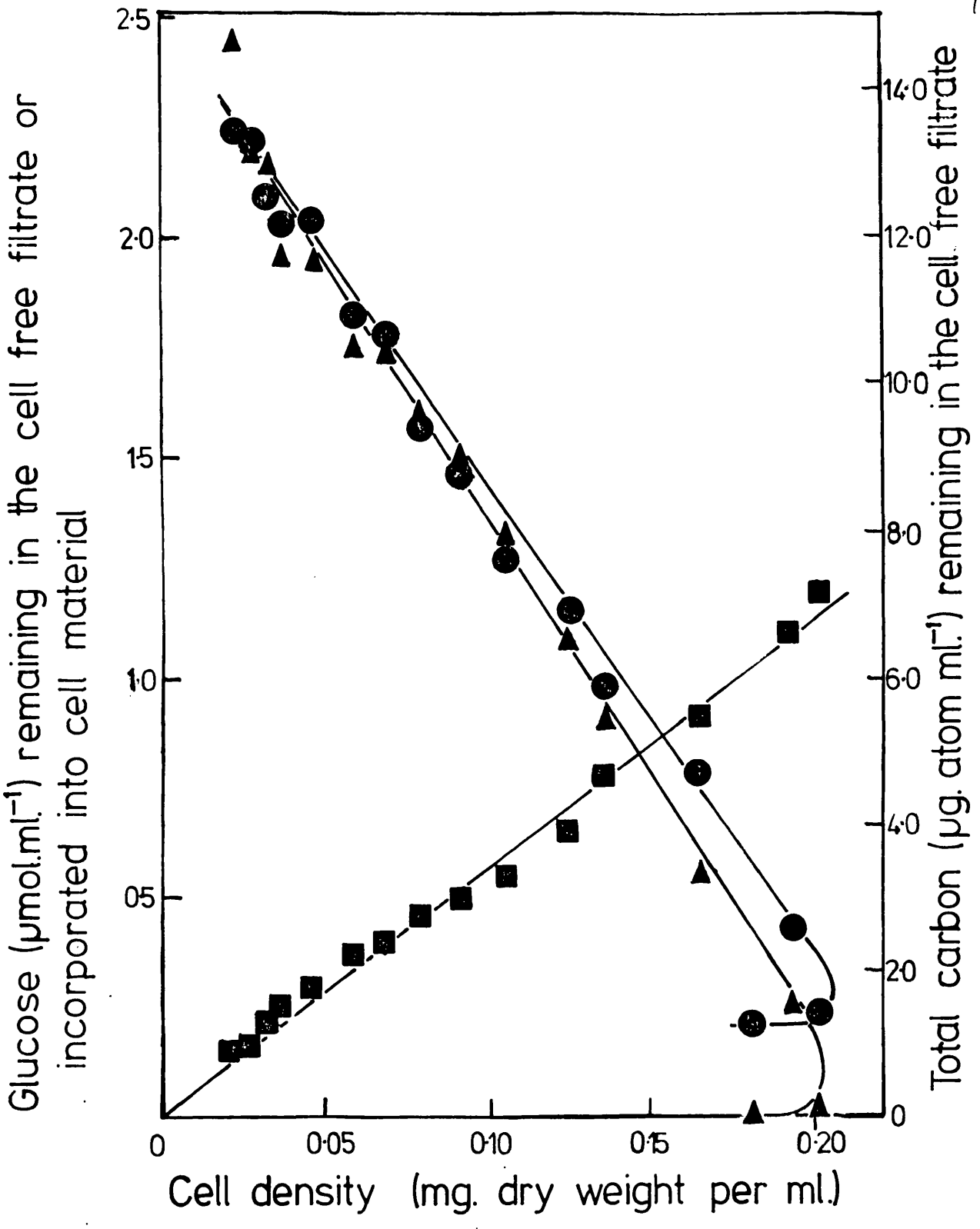


Figure 3.10. The fate of glucose consumed during growth at 43.5°. Samples were removed from the culture during growth and the concentrations of glucose (triangles) and total carbon (circles) in the supernatent, and the incorporation of glucose into cell material (squares) measured as described in the text.

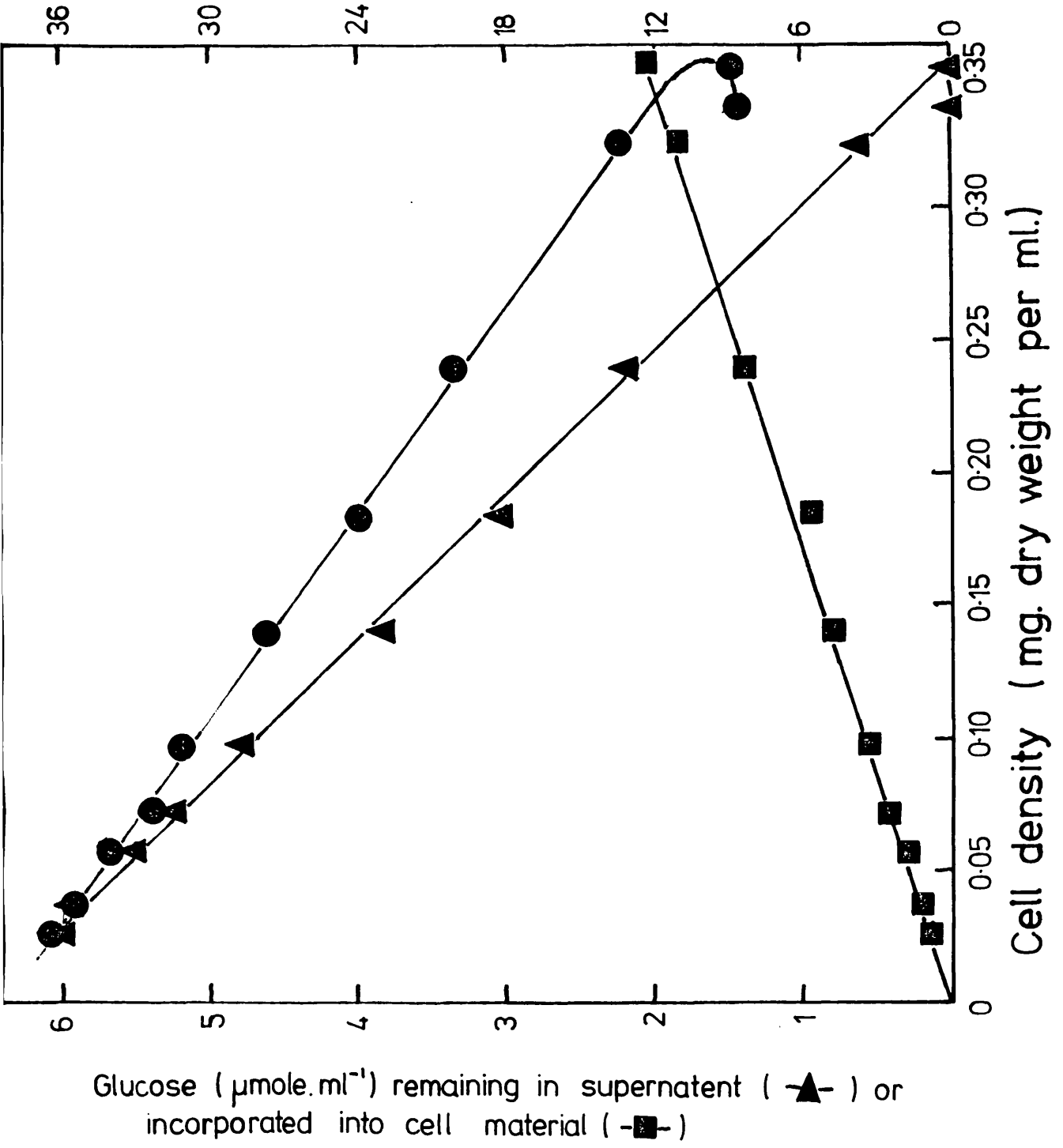


Figure 3.11. The fate of glucose consumed during growth at 57.0°. Other details are as given in the legend to Figure 3.10.

Table 3.2 The fate of glucose consumed during growth at high and low temperatures. Data I - VI were derived from the results presented in Figures 3.10 and 3.11 and correspond throughout to an increment of cell mass of 0.1 mg dry weight per ml.

	Experimental temperature	43.5°	57.5°
I	Glucose consumed (umoles.ml^{-1})	1.15	1.81
	Y_G (g.mole^{-1})	87.0	55.3
II	Glucose incorporated into cell material, (μmoles.ml^{-1})	0.580	0.564
	II as a percentage of I	50.4	31.1
III	Total carbon removed from the medium, (μgatoms ml^{-1})	6.36	7.62
IV	III expressed as glucose, (μmoles ml^{-1})	1.06	1.27
V	Glucose returned to the medium in non-utilizable form i.e. I-IV, (μmoles ml^{-1})	0.09	0.54
	V as a percentage of I	7.8	29.8
VI	Glucose unaccounted for as either waste (V) or cell material (II) i.e. I-(II+V), (μmoles ml^{-1})	0.48	0.71
	VI as a percentage of I	41.7	39.2

Table 3.3 Oxidation of glucose to CO_2 at high and Low temperatures. The CO_2 evolved from the amounts of U-C14-glucose indicated was collected as described in the text and, from its radioactivity, the proportion of glucose oxidised to CO_2 was calculated.

Radioactive glucose supplied (μmoles)	Glucose oxidised to CO_2 (μmoles)		Percent. glucose oxidised	
	Temperature			
	43.5°	57.5°	43.5°	57.5°
25	10.7	-	40.6	-
50	19.9	18.6	39.7	37.1
75	33.5	30.5	44.7	40.6
100	-	32.2	-	32.2
125	52.4	46.3	41.9	37.0
	Average -		42.9	36.4

glucose under conditions in which the use of glucose for processes other than the provision of energy was restricted by the inclusion in medium of casamino acids (0.2% w/v) and yeast extract (0.1% w/v). The results of this experiment are presented in Figure 3.12. It is seen that for each 0.1 mg increment of cell mass, during growth at 57.5°, 0.78 μmole of glucose (represented as 100%) disappeared (giving a Y_G value of 128.2). Of this 0.19 μmole (24.4%) was incorporated into cell material, the equivalent of 0.37 μmole (47.4%) was returned to the medium in a non-utilizable form and the remaining 0.22 μmole (28.2%) was

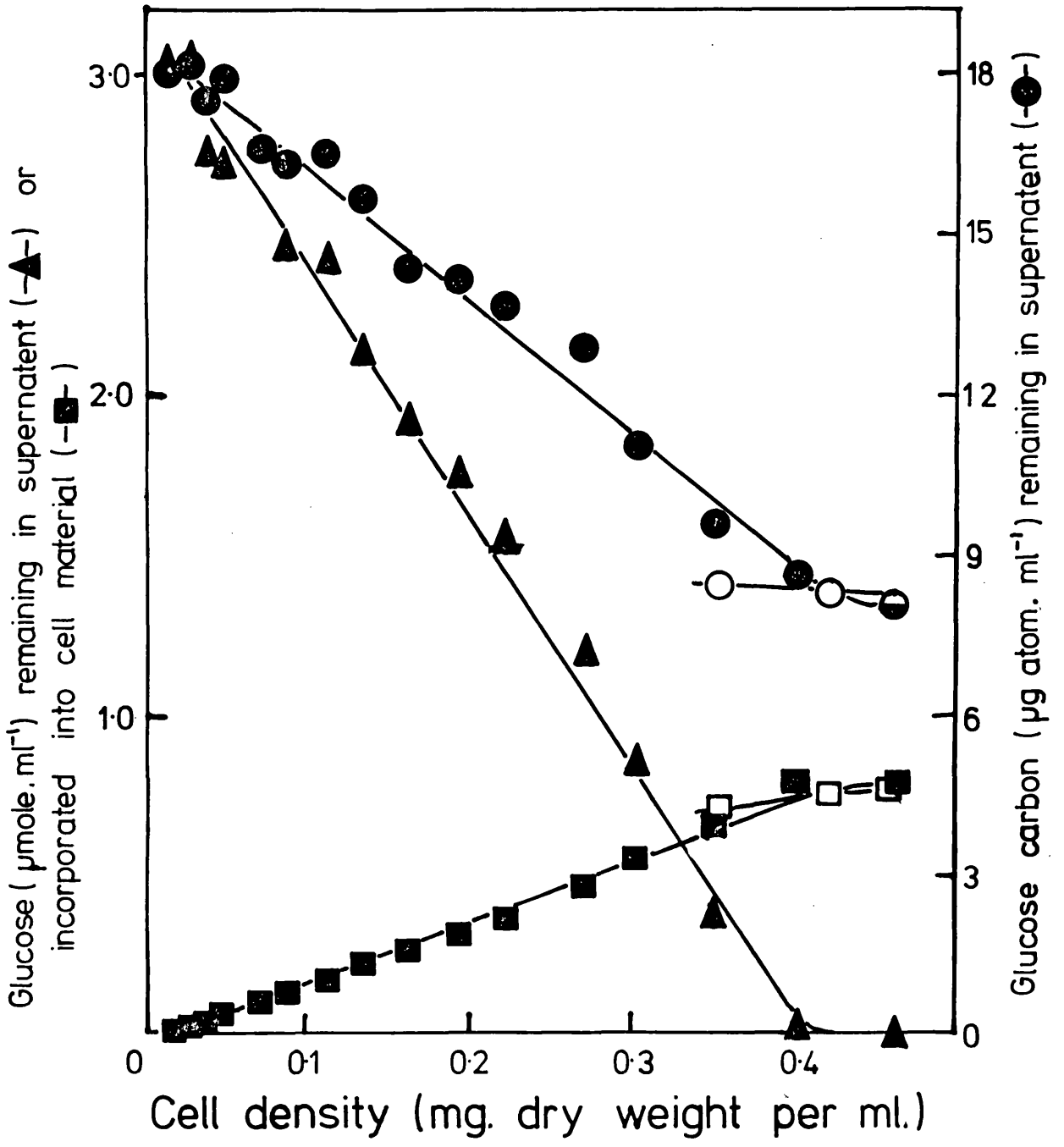


Figure 3.12. The fate of glucose consumed during growth at 57.5° in the prescence of casamino acids (0.2%) plus yeast extract (0.1%). For data obtained after the cessation of growth is plotted as open symbols.

presumably disposed of as CO_2. This suggests again that a considerable proportion of the glucose is degraded in the initial (non-oxidative) phase of metabolism and then discarded in the medium. The amount of glucose incorporated into cell material is, as might be expected, lower in this experiment than when the organism grows on glucose prototrophically (see Table 3.2). However the proportion of glucose carbon left unutilized in the medium is somewhat greater when the medium is supplemented with the amino acids and yeast extract (47.4%) than under prototrophic growth conditions (29.8%). Obviously, the provision of amino acids and other metabolites reduces the demands on glucose carbon as a source of cellular material but the inefficient control of the initial, non-oxidative phase of glucose degradation results in an even greater proportion of glucose being "wasted" in the medium largely in the form of acetate (see Section 3.3.5).

3.3.4 The oxidation of 6-Cl4-glucose during growth.

The discussion above on the co-ordination of the non-oxidative and oxidative phases of metabolism has tacitly assumed that the thermophile metabolizes glucose via the tricarboxylic acid cycle and that an electron transport system coupled to phosphorylation was available to facilitate the transfer of electrons from substrates to oxygen. This is certainly warranted by the magnitude of Y_G which is similar (especially at lower temperatures) to the Y_G values reported for mesophiles growing under similar conditions where the tricarboxylic acid (T.C.A.) cycle is known to be

Table 3.4 The production of 14-CO_2 from 6-C14-glucose and U-C14-glucose during growth.

Substrate	Radioactivity of CO_2 liberated from 20 nCi; 20 µmole of labelled glucose
6-C14-glucose	5175 c.p.m. av. of 4 determinations
U-C14-glucose	9783 c.p.m. av. of 3 determinations.

operating and furthermore is considerably greater than would be expected if glucose underwent a purely fermentative metabolism.

Further support for the assumption of an operative T.C.A. cycle is provided by the data in Table 3.4 which shows the production of 14-CO_2 during growth of the thermophile on 6-C14-glucose. In spite of the potential production of 14-CO_2 from 6-C14-glucose by the complete oxidation of glucose ~~hexose~~ in the hexose monophosphate phosphate pathway the production of 14-CO_2 from 6-C14-glucose is widely regarded (e.g. see Goldman and Blumenthal, 1964) as evidence of the operation of the T.C.A. cycle.

3.3.5 The identification and determination of "waste"products. An overnight (57°) culture having 5 mM U-C14-glucose as sole carbon source was the subject of these experiments. Determination of the radioactivity of the cell free filtrate revealed a total carbon concentration of 11.80 µg atom.ml^{-1}. The residual glucose concentration was 1.05 mM indicating a total concentration of non-glucose carbon of 5.46 µg atom.ml^{-1}.

No lactate (D or L) or pyruvate was detected in

spite of the sensitivity of the methods used. (Concentrations of 0.02 mM should be detectable in assays coupled to the oxidation or reduction of nicotinamide coenzymes.) However the acetate determination revealed a concentration of 2.18 mM. This is equivalent to 4.36 μg atom.ml^{-1} of carbon accounting for 80% of the total non-glucose carbon.

3.3.6 Respiration. Arrhenius plots of the relationship between temperature and the rate of oxygen uptake are shown in Figure 3.13. Values for the Arrhenius constant in the temperature range 40-55^{o} were 17.1 kcal mole^{-1} when glucose was the substrate and 13.8 kcal mole^{-1} with succinate as substrate. The most striking feature of these plots is their linearity down to a temperature of 20^{o} or lower, and some 20^{o} below the minimum for growth. This was in sharp contrast to the plots obtained by Stokes and Larkin (1968) with the psychrophile B. psychrophilus and the mesophile B. thirugensis. Both these organisms, but particularly the mesophile, gave continuously curved Arrhenius plots when whole cells oxidised glucose.

The response of the respiration of another mesophile B. lichenformis to temperature was therefore tested to examine the possibility that such curved Arrhenius plots were a general feature of mesophilic Bacilli. With exception of temperature procedures for the growth of this organism (strain A5- a generous gift of Mrs. E. Dickenson) preparation of the cell suspension and measurement of respiration were identical to those used for the thermophile. The same minimal salts medium, with 50 mM succinate as carbon source, was also used. The resulting Arrhenius

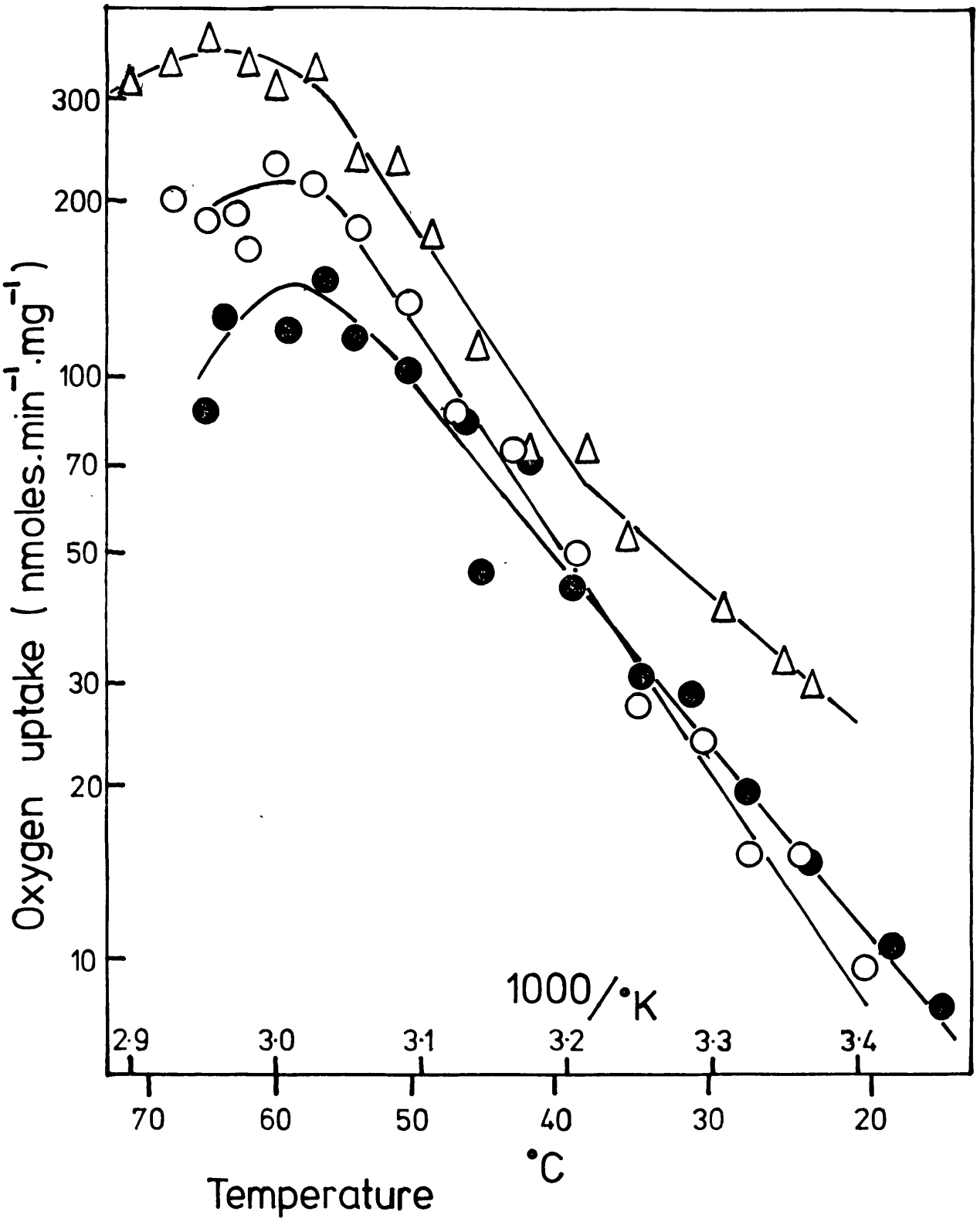


Figure 3.13. The effect of temperature on the respiratory activity of intact cells (plotted according to the Arrhenius relationship. Cells had been grown at either 42° (triangles) or 55° (circles). The carbon sources (for growth and as respiratory substrates) were either glucose (open symbols) or succinate (closed symbols).

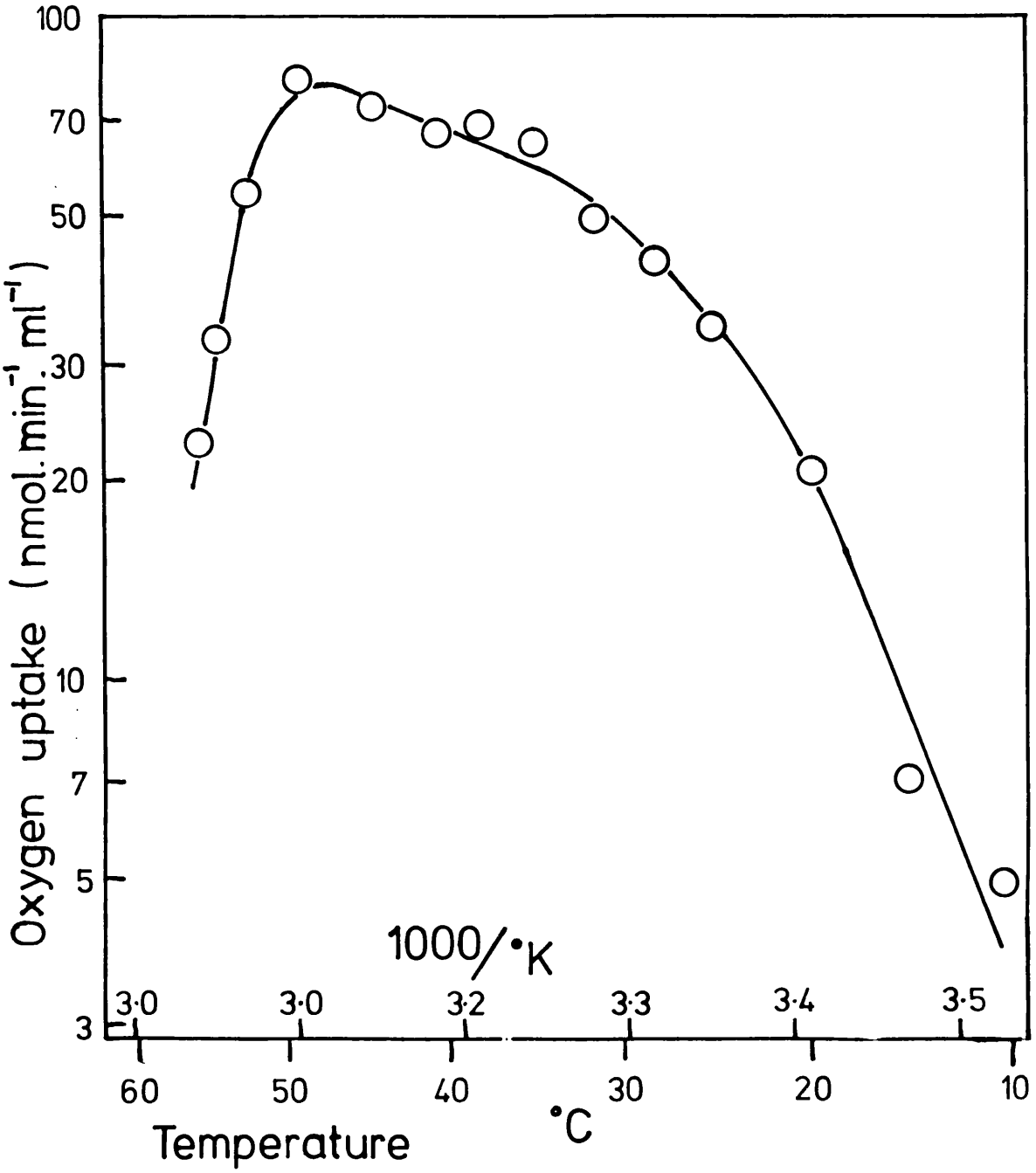


Figure 3.14. The effect of temperature on the respiratory activity of intact cells of B. licheniformis. In the absence of an established relationship between the absorbance of the culture and the cell density the rate of oxygen uptake has been referred to 1 ml of the original culture.

plot (Figure 3.14) is strikingly similar to that given by Stokes and Larkin (1968) for B. thirugensis. No attempt was made to assign values for the Arrhenius constant to this curved plot.

A comparison may be made between the results in Figure 3.13 and data for the rate of CO_2 production derived from Figures 3.10 and 3.11 and Table 3.3. by the following procedure. $Sene_Z$ (1962) suggested the use of the equation

$$Qt = (C) \int_{t_o}^{t} x.dt$$

(where (C) is the cellular rate of catabolic activity, i.e. the quantity of substrate utilized per gram of cells per hour, Qt is the amount of substrate utilized by the culture in time t, and the definite integral of the bacterial population during this time interval is obtained graphically from the area circumscribed by the growth curve) for deriving the rate of metabolic processes in growing cultures of microorganisms. Figure 3.15 shows the linearly plotted growth curves of the experiments presented in Figures 3.10 and 3.11. The areas under the curves, obtained as indicated in Figure 3.15 are presented in Table 3.5 together with the data for glucose consumption in the corresponding periods derived from Figures 3.10 and 3.11. At 57.5^{o} cells grown at 55^{o} on glucose as carbon source consume oxygen at a rate of 214 n mole.$min^{-1}mg^{-1}$. The data in Table 3.5 give a corresponding figure for glucose consumption of 270 n mole.$min^{-1}mg^{-1}$ of which 36.4% (Table 3.3) is oxidised to give 590 n mole.$min^{-1}mg^{-1}$ of CO_2. Similarly cells grown at 42^{o} gave a rate of

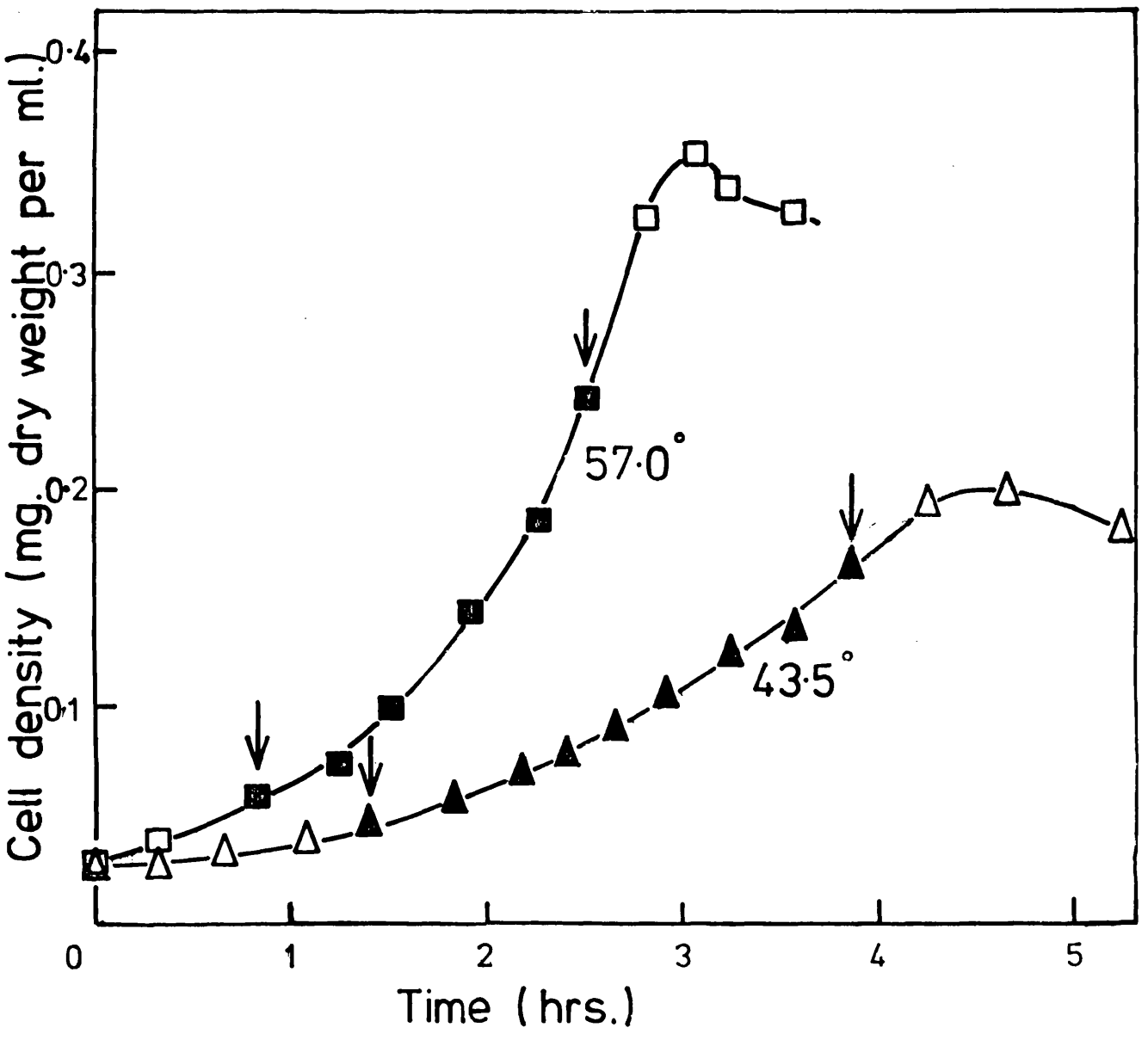


Figure 3.15. Linear plots of the growth curves from the experiments depicted in Figures 3.10 and 11. for the determination of the rate of glucose consumption (see the text) the areas under the portions of the curves delineated by arrows and closed symbols were obtained by mensuration.

oxygen consumption at 43.5° of 106 n mole $min^{-1}mg^{-1}$ which may be compared with the production of CO_2 of 220 n mole $min^{-1}mg^{-1}$. Both cell preparations consumed oxygen at only half the expected rate in spite of the care taken to avoid cell damage and provide an environment for respiration measurements closely resembling those associated with rapid growth. There was no significant difference between the rate of oxygen uptake at a standard temperature observed when a cell suspension was freshly prepared and some hours later at the completion of an experiment indicating that, once cooled, cells of this organism were not adversely affected by storage at low temperatures.

Table 3.5 Cellular rates of catabolic activity of cells at high and low temperatures. The rate of catabolic activity was calculated using the equation of Senez (1962) as discussed in the text. The quantities of glucose consumed (C) are taken from Figures 3.10 and 3.11. The integrals of the bacterial population are obtained from Figure 3.15.

	Temperature	
	43.5°	57.5°
Glucose consumed (umole ml^{-1} of culture)	1.38	3.30
Integral of bacterial population ($mg.ml^{-1}.min$)	16.14	12.20
(C) (μmole $mg^{-1}min^{-1}$ glucose consumed)	0.0855	0.270

3.4 Discussion

Payne's review (1971) provided an extensive survey of the results of molar growth yield determinations performed on a range of mesophilic heterotrophic organisms under conditions similar to those employed in the experiments reported here. Payne cites values of Y_G ranging from 69.5g.mole^{-1} for Pseudomonas fluorescens (Hernandez and Johnson, 1967) to 94 g.mole^{-1} for E. coli (Ribbons, 1969). In particular a value of 81.3 g.mole^{-1} for B. subtilis (Baschnagel-De Pamphilis and Hanson, 1969) is recorded. The values obtained in this work with B. stearothermophilis at low temperatures (Y_G 87.0 g.mole^{-1} at 43.5°) are clearly in accord with these values indicating that fundamental differences in the energy metabolism of mesophiles and this strain of B. stearothermophilis are unlikely. The value for $Y_{succ.}$ of 54.0 g.mole^{-1} is also of the same order as the values of 42.3 and 41.6 g.mole^{-1} reported for species of Pseudomonas cited by Payne.

As has already been mentioned there are two basic explanations for the fall in the molar growth yield of this organism from the "normal" values observed at the bottom end of its temperature range to the low values shown at the optimum temperature for growth rate. The determinations of the fate of glucose show that a large part of this fall is accounted for by the change in the proportion of glucose that was returned to the medium in a form that the organism was unable to utilize for growth. This "wastage" of glucose by aerobically growing organisms has been noted by other

workers. Ng (1969) found that about 20% of the glucose supplied to *E. coli* ML 30 was returned to medium as non-utilizable (and also unidentified) compounds. Nakata and Halvorson (1960) found that at the point of exhaustion of glucose from the medium *B. cereus* had converted approximately $\frac{3}{4}$ of the glucose to acetic and pyruvic acids. These observations suggest that it may not be unusual for the oxidative and non-oxidative phases of glucose metabolism to be poorly co-ordinated. The important feature of this phenomenon for the thermophile is that the degree of co-ordination is apparently temperature dependent. Hanson and Cox (1967) reported that the addition of glutamate to a minimal salts/glucose growth medium repressed key T.C.A. cycle enzymes of *B. subtilis*, *B. licheniformis* and *E. coli*. The probable occurence of this phenomenon in *B. stearothermophilis* also would account for the greater "wastage" of glucose in the presence of amino acids and other metabolites further accentuating the relative inefficiency in the co-ordination of glucose metabolism.

There is some evidence that the change in the extent of glucose oxidation is not the sole cause of the change in yield with temperature. The values of $Y_{av.e-}$ may be recalculated taking into account the product of glucose metabolism left in the medium unutilized and assuming this to be entirely acetate. The conversion of one molecule of glucose to 2 molecules of acetate plus 2 of CO_2 makes available 8 electrons. We may therefore derive modified values of $Y_{av.e-}$

from the data in Table 3.2 as shown in Table 3.6. There still remains a fall in yield of 24.8% as the temperature is raised from near the minimum for growth to a temperature in the range where growth is most rapid. One possible explanation of this that as the temperature is raised the coupling between electron transport and ATP generation becomes less efficient.

Although direct measurements of Y_{O_2} were not made in this study an insight into the relationship between oxygen uptake and the increase in cell mass may be gained by comparison of the Arrhenius plots for growth rate and respiration rate. Ng (1969) found with E. coli ML30 that, not unexpectedly, the two plots were parallel (i.e. gave identical Arrhenius constants) in the temperature range where molar growth yield was constant. The plot for oxygen uptake rate remained linear to low temperatures whereas the plot for growth rate deviated from linearity at the same temperature as that at which the molar growth yield began to fall from its constant value. The growth rate of this strain of B. stearothermophilus reaches a plateau at about 53°-58° (Epstein and Grossowicz, 1969a). Nevertheless the growth curves in Figure 3.4 indicate that the growth rate increases by a factor of 1.83 in the temperature range 43.5°-57.5° while the rate of oxygen uptake, shown in Figure 3.13, increases by a factor of 2.80 in the same interval. We thus have strong circumstantial evidence for a deleterious effect of high temperature on energy coupling in the organism.

Table 3.6 The derivation of values for $Y_{av.e-}$ modified by an allowance for the production of acetate. As in Table 3.2, from which the data below are obtained all figures refer to an increment of growth of 0.1 mg in a volume 1 ml.

	Experimental temperature	43.5°	57.5°
I	Total carbon removed from the medium expressed as glucose (μmoles ml^{-1})	1.06	1.27
II	Available electrons (μmole equivalents) i.e. (I) x 24	25.44	30.48
III	Glucose converted to acetate (μmoles ml^{-1})	0.09	0.54
IV	Available electrons (μmole equivalents) i.e. (III) x 8	0.72	4.32
	Total available electrons (μmole equivalents) i.e. (II + IV)	26.16	34.80
	$Y_{av.e-}$ ($g.(av.e-)^{-1}$)	3.82	2.87

There are some reports in the literature of studies of the effect of temperature on oxidative phophorylation in mitochondrial preparations from mammals (Kemp et al., 1969 and Horowitz et al., 1967) and insects (Davison, 1971). No general conclusions can be drawn from the small changes in P:O ratios and respiratory control indices that were found.

Two other features of the effect of temperature on respiration of the thermophile are worthy of comment. Firstly the values of the Arrhenius constant of 17.1 and 13.8 k cal.mole^{-1} obtained when glucose and succinate respectively served as the substrate are quite similar to the corresponding values reported for mesophilic bacteria. Values have been obtained for the Arrhenius constant (or the activation energy) for respiration of 13.4 k cal.mole^{-1} for a species of Rhizobium (Koffler et al., 1947), 18 k cal.mole^{-1} for E. coli ML 30 (Ng, 1969) and 12.8 k cal.mole^{-1} for A. aerogenes (Senez, 1962). There is thus no support here for the suggestion that high Arrhenius constants are a characteristic of thermophiles (Ingraham, 1958). A second notable feature of the Arrhenius plots in Figure 3.15 is their reasonably linear character. Lyons and Raison have shown that breaks in the Arrhenius plots of respiratory rate of mitochondrial preparations from a number of both plant and animal tissues possibly may be correlated with phase changes in the membrane lipids (Raison et al., 1971a). In particular they have shown that the breaks in Arrhenius plots are characteristic of prepartions from higher plants sensitive to chilling injury (Lyons and Raison, 1970a) and

homeothermic animals (Lyons and Raison, 1970b). Plants and animals less sensitive to temperature changes gave continuous, linear Arrhenius plots (Raison et al., 1971b). An extension of their reasoning to the results obtained with B. stearothermophilus would support the suggestion, made in the General Introduction (Section 1.1) that the character of the membrane lipids may not be an important factor in the thermophilic habit of this organism.

4. ENZYME STUDIES

4.1 Introduction

Two lines of argument prompted an examination of the possible effects of temperature on a key regulatory enzyme in B. stearothermophilus. First is the attractiveness of the suggestion, first made by Ingraham and Maaløe (1967), that it is the derangement of regulatory mechanisms at extremes of temperature that establishes the limits of the temperature range within which the growth rate of a microorganism obeys the Arrhenius equation. The study of the response to temperature of a key regulatory enzyme in the thermophile would, it was thought provide a test of this hypothesis. It has been shown (Sundaram et al., 1969 and Sundaram 1973) that pyruvate carboxylase is the enzyme responsible for the anaplerotic fixation of CO_2 during growth of B. stearothermophilus on glucose. This means that the pyruvate kinase reaction has a role in both glycolysis and the anaplerotic pathway of CO_2 fixation in this organism. Any derangement at extremes of temperature in the regulation of this enzyme would obviously have repercussions for energy metabolism and the provision of intermediates for biosynthesis derived from pyruvate.

A second argument which prompted this study was the indication from the preceeding phase of this work that the co-ordination between the oxidative and non-oxidative phases of glucose metabolism was adversely affected by high temperature. The regulation of the pyruvate kinase reaction is clearly a crucial part of this co-ordination as has been demonstrated in many

other microorganisms (see Sections 4.3.3. and 4.4)

For the studies to be described here the pyruvate kinase of B. stearothermophilus was partially purified so as to eliminate the interference of the adenylate kinase activity present in crude cell extracts. A thirty fold purification was achieved by the use of ion exchange chromatography and gel filtration.

No attempt has been made to obtain a comprehensive description of the kinetic and regulatory properties of the enzyme. The work to be described here has been directed mainly towards revealing which, if any, of the features of the enzyme regulation are sensitive to temperature change. In general the pattern of the regulation of the pyruvate kinase was found to be that expected of the enzyme from a heterotrophic, obligate aerobe, i.e. a sigmoid response of activity to the concentrations of the substrates phospho enol pyruvate (PEP) and ADP, activation by AMP(as in this case) or fructose 1,6 diphosphate (FDP) and inhibition by ATP. This pattern of positive and negative effectors was very similar to that recently established for the pyruvate kinase from B. licheniformis (Tuominen and Bernlohr 1971 a and b). The effects of temperature on the kinetic and regulatory parameters were small, rather contrary to expectations. However the general trend of all those variations that did occur was towards less effective regulation at high temperatures.

During the course of preliminary studies a number of substrates cofactors and effectors were found to enhance the thermolability of the enzyme and this

phenomenon was examined in some detail. Tuominen and Hernlohr (1971 a and b) reported a related phenomenon with the B. licheniformis enzyme. The effect of the allosteric activator FDP on the cold lability of the yeast pyruvate kinase (Kuczenski and Suelter, 1970) however provided a closer analogy.

4.2 Methods

4.2.1 Assay systems for pyruvate kinase. The assay system employed was essentially that of Bücher and Pfleiderer (1955) which uses the lactate dehydrogenase reaction for the determination of the pyruvate formed by the kinase from PEP. For the determination of the total enzyme activity of fractions obtained during purification and after heat treatment in the thermal inactivation experiments the assay system was as follows.

In a final volume of 1.0 ml, contained in a semi-micro glass cuvette of 1 cm pathlength were mixed: .10 μmoles of magnesium sulphate; 2 μmoles of PEP; 5 μmoles of ADP; 0.25 μmoles of NADH; 100 μmoles of potassium chloride; 50 μmoles of imidazole-HCL pH 6.8, 4 units of lactate dehydrogenase and the enzyme sample.

The PEP was found to contain variable small amounts (1-2%) of free pyruvate and it was therefore necessary to ensure the complete reduction of this to lactate before the assay commenced. This was achieved by always pipetting the PEP, NADH, lactate dehydrogenase and buffer into the cuvette before the other reagents and never less than one minute before the addition of the enzyme sample to initiate the reaction. No attempt was made to correct the recorded PEP concentration for this

contamination. The oxidation of the NADH was followed at 340 nm using a recording spectrophotometer with a water jacketed cuvette compartment. The circulating water was maintained at a temperature of 30°.

For preliminary kinetic studies the concentrations of reagents were varied from those given above according to the requirements of the particular experiment. This was of course true also of the experiments designed to test the effect of temperature on the response of the enzyme to substrates and effectors; in addition for this work imidazole-HCL buffer was not used. In its place piperazine ethane sulphonate (Pipes)-NaOH was used at a final concentration of 20 mM. This buffer, unlike imidazole-HCL or tris-HCL, has a very small temperature coefficient (Good et al., 1966) making it better suited to this work. For each experimental temperature a concentrated stock buffer was prepared with its pH at laboratory temperatures adjusted so that it would give a pH of 6.8 at the experimental temperature.

Accurate control of the temperature of reaction mixtures during continuous spectrophotometric assays presents particular problems at the high temperatures involved in these experiments. For the determination of the temperature of the small volume contained within a semi-micro cuvette a thermocouple or thermistor is convenient. An apparatus constructed in this laboratory by Mr. R. Parkin which employed a thermistor was used in this work. The circuit diagram is shown in Figure 4.1. The instrument was used with switch (a) in the "READ" position except when the thermistor was bypassed for

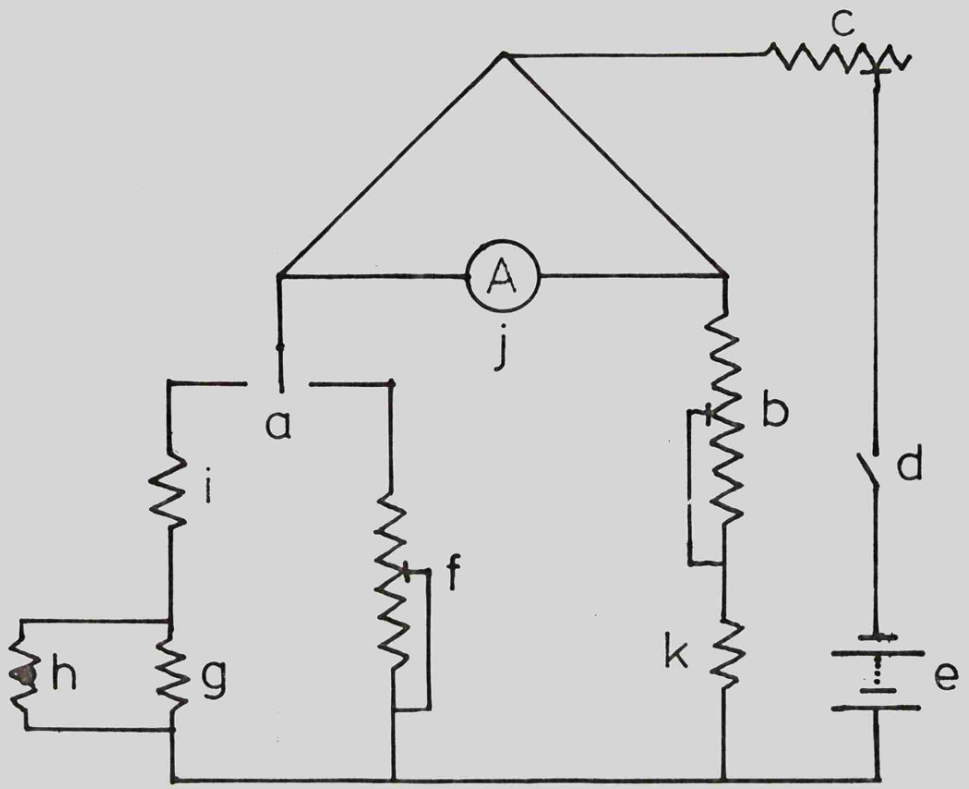


Figure 4.1. Circuit diagram of the apparatus for the measurement of the temperature of reaction mixtures.

Key:- a) Double pole double throw switch.
b) 100 Ω linear potentiometer.
c) 1 k Ω linear potentiometer
d) On-off switch.
e) 6-volt dry battery.
f) 500 Ω linear potentiometer.
g) 390 Ω resistor.
h) Thermistor (Mullard VA 3700).
i) 330 Ω resistor
j) 0-50 μA F.S.D. moving coil meter.
k) 620 Ω resistor.

a check on the condition of the battery. Crude calibration was achieved as follows. With the thermistor immersed in the cooler of two water baths at known temperatures the variable resistor (b) was adjusted to bring the meter reading to zero. The thermistor was then transferred to the warmer bath and the variable resistor (c) adjusted to define the full scale deflection of the instrument. For accurate work a fine calibration was obtained by noting the meter readings given by immersion of the thermistor in reference water baths maintained at temperatures that spanned a range from a few degrees above to a few degrees below the expected temperature of the cuvette contents. The test temperature was then obtained from the meter reading by interpolation. The relationship between the meter reading and temperature to be linear to within 0.05° over a range of 5°. The thermistor was sealed together with its leads into a thin walled glass capillary of no more than 1 mm outside diameter. This meant that the response of the meter reading to temperature change was virtually instantaneous and that the insertion of the cold thermistor to the cuvette had no significant cooling effect on the contents.

The temperature of the cuvettes was maintained during spectrophotometeric assay by the use of a water jacketed cuvette carriage. Water was circulated from a bath maintained $1\text{-}2^{\circ}$ above the experimental temperature to allow for cooling in transit to the spectrophotometer. The cuvette temperature rather than that of the circulating water was carefully maintained.

The time taken for the cuvette reaction mixtures to reach the experimental temperature was reduced in a number of ways. The completely thermostable reagents, buffers, water for volume corrections and potassium chloride solutions were kept in the water bath at the experimental temperature. The other reagents were kept in ice. During the addition of all reagents except the pyruvate kinase the cuvettes were held in a second water jacketed cuvette carriage heated by a parallel water supply to that used for the spectrophotometer. This mixture, which lacked only pyruvate kinase, was then transferred to the spectrophotometer and allowed to reach the experimental temperature. The time necessary for this equilibriation had been established previously using the thermistor with cuvettes containing only buffer.

Pyruvate kinase was then added from a disposable micropipette (Drummond Microcap) to start the reaction, the cuvette contents were mixed by inversion and the cuvette replaced rapidly in the spectrophotometer. The volume of pyruvate kinase was kept to 10 μ l or less to facilitate rapid addition and to reduce to a negligible degree the cooling effect of the addition of the cold enzyme preparation.

One unit of pyruvate kinase is defined that amount of enzyme which catalyses the production of 1 μmole of pyruvate from PEP per minute at 30^{o} in the standard assay system (with imidazole-HCL buffer) described above.

4.2.2 <u>The determination of contaminating enzyme activities in pyruvate kinase preparations.</u>

(a) Adenylate kinase was assayed in the standard reaction mixture for pyruvate kinase except that the ADP was replaced by ATP (5 mM) and AMP (5 mM); 0.5 units of purified B. stearothermophilus pyruvate kinase was used.

(b) Fructose 1,6 diphosphatase (FDPase) was assayed in a system based on that of Fernando and co-workers (1969). The reaction mixture contained, in a volume of 1 ml, 50 µmoles of tris-HCL pH 7.5, 5 µmoles of magnesium sulphate, 2 umoles of fructose diphosphate, 0.5 µmoles of NADP and 5 µg each of glucose-6 phosphate dehydrogenase (G6PDH) and hexose phosphate isomerase (HPI). The reaction was initiated by the addition of the pyruvate kinase sample and monitored by the reduction of NADP to NAPDH (observed spectrophotometrically at 340 nm) that forms the final step in the coupled reaction system:

$$\text{Fructose diphosphate} \xrightarrow{\text{FDPase}} \text{Fructose-6-phosphate} + \text{P};$$

$$\text{Fructose-6-phosphate} \xrightarrow{\text{HPI}} \text{Glucose-6-phosphate}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^{+} \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^{+}$$

4.2.3 Protein determination. The determination of the protein content of crude cell extracts was by the biuret method (Gornall et al., 1949). During the later stages of purification the spectrophotometric method of Warburg and Christian (1942) was used.

4.2.4 Thermal inactivation studies. Partially purified pyruvate kinase, free from adenylate kinase activity and having a specific activity of about 20-25 units per mg. protein was used in all these experiments. Before use in an experiment the enzyme was dialysed in

the cold room in two stages to remove the ammonium sulphate, EDTA and phosphate contained in the preparation. In the first stage the enzyme was dialysed overnight against 1000 volumes of 1 mM potassium phosphate buffer pH 6.8 containing 0.1 mM EDTA and 1 mM mercaptoethanol. The second stage of the dialysis was conducted for 4 hr. against 100 volumes of 1 mM Pipes-NaOH (pH 6.8) also containing 1 mM mercaptoethanol.

The heat treatment of the enzyme was carried out in 100 x 12 mm test tubes (closed with loosely fitting aluminium caps) held in a water bath at the appropriate temperature. 0.9 ml of 1 mM Pipes-NaOH buffer (pH 6.8) containing the desired supplement at the appropriate concentration was placed in the test tube and allowed to come to the experimental temperature. The enzyme (0.10 ml) was then added, the contents of the tube were mixed with a vortex mixer, the tube replaced in the water bath and the clock started. Immediately, and at suitable intervals thereafter, samples (approximately 0.06 or 0.11 ml) were withdrawn into a cold pipette and precisely 0.05 or 0.10 ml delivered immediately into a previously prepared reaction mixture for the determination of the pyruvate kinase activity remaining.

4.3 Results

4.3.1 Enzyme purification

Step 1. The production and harvest of cells.

Nutrient broth (120 ml) was inoculated from a nutrient agar slope of the organism and incubated (with shaking) at 55° until the culture reached an absorbance of about 0.6 at 680 nm. 10 mls of this culture then served as

an inoculum for each of 12 2 litre Erlenmeyer flasks, each containing 1500 ml of minimal salts medium with 50 mM glucose as carbon source. The flasks had been brought to the incubation temperature of 55° before inoculation. These were then incubated for about 18 hours at 55° in the New Brunswick Gyrotary Shaking Incubator. The poor air circulation in the incubator resulted in temperature differences of up to 5° between flasks in different parts of the shaker and it was necessary to pool and redistribute the flask contents after about 12 hours to ensure even growth in all flasks. The flasks were removed from the shaker and cooled to room temperature when their absorbance reached about 0.9 (i.e. a cell density of 0.4 mg dry weight per ml) and harvested in a Sharples continuous centrifuge. The packed cells were resuspended for washing in approximately 1 litre of phosphate/EDTA buffer (10 mM potassium phosphate, 1 mM EDTA pH 6.8) and centrifuged at 5000 x g for 10 minutes in a M.S.E. 18 centrifuge run at 5°. Unless required for use immediately the packed cells were then stored at -20°.

Step 2. The preparation of a crude cell free extract. In a typical preparation 27.2 g. (wet weight) of cells were resuspended in 190 mls of phosphate/EDTA buffer. Lysozyme (20 mg.) was added and after mixing the suspension was incubated at 37° for 30 minutes. The lysate was then centrifuged in the M.S.E. 18 centrifuge at 38000 x g for 30 minutes at 5°. The glutinous DNA rich supernatent was carefully separated from the cell debris and the protein concentration determined. To this supernatent was

added, with constant stirring, 284 mg of protamine sulphate (15 mg per 100 mg protein) as a 5% (w/v) solution. After the addition was complete stirring was continued for 30 minutes to complete the precipitation of nucleic acid material. The precipitate was removed by centrifugation at 40,000 x g for 30 minutes at 5°. This supernatent was then dialysed overnight against 10 litres of phosphate/EDTA buffer containing 1 mM mercaptoethanol.

In all subsequent steps in the purification the buffer contained (except where otherwise indicated) 10 mM potassium phosphate, 1 mM EDTA and 1 mM mercaptoethanol, pH 6.8.

Step 3. Ion-exchange chromatography. Early attempts at the purification of pyruvate kinase had shown that approximately 50% of the enzyme activity was lost on each occasion that the enzyme was precipitated with ammonium sulphate. It was therefore considered inadvisable to include such a step in the purification procedure.

For ion-exchange chromatography a column of DEAE-cellulose (Whatman DE52) was prepared according to the manufacturers recommendations. The ion-exchanger was resuspended in phosphate/EDTA buffer and, after allowing a suitable time for the suspension to settle the fines were removed by decantation of the supernatent liquid. Equilibriation was then achieved by repeated resuspension of the ion-exchanger in phosphate/EDTA buffer of 5 times the usual concentration, i.e. containing 50 mM phosphate and 5 mM EDTA. After prolonged washing in 10 mM phosphate/EDTA/Mercaptoethanol

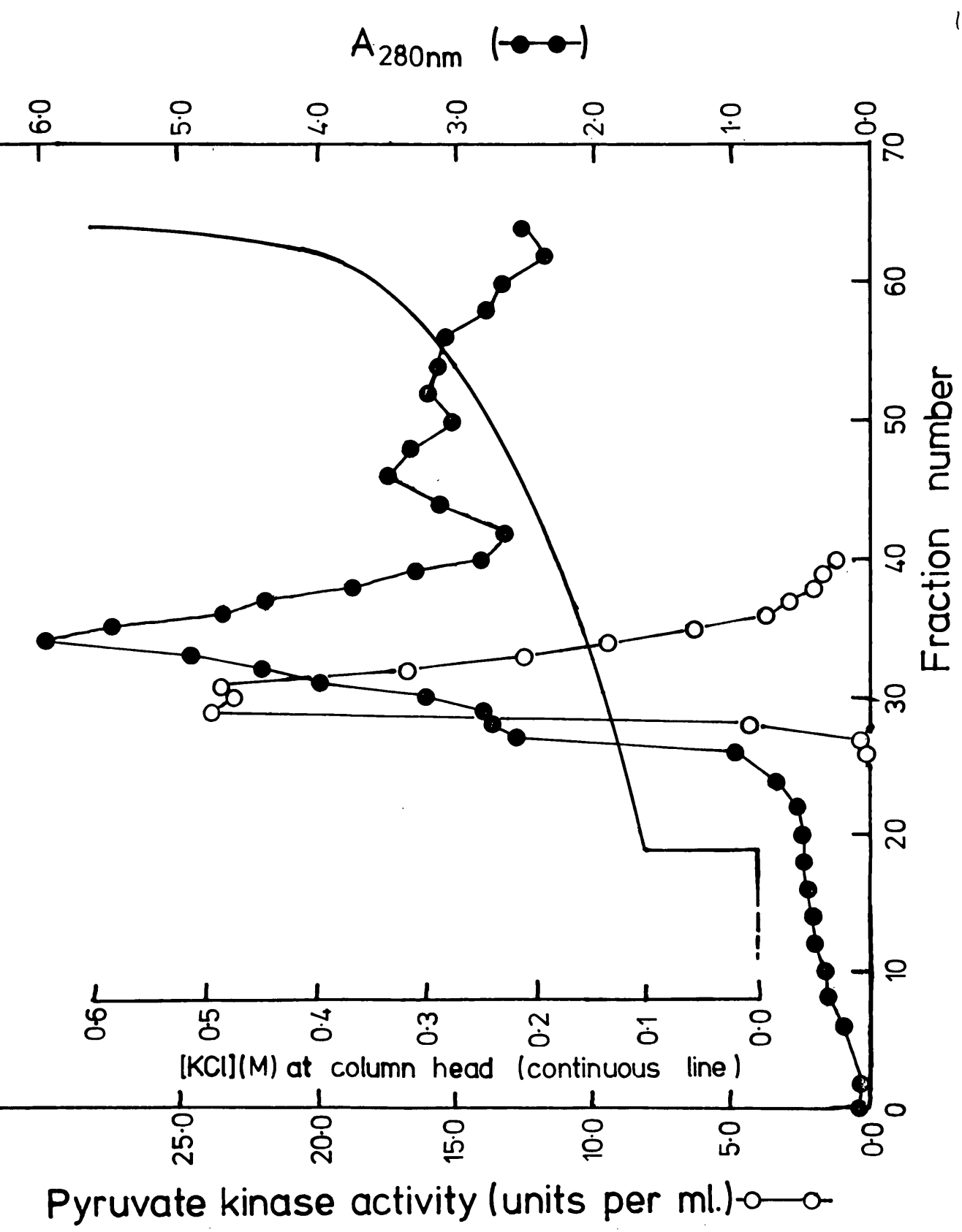


Figure 4.2. The elution of pyruvate kinase from DEAE-cellulose. The pyruvate kinase activity of the (10 ml) fractions was measured as described in the text. The concentration of KCl at the head of the column was calculated using the equation of Bock and Ling (1954). The protein concentration in the eluate was monitored as the absorbance at 280 nm.

buffer the column of the DEAE-cellulose was prepared (15 cm high, 3 cm diameter, bed volume 106 cc) and transferred to the cold room where all chromatographic procedures were conducted. The entire supernatent liquid from Step 2 was applied to the column followed by 20 mls of buffer. The column was then eluted with a non-linear gradient of potassium chloride in phosphate/EDTA/mercaptoethanol buffer. The concentration of potassium chloride in the mixing vessel (350 ml) was 0.1 M and in the reservoir (100 ml) was 0.6 M. The advantage of this scheme was that the potassium chloride gradient remained shallow in the concentration range where the pyruvate kinase was eluted but rose sharply after this point to strip all the remaining protein from the column. 10 ml fractions were collected and the pyruvate kinase activity and protein concentrations determined. Figure 4.2 shows the elution pattern obtained. Superimposed upon the figure is a curve indicating the KCL concentrations expected at the head of the column calculated from the equation

$$c = c_2 - (c_2 - c_I)\,(I - {}^{u}/_{v})^{\,A_r/A_m}$$

where c is the concentration in the eluant when the volume (Bock and Ling, 1954) u has emerged from the mixing vessel, out of a total volume v, c, is the initial concentration in the mixing vessel, c_2 is the concentration in the reservoir and A_m and A_r are respectively the areas of the horizontal cross sections of the mixing and reservoir vessels. Fractions having a specific activity greater than 4 (i.e. 28-35 inclusive) were pooled.

A second DEAE-cellulose column was used for concentrating these fractions. This column was prepared from the same batch of DEAE cellulose as the preparative column and was 7.3 cm high and 2 cm in diameter (bed volume 23 cc). The pooled fractions from the first column were diluted with an equal volume of buffer (to reduce the potassium chloride concentration) and run onto the concentrating column. After washing with buffer the enzyme was eluted with buffer containing 0.5 M potassium chloride. The proteins were collected in a 7 ml fraction. The identification and collection of this fraction was greatly facilitated by the occurence in the pyruvate kinase containing fractions of a yellow coloured protein.

Step 4. Gel Filtration. Sephadex G-200 (Pharmacia, Upsala) was swollen by boiling in phosphate/EDTA buffer containing 0.3 M ammonium sulphate. After cooling in a closed vessel the slurry was poured into a K25/45 Sephadex chromatographic column tube. The resulting gell bed had a length of 390 mm and a diameter of 25 mm. Before use the column was equilibriated with more buffer containing 0.3 M ammonium sulphate and 1 mM mercaptoethanol. 2.5 mls of the concentrated enzyme from Step 3 was then applied to the column after the addition of sufficient solid potassium chloride to ensure that its density was greater than that of the equilibriating buffer. The column was then eluted with buffer (phosphate/EDTA/mercaptoethanol/ammonium sulphate) and 2 ml fractions were collected. Figure 4.3 shows the elution pattern

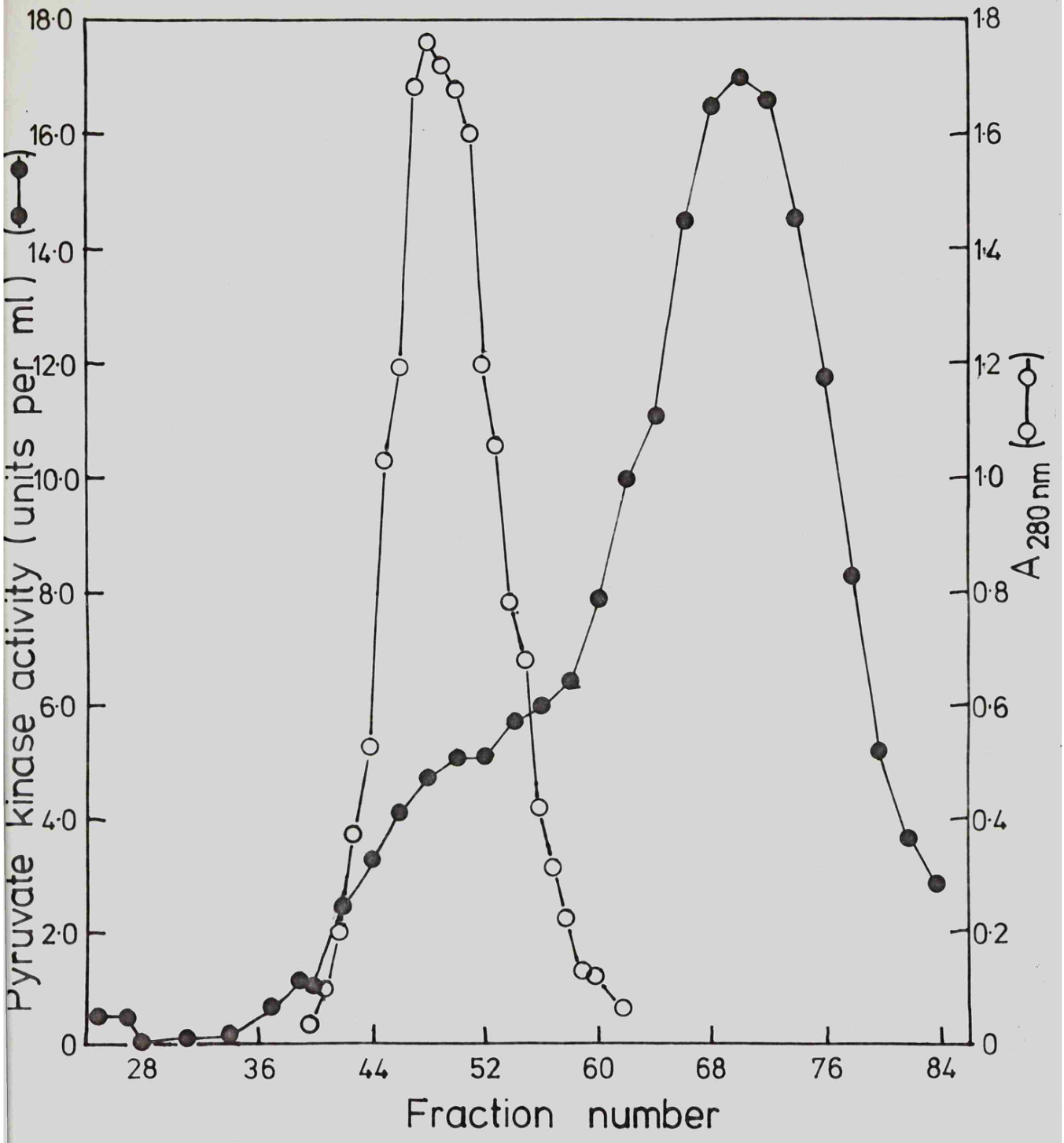


Figure 4.3. The elution of pyruvate kinase from Sephadex G-200. The pyruvate kinase activity of the (2 ml) fractions was measured as described in the text. The protein concentration in the eluate was monitored as the absorbance at 280 nm.

Table 4.1. Summary of the partial purification of pyruvate kinase.

Step	Fraction	Volume ml.	Protein mg.	Total pyruvate activity units [a]	Specific activity units per mg protein	Purification	Yield %
1	Crude extract	180	1890	1421	0.751	1	100
2	Supernatent from protamine sulphate after dialysis	190	-	1384	-	-	97.4
3	Pooled fractions from DEAE-cellulose	80	277	1189	4.30	5.73	83.5
	Ditto after concentratio n	7	261	962	3.68	4.90	67.6
	Fraction appl ied to Sephadex G200	2.5	93.3	343	3.68	4.90	-
4	Pooled fractions from Sephadex G200	26	13.13	303	23.1	30.7	59.7[b]

(a) The unit of pyruvate kina se aotivity is defined in Section 4.2.1.

(b) This figure is calculated on the assumption that all 7 ml of concentrated enzyme had been submitted to gel filtration.

obtained. Fractions having a specific activity greater than 9 (i.e. 43-55 inclusive) were pooled. This product was the partially purified pyruvate kinase preparation used in the experiments that follow. A summary of the purification procedure is shown in Table 4.1.

The product from Step 3 contained no fructose diphosphatase activity but adenylate kinase cochromatographed with the pyruvate kinase on DEAE-cellulose. However during gel filtration the adenylate kinase activity moved with the bulk of protein (i.e. fractions number 64-76, Figure 4.3) and could not be detected in the pooled, pyruvate kinase containing fractions.

4.3.2 Thermal stability studies. During the course of preliminary experiments on the purification of the pyruvate kinase the effect of a number of substrates and cofactors on the enzyme's thermostability in crude extracts was examined. The most unexpected result of these experiments was that far from exerting a stabilising influence, many of these substances had the reverse effect. The results, presented in Table 4.2, showed that, of the compounds tested, ADP-Mg had the most labilizing effect. This effect was therefore examined in more detail. The later identification of AMP as a positive effector of this enzyme and the finding that it had a similar destabilizing effect on the enzyme prompted a comparison of the effect of this activator with that of the substrate ADP-Mg.

Table 4.2 The effect of substrates, cofactors, and allosteric effectors on the stability of pyruvate kinase at 65°. The enzyme used was a redissolved ammonium sulphate precipitate obtained during a preliminary purification experiment. With this exception the procedure was the same as described in the text for the later experiments.

Additive	Activity remaining after 7 minutes. %
None	80.4
$MgSO_4$ (10 mM)	89.9
KCl (100 mM)	66.9
PEP (2 mM)	74.1
ADP (5 mM) + $MgSO_4$ (10 mM)	35.1

The labilizing effect of AMP on pyruvate kinase at 55° is illustrated in Figure 4.4. The linearity of these semi-logarithmic plots clearly demonstrates that the enzyme activity decayed at a constant exponential rate. Figure 4.5 shows the relationship between the concentration of AMP and the rate of inactivation. Although the affinity of the enzyme for AMP appears to be very great under these conditions the tendency of the rate of inactivation to fall to a limiting value at high AMP concentrations is curious. Non-cooperative binding to two kinetically distinct sites may be involved. The first phase of the interaction of AMP with the enzyme is characterised by a half saturating AMP

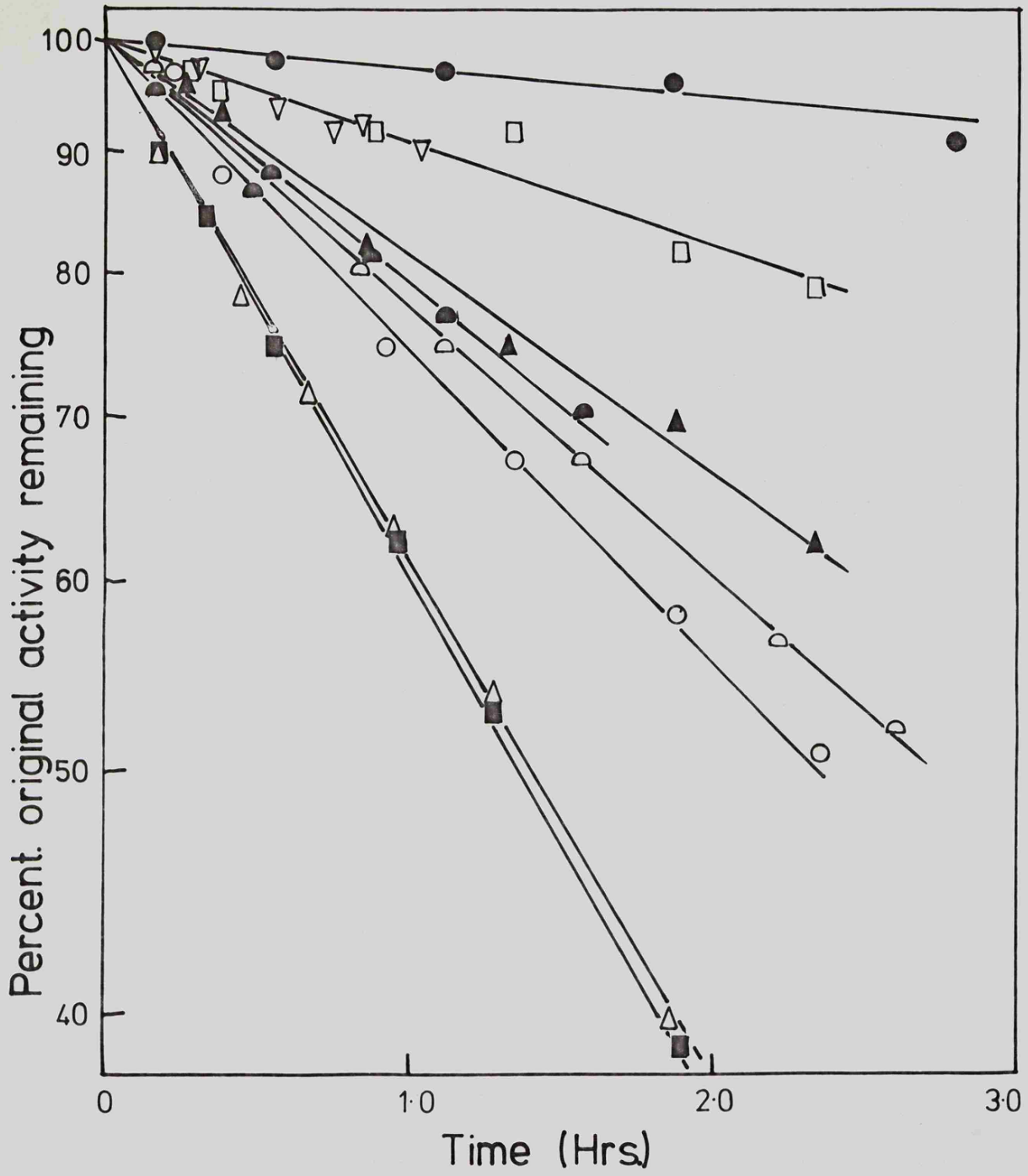


Figure 4.4. The effect of AMP on the stability of pyruvate kinase at 55°. The concentrations of AMP employed were; zero (●), 0.01 mM (□), 0.02 mM (▽), 0.05 mM (○), 0.10 mM (△), 0.20 mM (■), 0.50 mM (◘), 1.00 mM (◓) and 3.00 mM (▲).

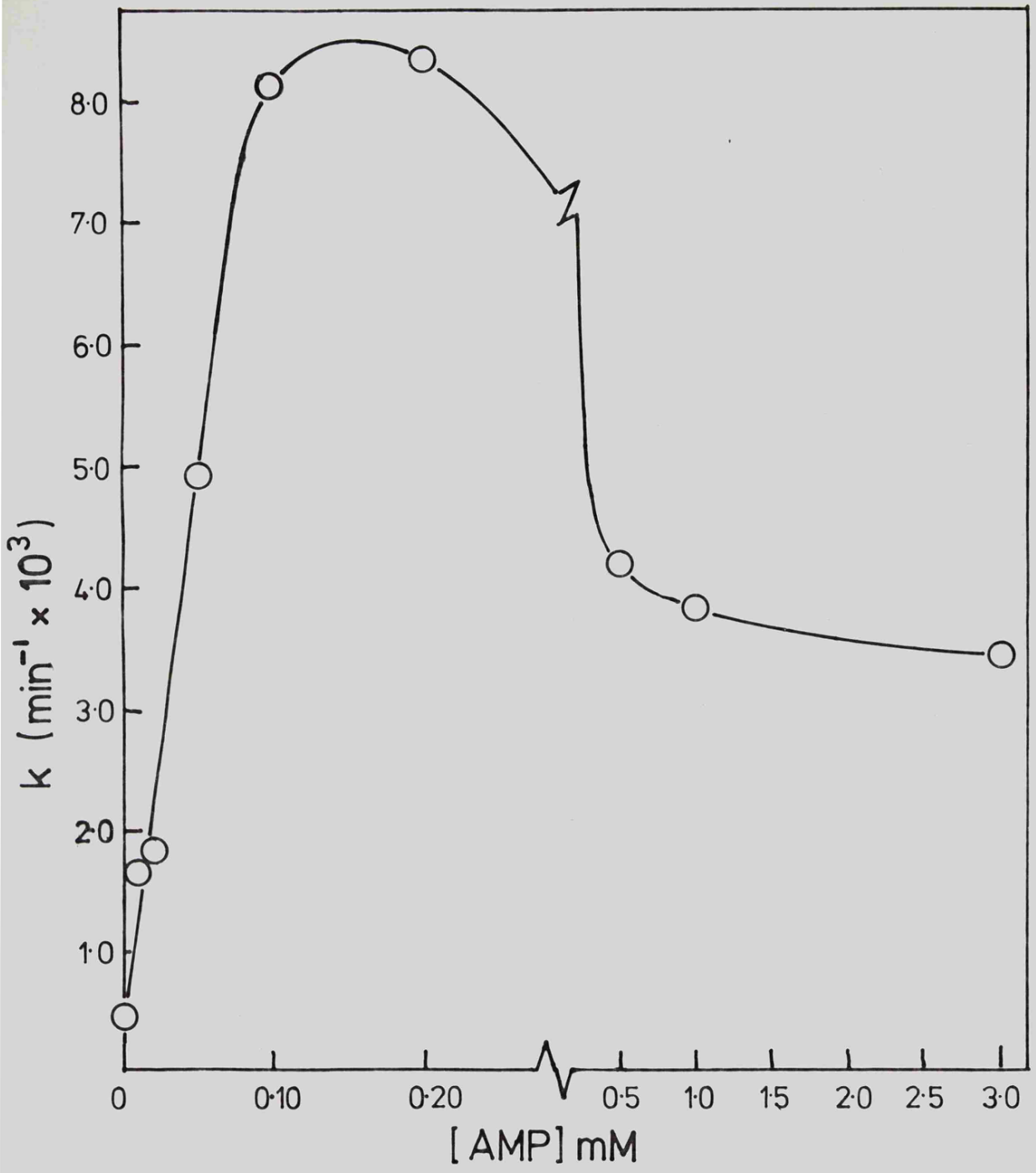


Figure 4.5. The rate of inactivation of pyruvate kinase as a function of AMP concentration. The values of k, the first order rate constant, were obtained from the plots in Figure 4.4.

concentration of 44 μM. The linearity, at low AMP concentrations, of the relationship between the rate of inactivation and the AMP concentration rules out the possibility of co-operativity in the binding of AMP.

Figures 4.6, 7 and 8 show the effect of ADP-Mg on thermal denaturation at 40°, 45° and 50° respectively. A number of striking features of the ADP-Mg mediated inactivation are revealed in these plots. The inactivation is clearly bimodal consisting of a rapid initial phase and a much slower second phase. Biphasic plots of inactivation processes are normally associated with heterogeneous systems, such as the occurrence of two isoenzymes differing in their thermolability. However such an explanation cannot be applied here. Extrapolation backwards to the ordinate of the lines describing the second phase of the inactivation shows that the extent of the first inactivation phase varies considerably, a function of both temperature and ADP-Mg concentration as shown by the plots in Figure 4.9.

The relationship between ADP-Mg concentration and the velocity of the second phase of inactivation was more amenable to analysis. The linearity of the semi-logarithmic plots implied that the loss of activity occurred exponentially and the first order rate constants (k) for the inactivation were therefore obtained for each ADP-Mg concentration at the three temperatures. The value of k at any one temperature proved to be related to the ADP-Mg concentration according to the expression

$$k \propto [\text{ADP-Mg}]^n$$

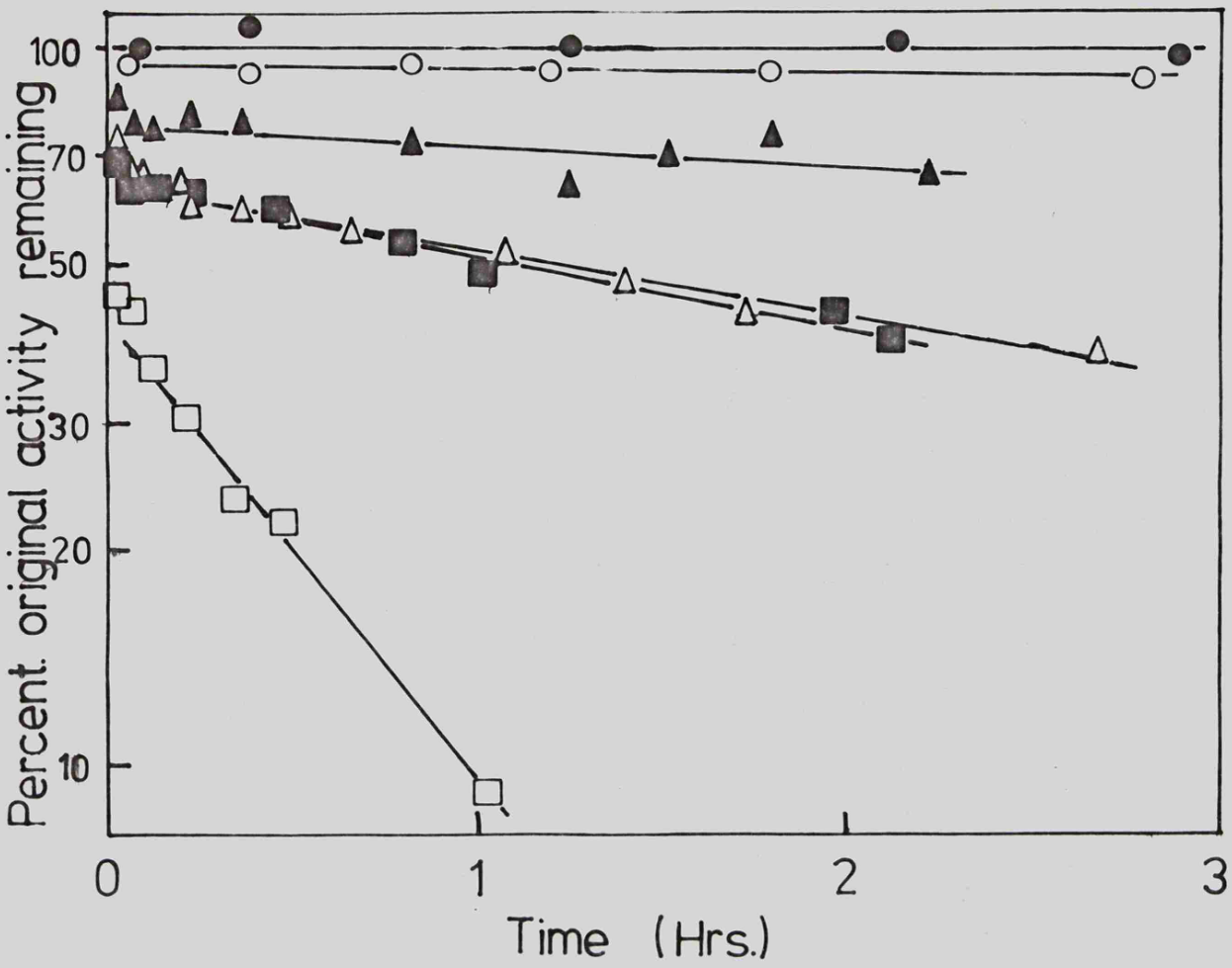


Figure 4.6. The decay of pyruvate kinase activity at 40° in the presence of the following concentrations of (ADP, Mg) : zero (●) , 2 mM (○) , 4 mM (▲) , 6 mM (△) , 9 mM (■) and 16 mM (□) .

The pyruvate kinase activities are expressed as a percentage of an unheated control which contained neither ADP nor Mg^{++} ions.

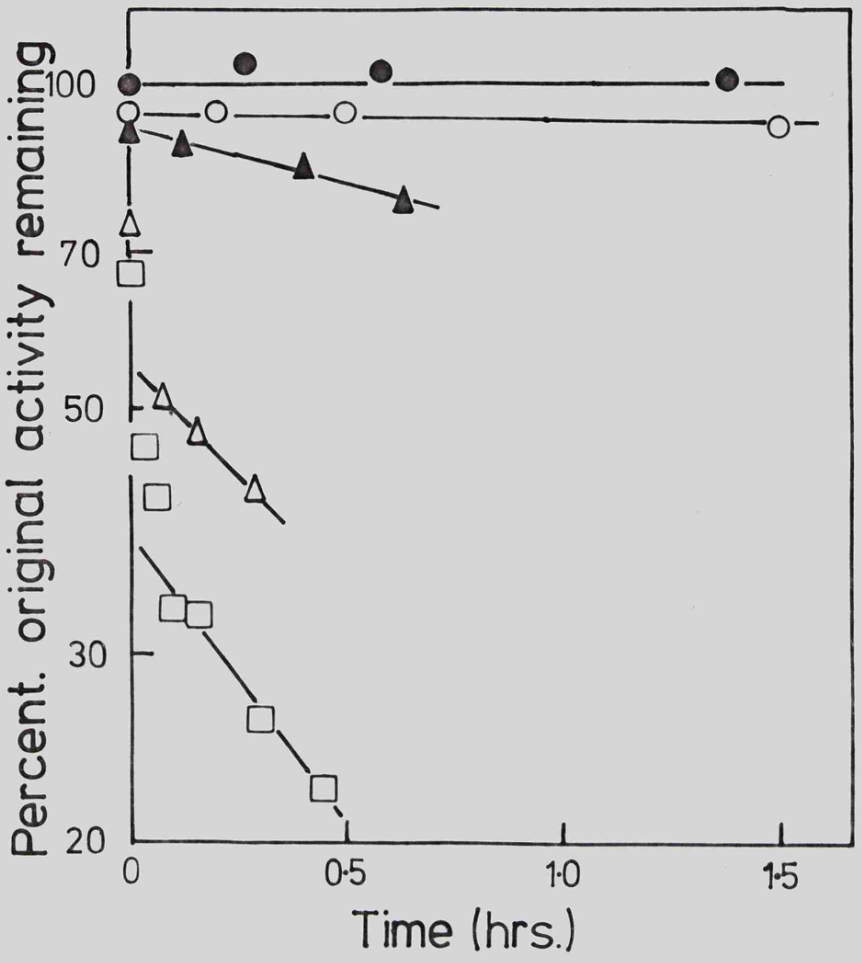


Figure 4.7. The decay of pyruvate kinase activity at 45° in the presence of the following concentrations of (ADP. Mg) : zero (●) , 2 mM (○) , 3 mM (▲) 6 mM (△) and 8 mM (□) . Other details are given in the legend to Figure 4.6.

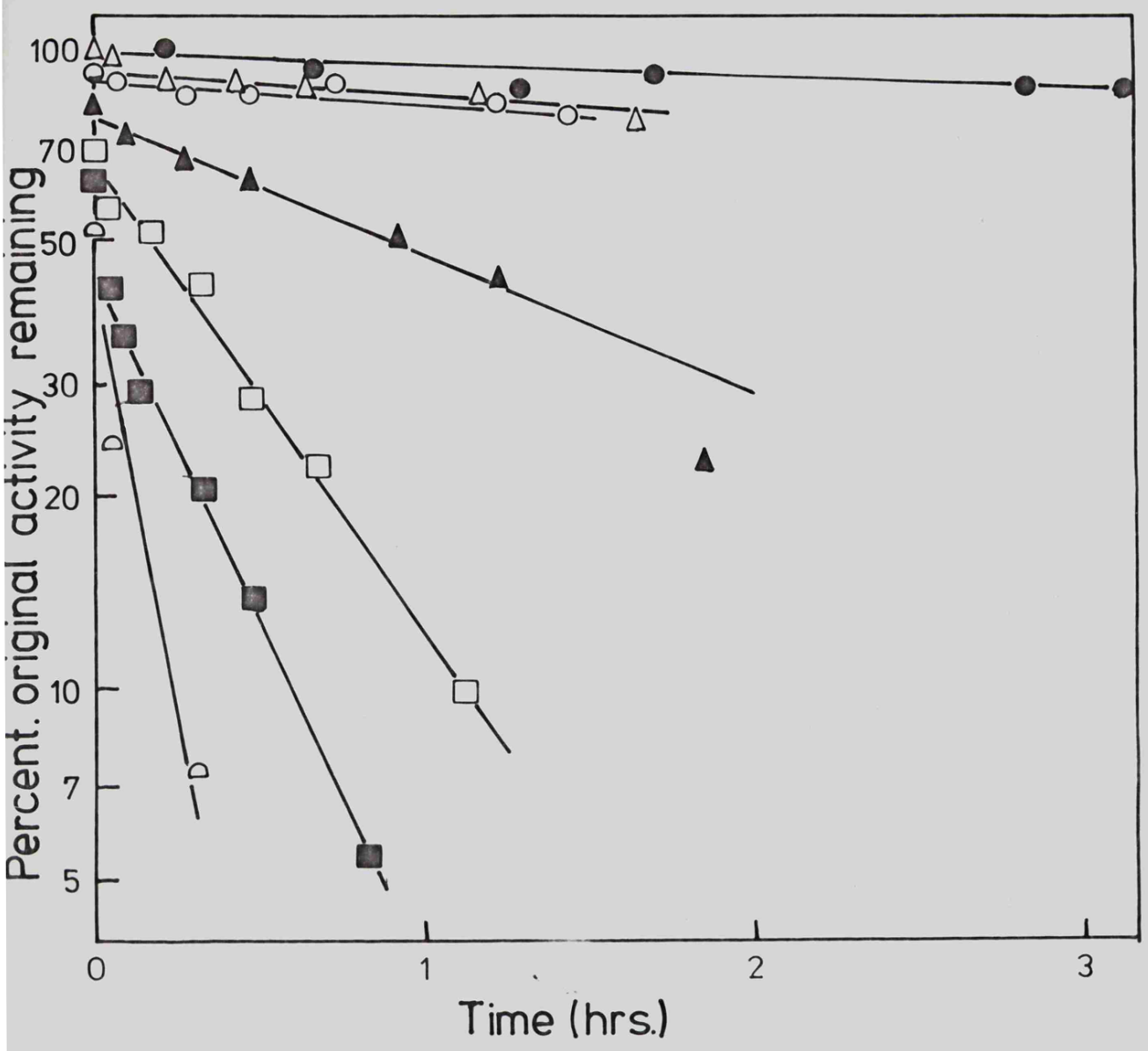


Figure 4.8. The decay of pyruvate kinase activity at 50° in the presence of the following concentrations at (ADP Mg): zero (●), 1 mM (○), 2 mM (△), 3.5 mM (▲), 5 mM (□), 7 mM (■) and 10 mM (⌓). A value at 0.33% activity after 40 minutes with 10 mM (ADP. Mg) was taken into consideration when that line was drawn. Other details are given in the legend to Figure 4.6.

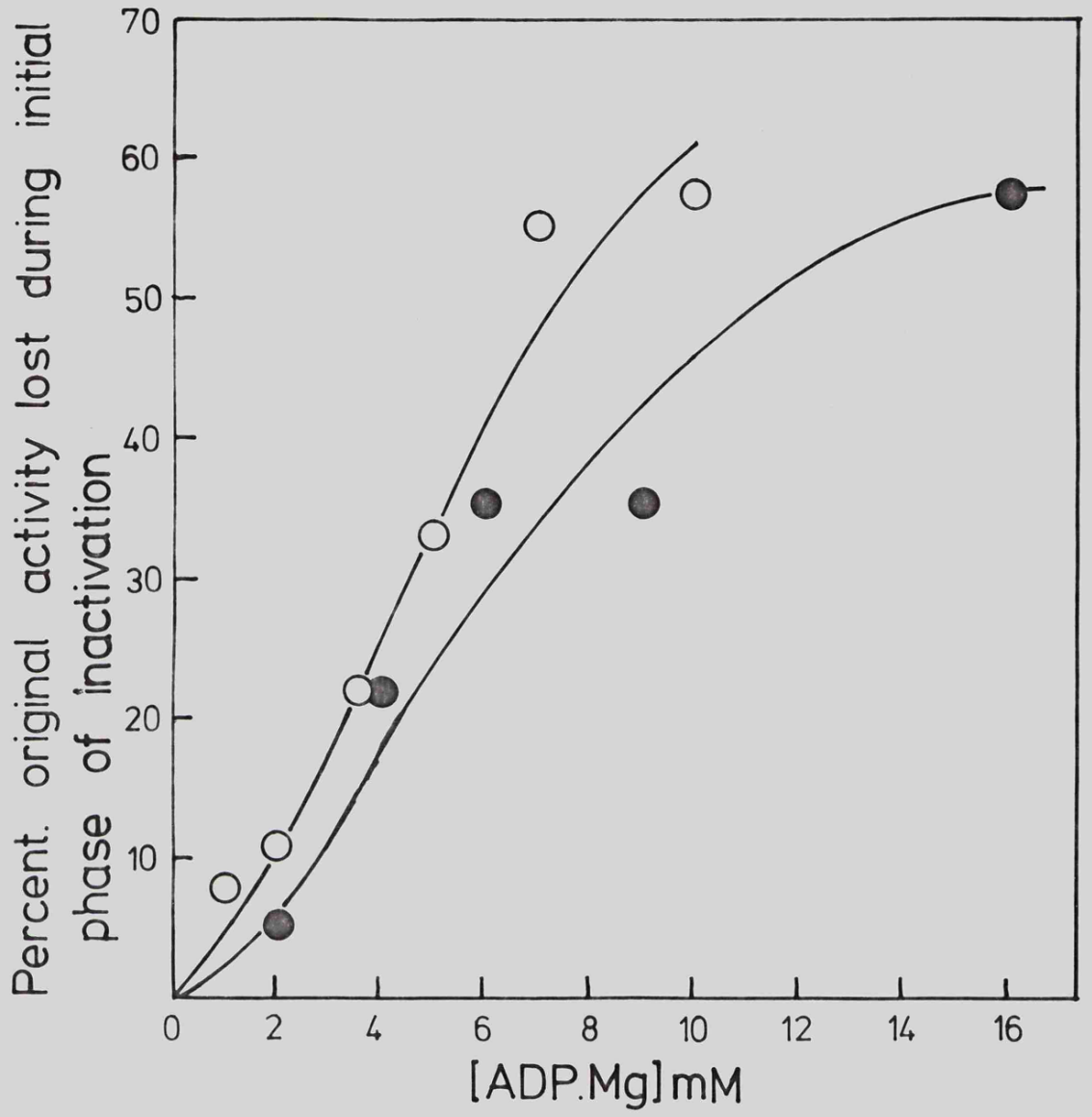


Figure 4.9. The relationship between (ADP.Mg) concentration and the extent of first phase of the inactivation. The losses of activity at 40° (closed circles) and at 50° (open circles) were obtained from Figures 4.6 and 8. as discussed in the text.

otherwise written in logarithmic form as

$$\log k = \log K + n[\text{ADP-Mg}]$$

where K is a constant. The relationship between the rate of inactivation and ADP-Mg plotted according to this equation is illustrated in Figure 4.10. It is not easy, and perhaps unwise, to attempt to provide a precise definition of the parameter "n" in a situation such as this. In the analogous situation presented by the allosteric regulation of enzyme acitivity the value of n is most frequently regarded as only a minimum value for the number of interacting sites at which a particular ligand may bind. Variations in the value of n do not imply changes in the number of binding sites but rather changes in the strength of the interaction between the binding sites i.e. the degree of co-operativity of binding (Newsholme and Start 1973).

4.3.3 Kinetic studies. A number of preliminary experiments established the basic properties of the pyruvate kinase preparation. When ADP was omitted from the reaction mixture there was no activity indicating the complete absence from the preparations of phosphatases able to hydrolyse phosphoenol pyruvate at neutal pH (pH 6.8) in this experiment. In the absence of K^+ ions in the reaction mixture the reaction rate was only 6% of that found when the potassium chloride concentration was brought to 50 mM. Activity was maximal over a broad pH range with an optimum pH 6.8 as Figure 4.11 illustrates.

The experiments described above were performed using a cell free extract (the product of Step 2 of the purification procedure). When partially purified

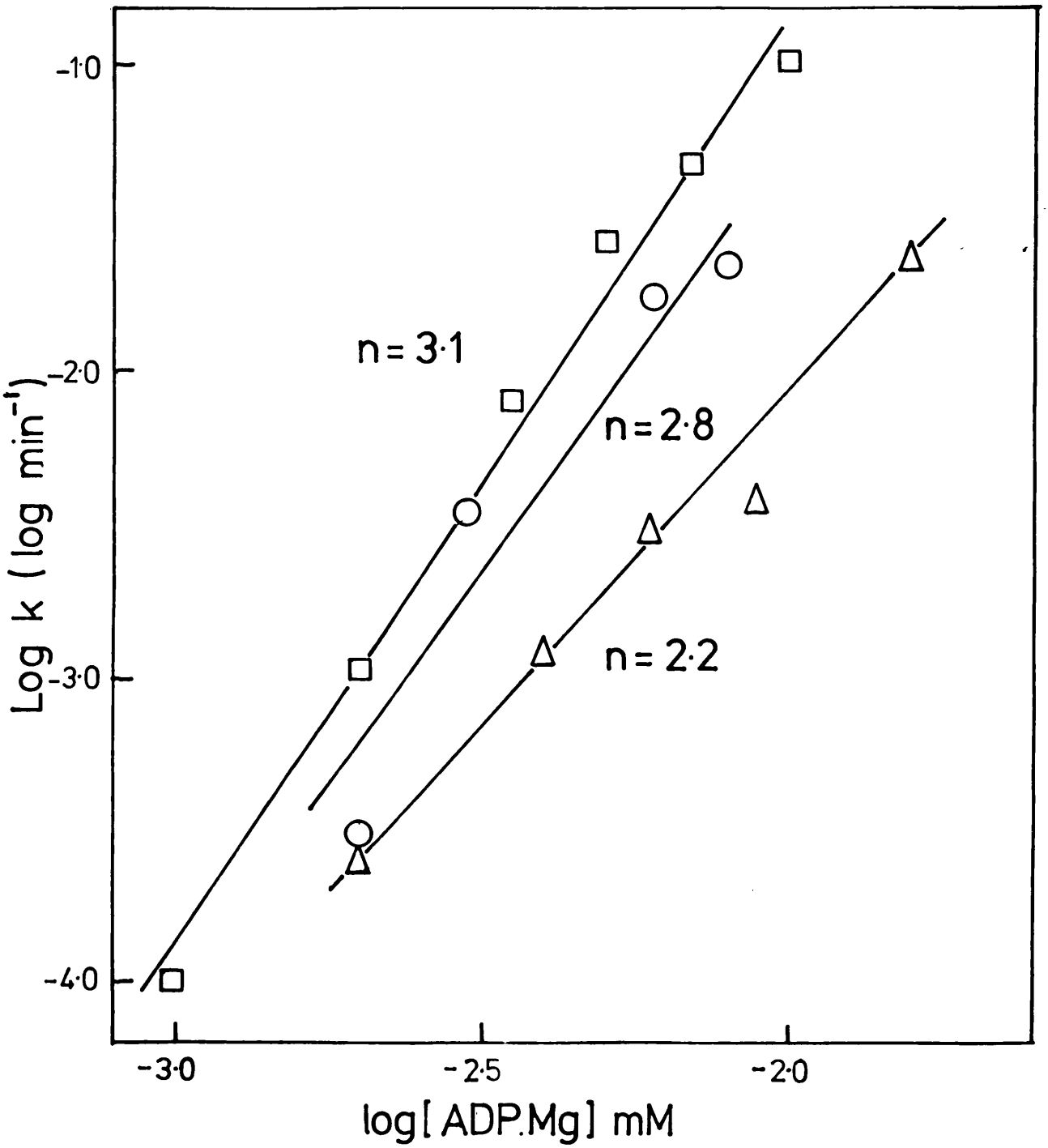


Figure 4.10. The relationship between (ADP.Mg) concentration and the velocity of the second phase of the inactivation at 50° (squares), 45° (circles) and 40° (triangles). The values of n, the slope of the plots, are shown on the figure.

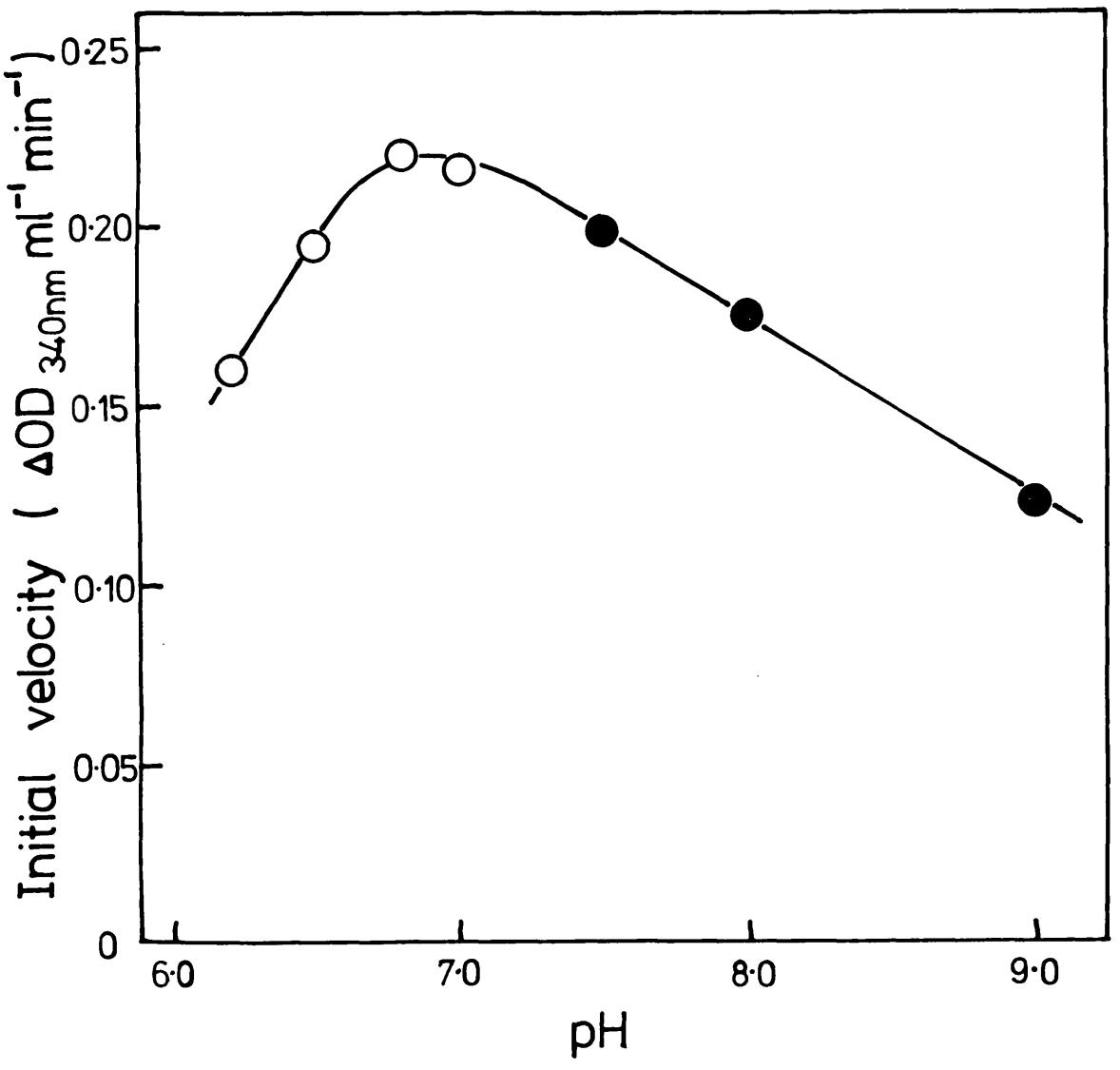


Figure 4.11. The effect of pH on pyruvate kinase activity. The reaction mixture used was as described in the text except for the variations in pH and the use of tris-HCl buffer at alkaline pH values (closed symbols).

enzyme preparations become available the sigmoid nature of the relationship between PEP concentration and reaction rate was established, and a number of potential allosteric effects of the enzyme were tested for their effect on the enzyme at high and low PEP concentrations. The pyruvate kinases of E. coli B (Maeba and Sanwal, 1968) E. coli K12 (Malcovati and Kornberg, 1969) and Azotobacter vinelandii (Liao and Atkinson, 1971) have been reported to be stimulated by low concentrations of fructose diphosphate (FDP) at low PEP concentrations. Similarly AMP has been reported to activate the pyruvate kinases of a number of microorganisms, E. coli B (Maeba and Sanwal, 1969) Brevibacterium flavum (Ozaki and Shiio, 1969), Brev. liquefaciens (Ide, 1970), A. vinelandii (Liao and Atkinson, 1971), Thiobacillus neopolitanus (Cornish and Johnson, 1971) and B. licheniformis (Tuominen and Bernlohr, 1971b). ATP has been found to inhibit the pyruvate kinases of all the microorganisms where this property has been sought, Brev. flavum (Ozaki and Shiio, 1969), Brev. liquefaciens (Ide, 1970) Acetobacter xylinum (Benziman, 1969) and B. licheniformis (Tuominen and Bernlohr, 1971b). Inhibition of the pyruvate kinase by inorganic phosphate (Pi) has been reported in three cases, Brev. liquifaciens (Ide, 1970), A. vinelandii (Liao and Atkinson, 1971) and B. licheniformis (Tuominen and Bernlohr, 1971b). In general the pattern of regulatory properties of the pyruvate kinases outlined above is not dissimilar from that of the L (i.e. liver) type enzyme from mammalian tissues (Llorente et al., 1970). Weber and co-workers (1968) have reported an

inhibition of liver pyruvate kinase by L-alanine and this amino acid and also L-aspartate were included in the list of potential allosteric effectors examined. The results recorded in Table 4.3 show that the thermophile pyruvate kinase has virtually the same pattern of regulation as that from the mesophile of the same genus B. licheniformis (Tuominen and Bernlorh, 1971b); i.e. activation by AMP and inhibition by ATP and inorganic phosphate.

The effect of PEP concentration on the initial velocity (v) of the reaction over the temperature range 15^{o}-55^{o} is shown in Figures 4.12 and 13. Analysis of these plots was complicated by the coincidence of inhibition by PEP at high concentrations (which ruled out any possibility of a meaningful estimate of the maximum velocity (V_{max}) directly from this data plotted as v vs. [PEP] and sigmoidicity of the relationship between v and [PEP] which meant that double reciprocal plots (i.e. 1/v vs. 1/ [PEP]) would be curved and also unusuable for the determination of V_{max}. The following procedure was adopted in order to circumvent this difficulty. Purely speculative values for V_{max} were derived from the plots in Figures 4.12 and 13 by the cautious extrapolation of the parts of the curves where v was increasing as the PEP concentration was raised. These values for V_{max} were then applied to the construction of Hill plots (i.e. log v/V_{max}-v vs. log [PEP]) from which a rough estimate of Hill coefficient, n, could be obtained (Atkinson, 1966). This approximate value of n was then used as a guide in the construction of Lineweaver-Bark plots from the

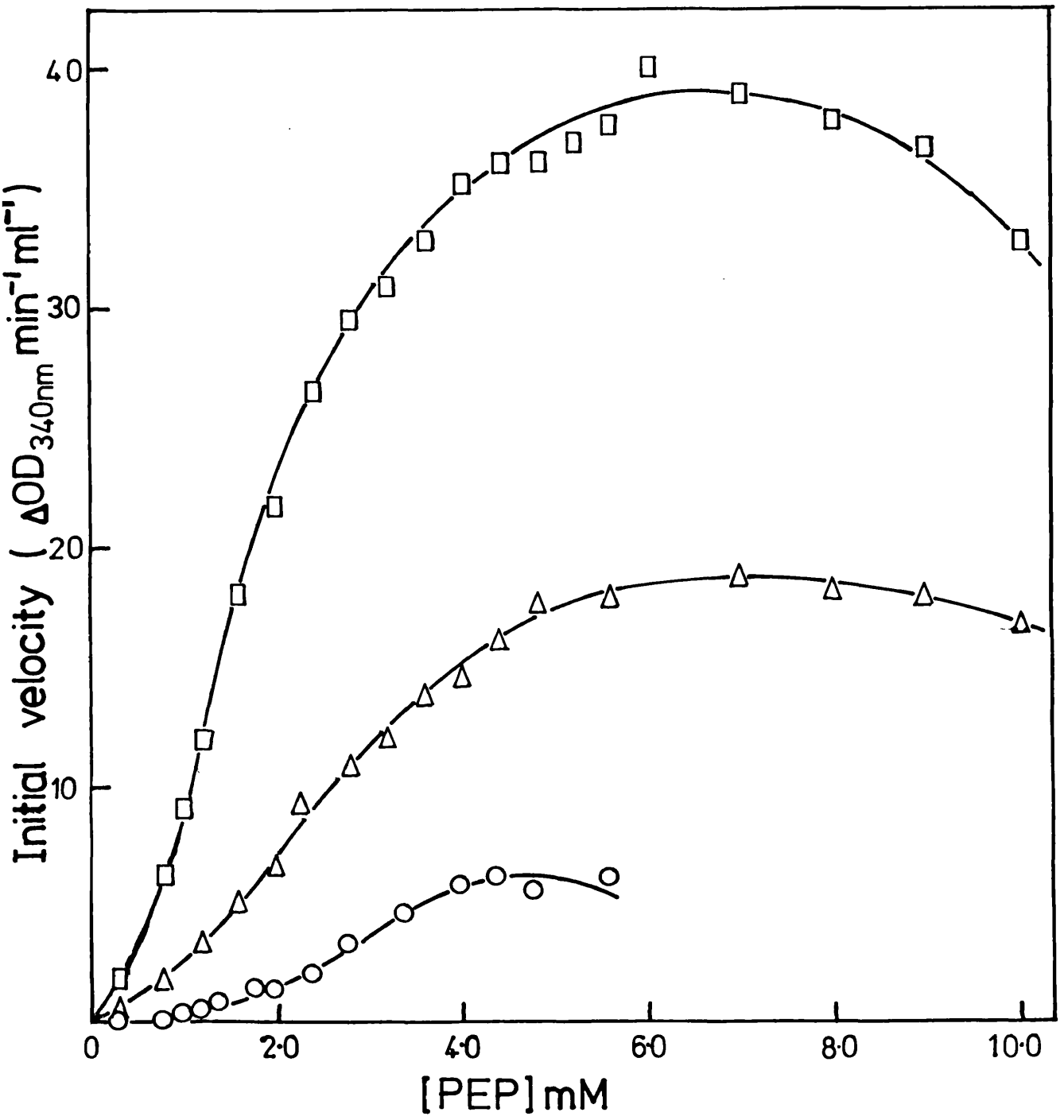


Figure 4.12 The relationship between PEP concentration and pyruvate kinase activity at 15° (circles), 25° (triangles) and 35° (squares). The assays were initiated by the addition of volumes of enzyme preparation appropriate to the temperature and the initial velocities are expressed in relation to 1 ml at enzyme preparation. All other reagents were at the concentration indicated in Section 4.2.1.

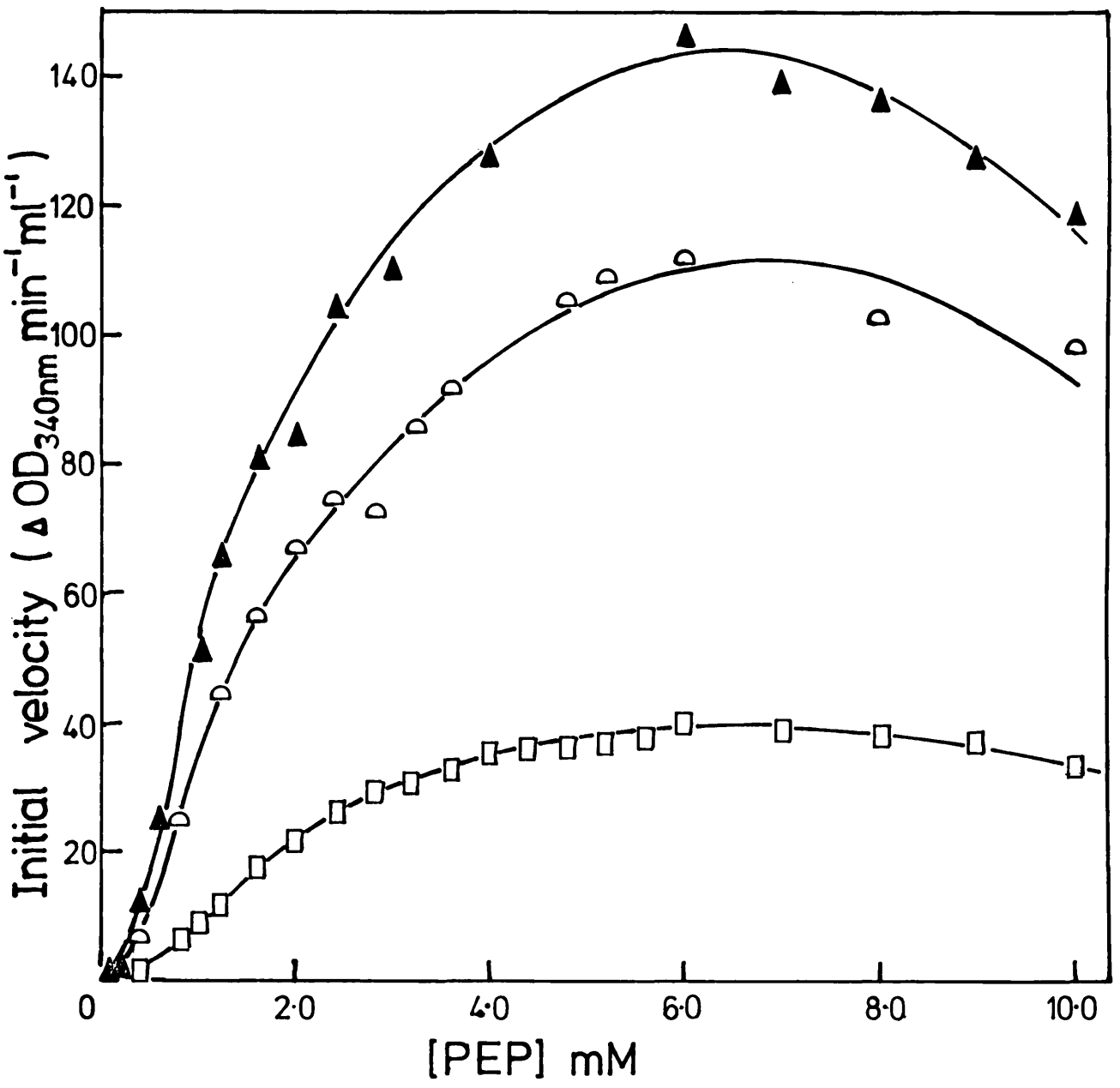


Figure 4.13. The relationship between PEP concentration and pyruvate kinase activity at 35° (squares), 45° (half-circles) and 55° (triangles). Other details are given in the legend to Figure 4.12.

Table 4.3 The effect of a number of potential activators and inhibitors on pyruvate kinase activity at 45°. The concentrations of these additives and also that of PEP are shown in the Table, the concentrations of all the other components of the reaction mixtures were as indicated in Section 4.2.1. Purified enzyme (10 µl) was used.

		Activity (ΔOD_{340nm} min^{-1})	
Effector	Concentration	0.2 mM PEP	1 mM PEP
None	-	.029	.604
AMP	1 mM	.220	1.074
FDP	1 mM	.023	.559
ATP	2 mM	-	.120
L-aspartate	10 mM	-	.444
L-alamine	10 mM	-	.535
Inorganic phosphate	10 mM	-	.358

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form $1/v$ vs. $1/[PEP]^n$. Different values were tested until values were arrived at with which the data gave straight lines for each temperature. The values of V_{max} at each temperature could then be obtained in the usual manner and were plotted according to the Arrhenius relationship (Figure 4.14). A value of 15.5 k cal $mole^{-1}$ was thus obtained for the activation energy of the reaction.

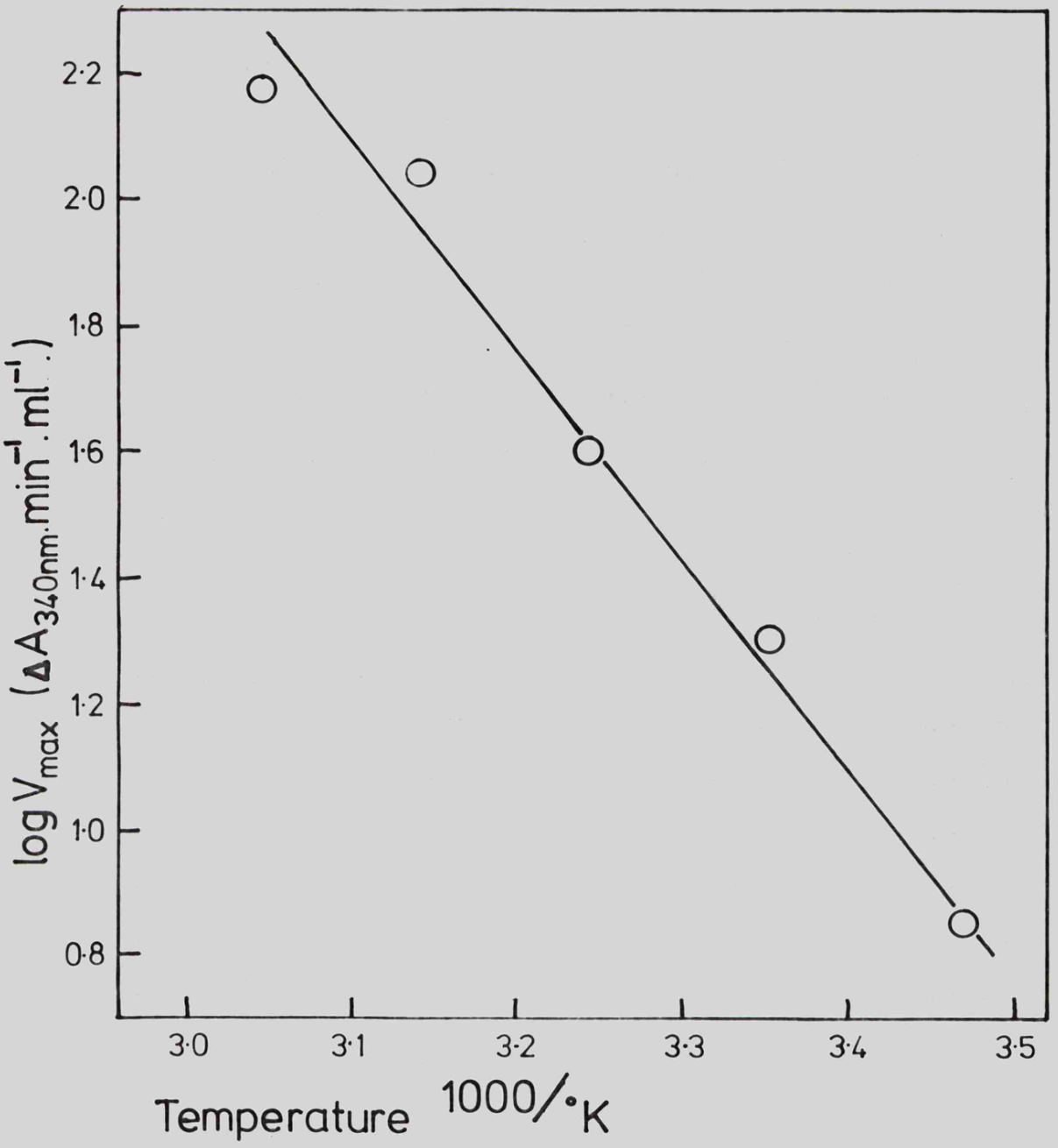

Figure 4.14. The effect of temperature on the maximum velocity of pyruvate kinase plotted according to the Arrhenius relationship values for V_{max} were derived from the data of Figures 4. 12. and 13. as described in the text.

Table 4.4 Summary of the effects of temperature on the values of the Hill coefficient and the half saturating concentration ($S_{0.5}$) for PEP. The final column contains the values of initial velocity at 2 mM PEP ($V_{2.0}$) relative to the value of V_{max} at the same temperature as discussed in the text.

Temperature	Hill coefficient	$S_{0.5}$(mM)	$v_{2.0}/V_{max}$
15°	3.20	2.86	0.211
25°	2.44	2.49	0.355
35°	2.14	1.72	0.548
45°	1.88	1.59	0.618
55°	1.82	1.47	0.618

These values of V_{max} were also applied to the construction of Hill plots (Figure 4.15) which provided an indication of the effects of temperature on both the Hill coefficient and the half saturating PEP concentration. The values of these parameters (as derived from Figure 4.15) are summarized in Table 4.4.

The influence of temperature on the regulatory characteristic of pyruvate kinase is manifested in the effects on the values of both n and $S_{0.5}$. A numerical expression of the effects of temperature on the regulation is provided by the ratio of the initial velocity at 2.0 mM PEP to the maximum velocity at the same temperature. This PEP concentration was chosen arbitrarily as a concentration where, throughout the temperature range covered in these experiments, the

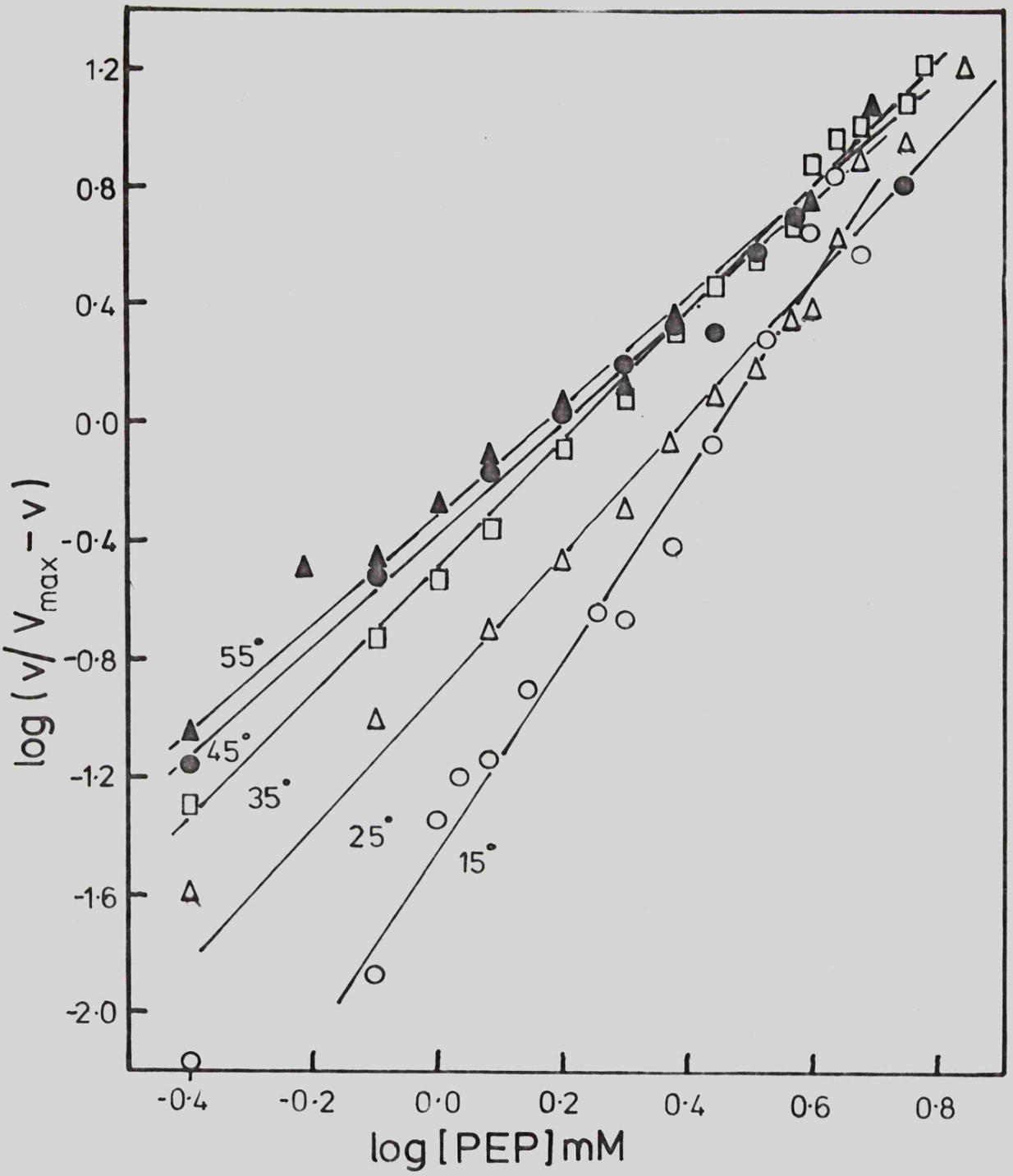

Figure 4.15. Hill plots of the data of Figures 4.12. and 13.

activity of the enzyme was most sensitive to changes in the PEP concentration. The value of $v_{2.0}/V_{max}$ for each temperature is recorded in Table 4.4 and shows a weakening of the regulation dependent on PEP concentration as the temperature is raised.

AMP at a concentration of 1 mM completely eliminated the sigmoidicity of the PEP saturation curve (Figure 4.16). The examination of this activation at different temperatures as a function of [AMP] was complicated by the fact that a given subsaturating concentration of the substrate PEP saturated the enzyme to different degrees at different temperatures, as is demonstrated, for example, by changes in the value of $v_{2.0}/V_{max}$ discussed above. A PEP concentration of 1 mM was therefore selected arbitrarily for these experiments and the initial velocity was determined at each concentration with a range of AMP concentrations. The degree of activation at a given AMP concentration was then expressed as a percentage of that observed saturating AMP concentrations. The resulting plots, presented in Figure 4.17 indicate that only below 25° is there a significant effect of temperature on the affinity of the enzyme for AMP. Thus at 15° the half saturating concentration of AMP was 0.09 mM, at 25° 0.24 mM and at higher temperatures, 0.18 mM.

The response of the initial velocity of the reaction to increasing concentrations of ADP at temperatures of 25°, 35° and 55° is shown in Figure 4.18. The procedure for the determination of kinetic parameters was identical to that described above for the

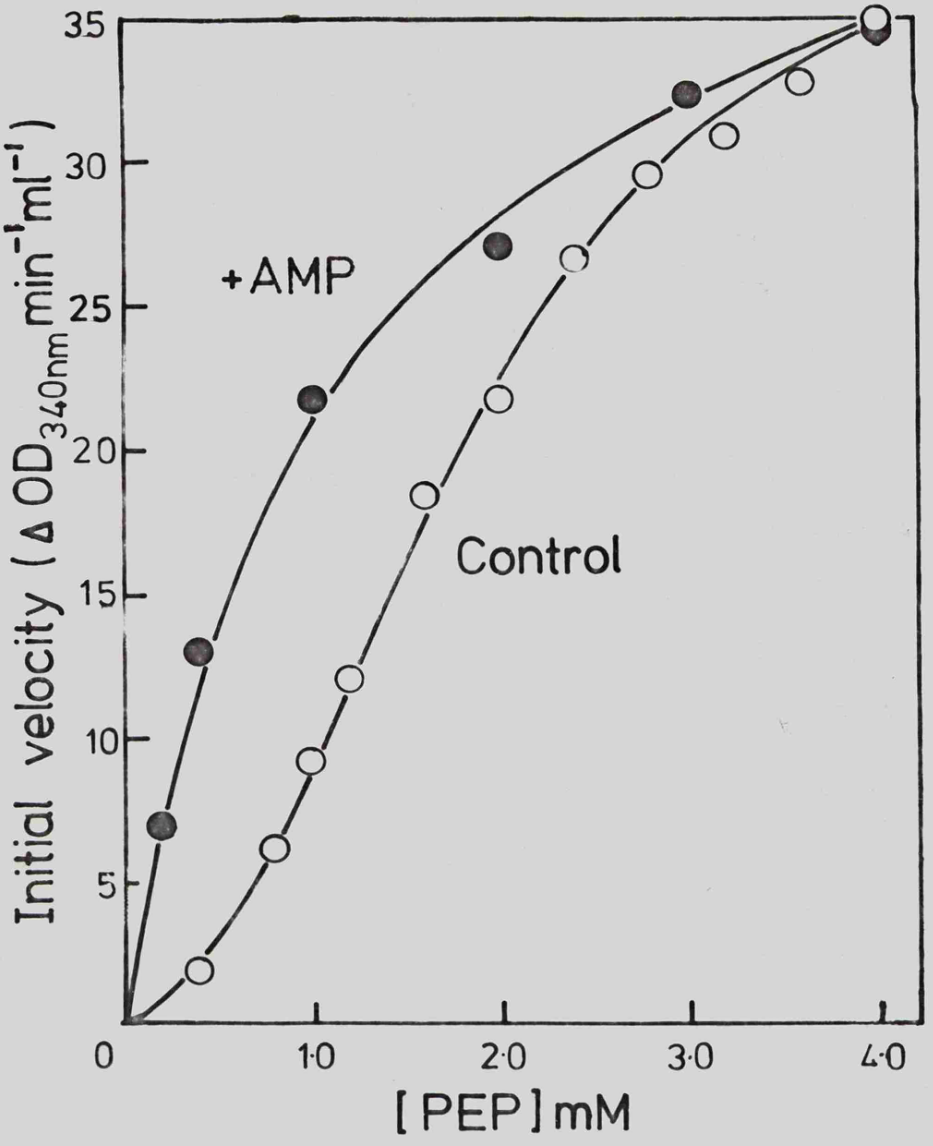

Figure 4.16. The effect of AMP on pyruvate kinase activity at 35°. AMP (1 mM) was included in reaction mixtures otherwise identical to those providing the data of Figure 4.12. from which the control curves is obtained.

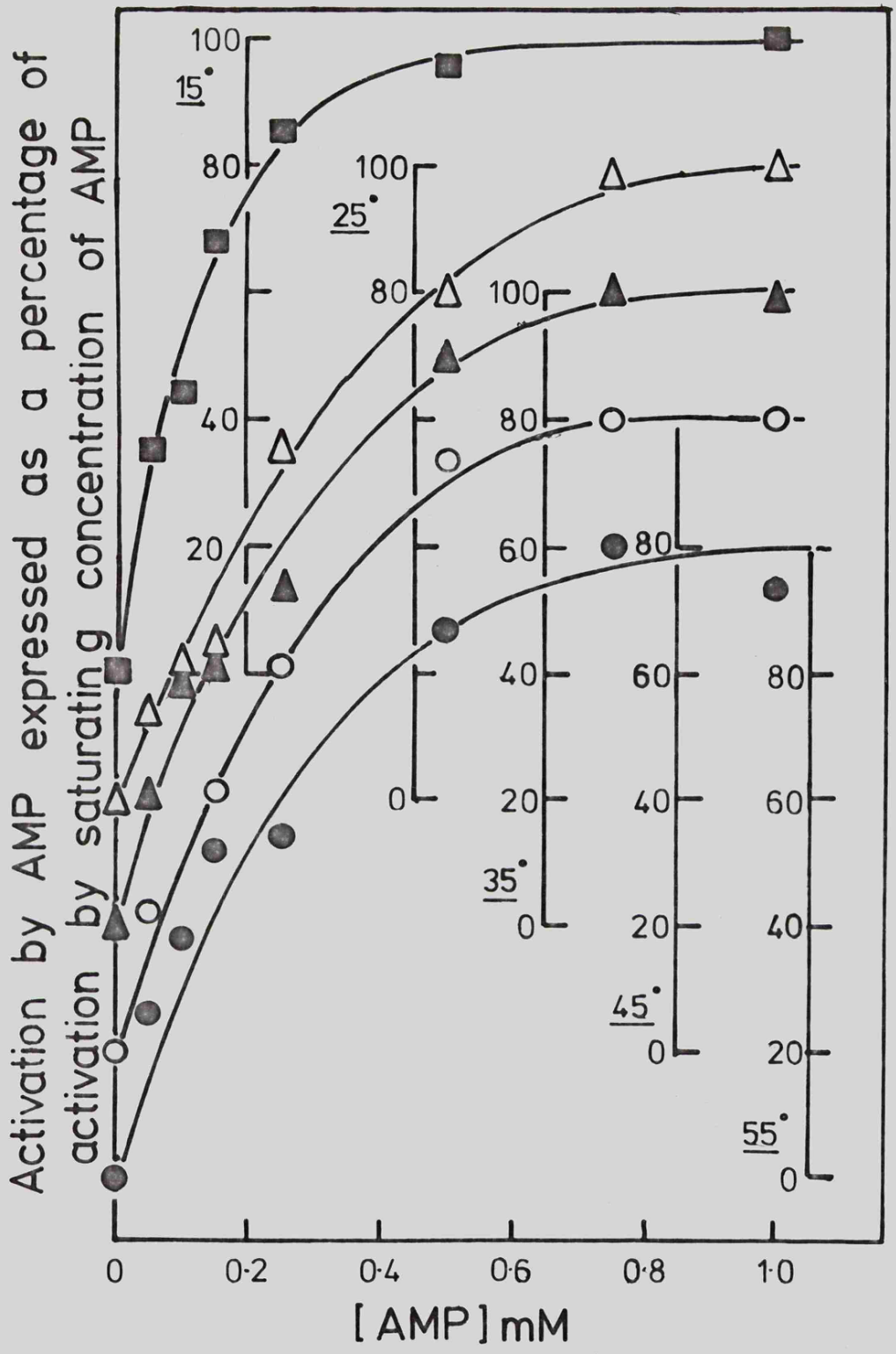

Figure 4.17. The ~~cul~~ activation of pyruvate kinase by AMP with 1 mM PEP at 15° (squares), 25° (open triangles), 35° (closed triangles), 45° (open circles) and 55° (closed circles). For clarity the different curves have been separated and individual ordinate scales are superimposed on the data plots.

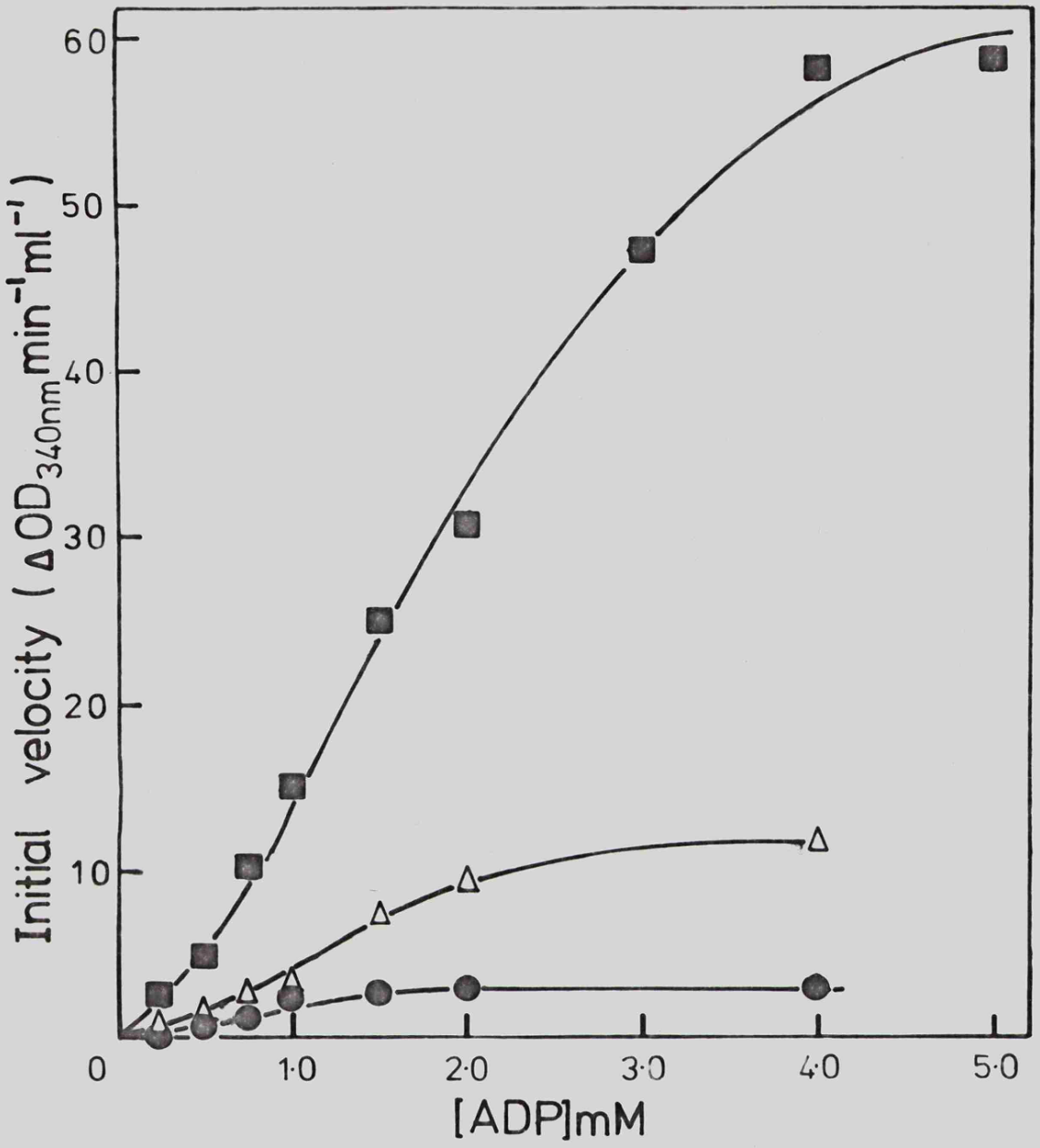

Figure 4.18. The relationship between ADP concentration and pyruvate kinase activity at 25° (squares, 35° (triangles) and 55° (squares). Assays were initiated by the addition of values of enzymes appropriate to the temperature and the initial velocities are expressed in relation to 1 ml of enzyme preparation. All other reagents were at the concentrations indicated in Section 4.2.1.

analysis of the data of Figures 4.12 and 13. The resulting Hill plots are shown in Figure 4.19 and the data is summarized in Table 4.5. As before a numerical expression of the effects of temperature on the regulation is provided by the ratio of the initial velocity at an arbitrarily selected ADP concentration (in this case 1.0 mM) to the maximum velocity. The temperatures effects on the ADP parameters differ from those on the PEP parameters. The affinity for ADP (represented by $S_{0.5}$) falls slightly as the temperature is raised while the Hill coefficient remains virtually unchanged. The resulting effects on $v_{1.0}/V_{max}$ shown in Table 4.5 indicate that regulation dependent on ADP concentration weakens as the temperature falls.

The potent inhibition of the enzyme by ATP would appear to have much greater significance for the control of pyruvate kinase activity. Figure 4.20 shows the inhibition as a function of ATP concentration at 25°, 35° and 55°. The values for $I_{0.5}$ (the ATP concentration giving 50% inhibition) and for n, the Hill coefficient, obtained from these curves and from the Hill plots (Figure 4.21) derived from the same data are presented in Table 4.6. The affinity of the enzyme for ATP (represented by the $I_{0.5}$ value) decreases by more than 3 times over the temperature range 25°-55° though there is only a small change in the degree of co-operativity in the binding of ATP (as indicated by the n values).

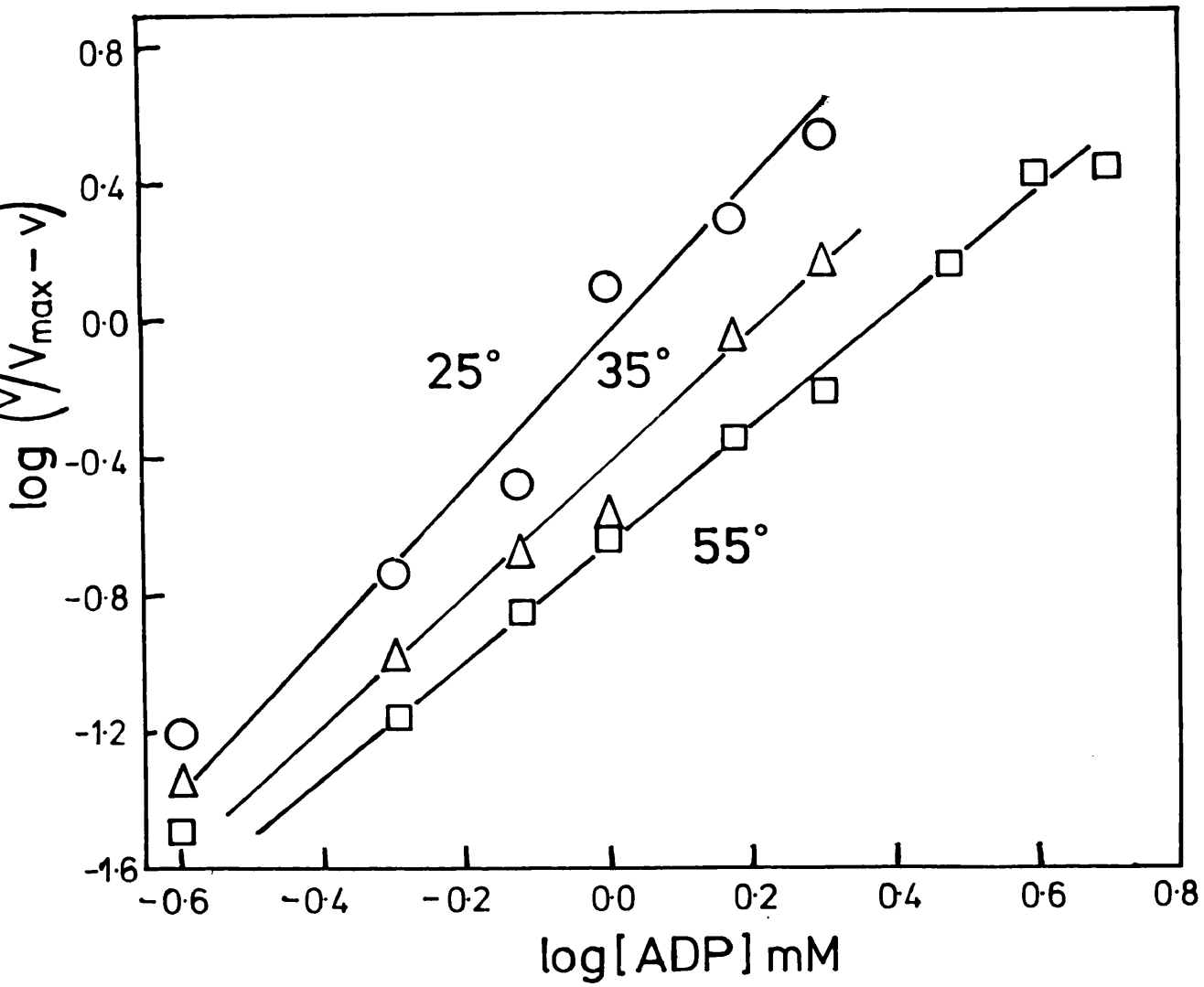

Figure 4.19. Hill plots of the data of Figure 4.18.

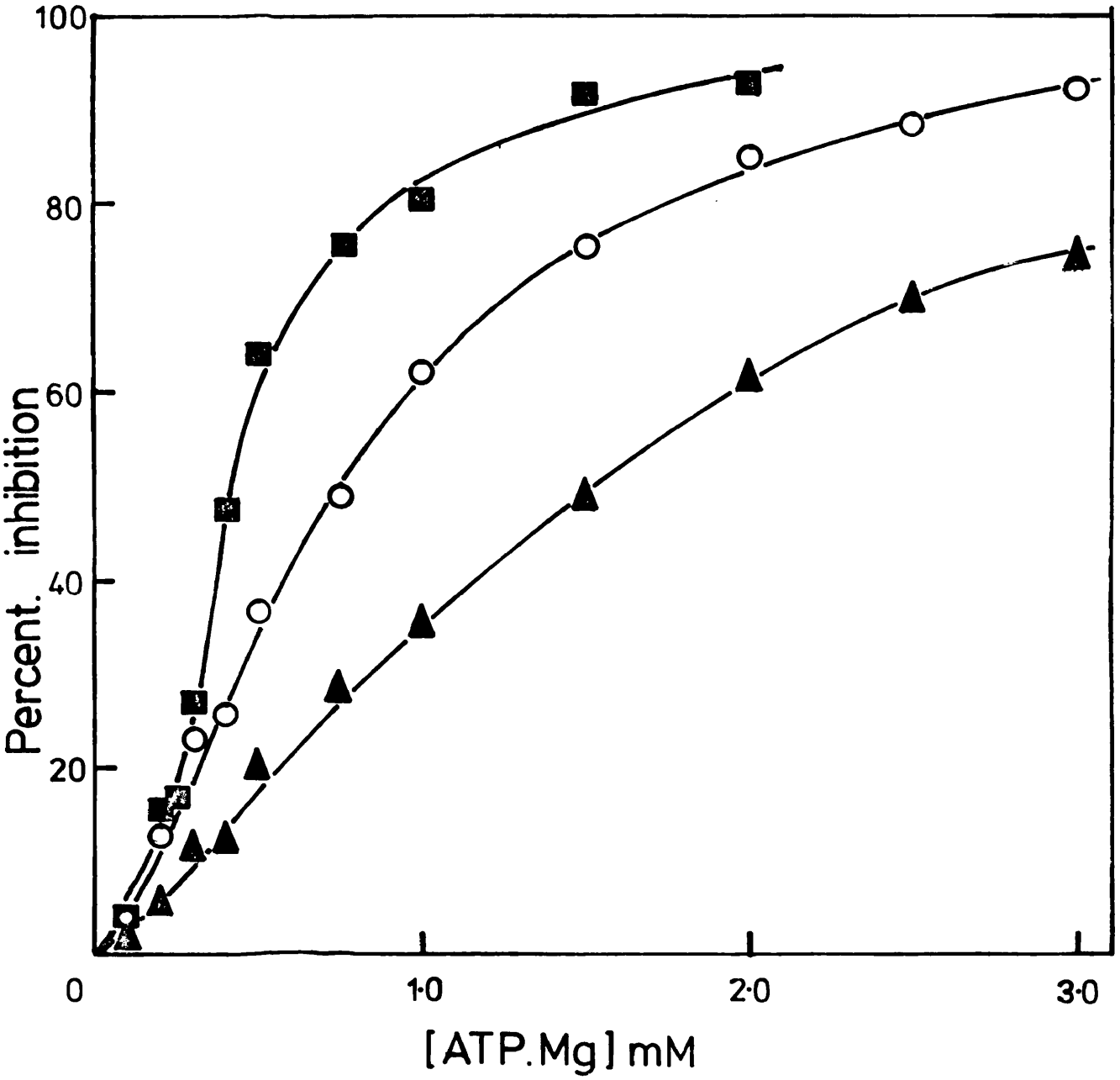

Figure 4.20. The inhibition of pyruvate kinase by ATP at 25° (squares), 35° (circles) and 55° (triangles). ATP additions to the standard reaction (see Section 4.2.1.) were accompanied by equimolar amounts of $MgSO_4$ in order that the possibility of ATP inhibition by Mg^{++} chelation could be ruled out.

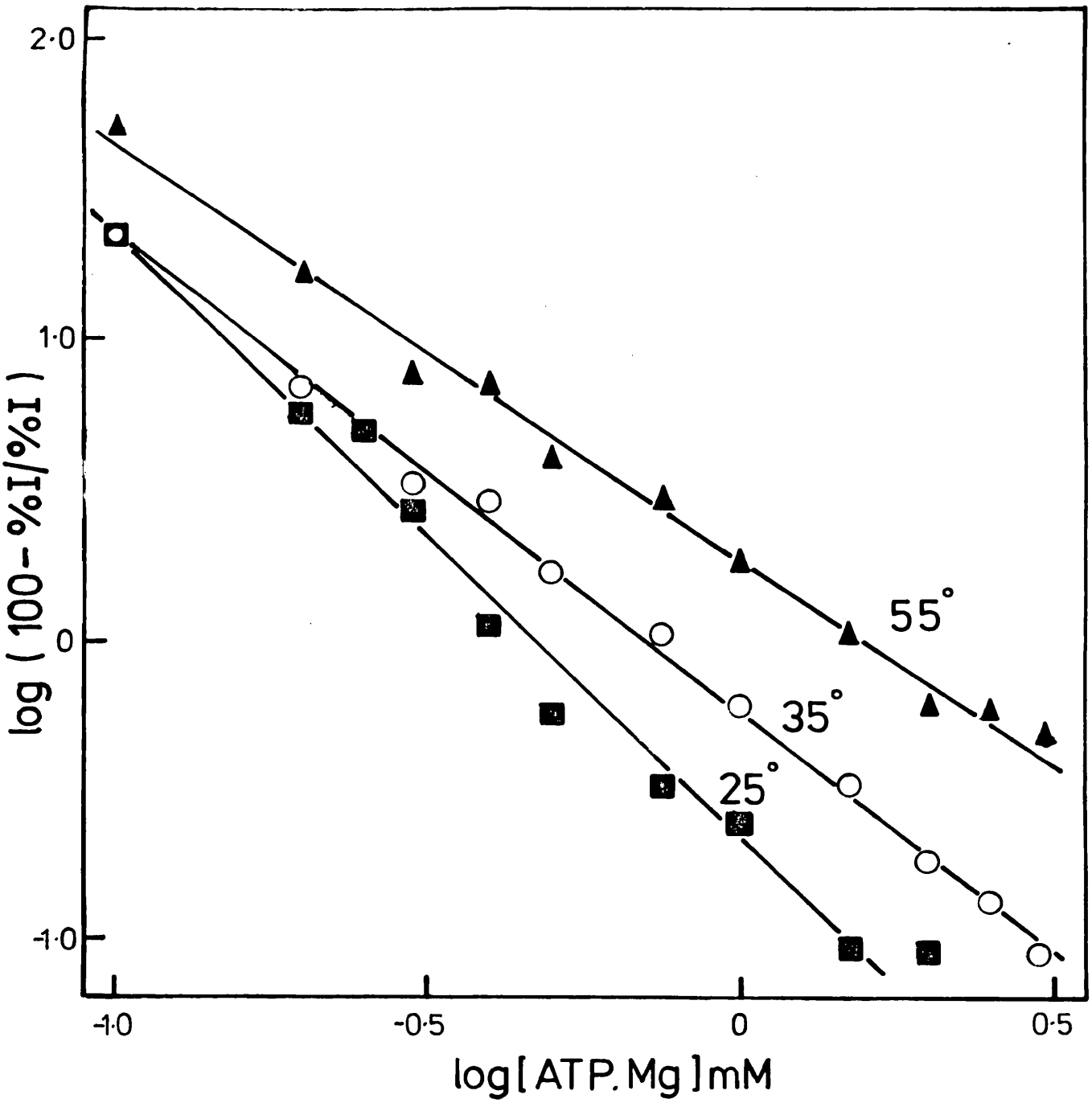

Figure 4.21. Hill plots of the data in Figure 4.20. Percent inhibition shown as %I.

Table 4.5 Summary of the effects of temperature on the values of the Hill coefficient and the half saturating concentration ($S_{0.5}$) for ADP. The initial velocities at 1.0 mM ADP ($v_{1.0}$) relative to the maximum velocity at the same temperature are shown in the final column.

Temperature	Hill coefficient	$S_{0.5}$(mM)	$v_{1.0}/V_{max}$
25°	2.24	1.03	0.300
35°	1.90	1.64	0.174
55°	1.70	2.40	0.120

Of the other effectors tested the only one to have any significant effect on the enzyme was inorganic phosphate. The results recorded in Table 4.7 show that temperature has no marked effect on this inhibition. The possible physiological significance of inorganic phosphate as an inhibitor of pyruvate kinase is obscure; one would reasonably expect it to fit into the pattern of regulation of this enzyme as a positive effector.

4.4 Discussion

Substrates or effectors frequently afford protection against the cold lability associated with many, allosteric and therefore presumably multisubunit enzymes (Stadtman 1966). Kuczenski and Suelter (1970) found the reverse to be true for the pyruvate kinase of baker's yeast. The yeast enzyme is susceptible to cold inactivation and this inactivation is enhanced 1000-fold by micromolar amounts of the allosteric

Table 4.6 Summary of the effects of temperature on the values of the Hill coefficient and half saturating concentration ($I_{0.5}$) for ATP.

Temperature	Hill coefficient	$I_{0.5}$(mM)
25°	-2.02	0.44
35°	-1.49	0.73
55°	-1.38	1.53

Table 4.7 The inhibition of pyruvate kinase by inorganic phosphate (Pi). The standard reaction contained potassium phosphate at the concentrations indicated.

Temperature	% Inhibition	
	1 mM Pi	10 mM Pi
25°	7.4	74.8
35°	2.7	60.7
45°	10.7	59.6
55°	7.0	60.1

---

activator FDP. The complete protection of the enzyme by Mn^{++} or Mg^{++} ions however suggested that, under physiological conditions, the enzyme would be stable in the presence of FDP. Tuominen and Bernlohr (1971 a and b) found that the pyruvate kinase of B. licheniformis was inactivated by cold and that divalent cat ions, PEP and either AMP or inorganic phosphate acted in concert to protect the enzyme against cold inactivation.

However at 30° low concentrations of Mg^{++}, Mn^{++} or PEP induced a loss of activity which was prevented by saturating concentrations of the same ligands.

Although no inactivation was involved it may be relevant that Llorente and co-workers (1970) have found that the pyruvate kinase of rat liver may be reversibly desensitized by low temperatures to the homotropic co-operativity of the substrate PEP, to the allosteric inhibition by ATP and alanine and to the activation by FDP.

The studies of the thermostability of the pyruvate kinase of B. stearothermophilus reported here indicate that the complexity of inactivation processes of the thermophile enzyme may be no less than in these mesophile systems. In the absence of ligands the enzyme is quite stable: the data of Figure 4.4 indicate that at 55° the loss of activity is about 6% per hour. At 65° the rate of inactivation is very much more rapid (Table 4.2) implying that in the absence of ligands the inactivation process has the high temperature coefficient characteristic of protein denaturation (Joly 1965).

The form of the enzyme resulting from binding AMP is clearly unstable at 55° but the present data give no further insight into the mechanism of the inactivation process. The very high affinity for AMP shown by the enzyme in these conditions is noteworthy. The half saturating concentration of 44 μM AMP is approximately one fifth of the concentration which elicits half-maximal activation of the enzyme in the complete assay system containing substrates and cofactors (Figure 4.17).

The apparent lack of co-operativity in the binding of AMP is in accord with the kinetics of the AMP activation of the enzyme's activity discussed below.

The concerted effect of ADP and Mg^{++} on enzyme stability appears to be more complex. The characteristics of both phases of the inactivation are dependent on both the temperature and the concentration of ADP.Mg. As has already been mentioned the extreme rapidity of the first phase of the inactivation made kinetic analysis of this phase of virtually impossible. The plots in Figure 4.9 show that the relationship between the extent of the first phase and the concentration of ADP.Mg is not simple. The sigmoidicity of these plots suggests that the co-operative binding of more than one molecule of ADP.Mg may be involved. It is also likely the product of saturation of the system with ADP.Mg does not entirely lack catalytic activity. Kuczenski and Suelter (1970) drew similar conclusions from their observations on the biphasic nature of the FDP enhanced cold inactivation of the yeast enzyme.

In contrast the second phase of the inactivation clearly leads to the formation of a totally inactive form of the enzyme. The results recorded in Figure 4.10 suggest the involvement of a minimum of three molecules of ADP.Mg. The concentrations of ADP.Mg effective in the inactivation of pyruvate kinase are of the same order as those effective as a substrate for the enzyme.

No systematic studies have been made on the protection of the enzyme against inactivation. Under normal assay conditions instability was only found when

the temperature was raised to 55°. At this temperature a slight falling off in the reaction velocity was observed during the course of rate determinations when the enzyme activity was low and the assay time therefore prolonged. The early part of the progress curve was nevertheless always sufficiently linear for accurate measurements of the initial velocity to be made. These observations provide considerable circumstantial evidence that one or more components of the reaction system afford complete protection to the enzyme at temperatures up to 55°, the optimum for growth of the organism. Experiments described elsewhere in this thesis (see Figures 2.6 and 7) also provide evidence for stabilization of the enzyme in the intracellular environment.

A rather similar finding has been made by Tuominen and Bernlohr (1971 a and b) in their study of the pyruvate kinase of B. licheniformis. This enzyme was found to require saturating levels of divalent cations or PEP for stability in vitro at 30° although the organism grows readily at 37°. It seems likely that the instability shown by the mesophile enzyme is a manifestation of a property similar to that of the thermophile enzyme, especially in view of the fact that most of the results on the thermophile enzyme were obtained at temperatures below the optimum for growth.

The availability of ultracentrifuge data might have helped in determining whether the inactivation process involved dissociation of the enzyme into subunits. In the absence of such data the scheme presented below appears to accommodate the results of

this study.

The two phases of the inactivation promoted by ADP.Mg appear to be quite independent (I and II) and it would be expected that their products (A_P and A_I respectively) are quite distinct. A_P probably has some catalytic activity and is convertible into a totally inactive form, A_I. In this scheme

$$A_D \xleftarrow{III} A_N \xrightarrow{I} A_P \xrightarrow{II} A_I \xrightarrow{III} A_D^1$$

the native enzyme is represented by A_N and A_D and A_D^1 are the thermally denatured, catalytically inactive end-products of the process. In the absence of evidence to contrary all stages of the inactivation are represented in this scheme as irreversible. I represents the very rapid first phase of the inactivation, II represents the slightly slower second phase and III the relatively slow thermal denaturation/inactivation that occurs in the absence of ligands and to which all forms of the enzyme are presumably susceptible. The nature of the involvement of ADP.Mg in these processes has already been mentioned but the basis of the dependence on ADP.Mg of phases I and II of this scheme must remain a subject for speculation at the present time. However it is clear from the great similarities between these results and those of Kuczenski and Suelter (1970) that a similar process of breakdown into sub-units, mediated the binding of a number of ligand molecules is a most probable explanation.

The AMP mediated inactivation differs in many respects from that involving ADP.Mg. It is a one stage process and the relationship of the inactivation

rate to the AMP concentration is quite different. The significance of these differences and their relationship to the structure of the enzyme must also remain unresolved for the time being.

The present studies of the kinetics of the thermophile pyruvate kinase do not, and were not intended to, provide a detailed analysis of the complexities of the regulation of the enzyme. No attempt has been made to analyse the dependence of the binding of one ligand on that of another, the complex interactions of substrates, positive and negative allosteric effectors and other ligands. The object of this study was to determine the part played by temperature in defining some of the regulatory parameters of the enzyme.

The recent reports by Tuominen and Bernlohr (1971 a and b) on the properties of the pyruvate kinase from a mesophilic member of the same genus, B. lichenformis, has provided a convenient basis for comparison of the basic features of the thermophile enzyme with those of an enzyme from a closely related mesophile. In most respects the requirements for activity and the pattern of regulation are similar in the two systems. Like its mesophilic counterpart the enzyme from B. stearothermophilus requires both monovalent and divalent cations for activity and the pH optima for the two enzymes are similar.

Both enzymes exhibit co-operative binding of the substrate PEP. Except at the lower extreme of the temperature range (15°) the Hill coefficient of thermophile enzyme for PEP is around 2 (Table 4.4).

Tuominen and Bernlohr (1971 b) reported the corresponding value for the mesophile enzyme to be 2.2. One difference between the t wo enzymes is in the half-saturating concentrations for PEP. In the physiological temperature range the values for $S_{0.5}$ recorded in Table 4.4 are some 6-fold greater than the value reported by Tuominen and Bernlohr. Of course the crucial factor here may be the concentrations of the other components of the assay systems, rather than a basic difference in the properties of the two enzymes.

Both high and low values of $S_{0.5}$ for PEP have been reported in the literature for the pyruvate kinases from microorganisms. The enzymes of E. coli K 12 (Malcovati and Kornberg, 1969), Acetobacter xylinum (Benzimqn, 1969) and Mucor rouxii (Passeron and Terenzi, 1970) give values for $S_{0.5}$ PEP of 2.5, 1.7 and 3.3 mM respectively while the enzymes of E. coli B (Maeba and Sanwal, 1968), Brev flavum (Ozaki and Shiio, 1969) and Thiobacillus neopolitanus (Cornish and Johnson 1971) all give values below 0.5 mM. No physiological significance is apparent in this variation.

With the exception of the A. xylinum enzyme all the pyruvate kinases so far examined from microbial sources have been activated by either AMP or FDP; the enzymes from Azotobacter vinelandii (Liao and Atkinson 1971) and E. coli B (Maeba and Sanwal, 1968) are activated by both AMP and FDP. In every case except these two last mentioned species the sigmoid PEP saturation curve is converted to a hypobolic curve by the presence of the activator. In the cases of A. vinelandii and E. coli B FDP enhances activity without eliminating

the sigmoidicity of the PEP saturation curve. Once again the physiological basis of the distinction between organisms having AMP or FDP activated pyruvate kinases is obscure. The FDP activated g roup includes all the eukaryotic organisms studied so far including Euglena (Ohmann 1969) Mucor rouxii (Passeron and Terenzi, 1970) Coprinus lagopus (Stewart and Moore 1969) and baker's yeast (Hess et. al, 1966 and Hunsley and Suelter 1969) together with the prokaryotes E. coli B, E. coli K 12 (Maluvati and Kornberg 1969) and A. vinelandii. Besides E. coli B and A. vinelandii the AMP activated g roup includes Brev. flavum (Ozaki and Shiio 1969), Brev. liquefaciens (Ide 1970), T. neopolitanus (Cornish and Johnson 1971) B. lichenformis (Tuominen and Bernlohr 1971 b) and the organism used in this work, B. stearothermophilus.

With the pyruvate kinase from B. stearothermophilus AMP has the expected effects of converting to a hypobola the sigmoid PEP saturation curve (Figure 4.16) and lowering the $S_{0.5}$ for PEP at 35° from 1.72 mM to 0.88 mM. There is no indication from the plots in Figure 4.17 of co-operativity in the binding of AMP, a result also obtained with the enzymes from B. lichenformis and A. vinelandii.

Tuominen and Bernlohr examined the saturation curve for AMP only under conditions of ATP inhibition and found the $A_{0.5}$ value to be 60 μM. This is lower than the values recorded with the thermophile enzyme (Figure 4.17) but is comparable with the half saturating concentration in the absence of other ligands for the thermal inactivation studies (Figure 4.5). This

apparent dependence of AMP binding on the presence of other ligands has already been discussed but it should be recalled that a lack of co-operativity in AMP binding is common to both the inactivation and assay systems.

The values of the Hill coefficient and $S_{0.5}$ for ADP recorded in Table 4.5 are similar to the values reported by Tuominen and Bernlohr for the B. licheniformis enzyme.

When sought inhibition by ATP has been found to be a property of the pyruvate kinases of all the microorganisms so far examined. The enzymes from Coprinus lagopus (Stewart and Moore 1969) and B. licheniformis have been shown to resemble the mammalian liver enzyme (Tanaka et al., 1967) in that the degree of inhibition shows a sigmoid response to increasing ATP concentration. The results obtained with the thermophile enzyme were not therefore unexpected. The degree of co-operativity in the binding of ATP and the values for the half saturating ATP concentration indicated in Table 4.6 are little different from the values Tuominen and Bernlohr have reported for the B. licheniformis enzyme.

In the absence of data on the relative concentrations of the adenine nucleotides and inorganic phosphate in the cytoplasm of the thermophile it is difficult to speculate on the exact metabolic significance of the various positive and negative effectors identified in this work. However, as has already been mentioned it seems unlikely that inhibition of the enzyme by inorganic phosphate (Table 4.7) can be

physiologically significant. Arguing from a metabolic standpoint one would expect inorganic phosphate to have an activating rather than inhibitory effect on this enzyme, as it does with some higher plant enzymes, where it fulfils the role generally played by AMP (Dennis and Coultate 1967) as a signal for ATP generation.

It thus appears that the pyruvate kinase from B. stearothermophilus is regulated by AMP activation, ATP inhibition and feed forward activation by PEP and ADP. The pattern of regulators for the thermophile is therefore essentially similar to that in most other microorganisms with the exception of those in which FDP replaces or supplements AMP as the positive effector.

Before proceeding to a discussion of the effects of temperature on the regulation of pyruvate kinase the effect of temperature on activity will be considered. The value of 15.5 k cal mole^{-1} for the Arrhenius constant of the thermophile pyruvate kinase is somewhat higher than the value (9.8 k cal mole^{-1}) recorded by Llorente and co-workers (1970) for the rat liver enzyme. However, the wide range of values found by Somero and Hochachka (1968) for the muscle pyruvate kinases of the rat, rainbow trout and the Antarctic fish Termatomous bernachi (10, 30 and 10 k cal mole^{-1} at their respective environmental temperatures) suggests that such differences may not be physiologically significant.

Few studies have been reported of the effects of temperature on the kinetic and regulatory parameters

of enzymes of carbohydrate metabolism. The muscle pyruvate kinases of the poikilothermic animals studied by Somero and Hochachka (1968) were not subject to feed forward regulation by PEP (although they were activated by FDP). The values of K_m for PEP were influenced by temperature to a limited degree and were minimal some 5^o above the environmental temperature. The K_m for PEP of the rat liver pyruvate kinase is largely unaffected by the fall in temperature from 38^o to 22^o (Llorente et al., 1970). The data accumulated by Behrisch and Hochachka (1969) and Behrisch (1969) on the effects of temperature on the allosteric inhibition by AMP of the fructose diphosphatase from marine poikilotherms is possibly more relevant to this discussion. The fructose diphosphatase of rainbow trout was found to be increasingly susceptible to inhibition by AMP as the temperature fell ($I_{0.5}$ for AMP was 130 μM at 10^o, 25 μM at 0^o) whereas the enzyme from the migrating salmon showed little change in susceptibility to AMP over a comparable temperature range ($I_{0.5}$ for AMP was 300 μM at 15^o, 200 μM at 5^o). Behrisch points out that the migrating salmon normally encounters a much wider range of environmental temperatures than the rainbow trout.

The data in Table 4.4 show that within the g rowth range of B. stearothermophilus the effects of temperature on the Hill coefficient and $S_{0.5}$ values for PEP are slight. Only at temperatures well below the minimum for growth is there any change in the relationship between activity at saturating PEP levels and

that at lower PEP concentrations (such as the arbitrarily selected figure 2 mM) where the activity of the enzyme is most sensitive to PEP concentration. Within the temperature range where the rate of growth increases with temperature (approximately 40-55°) the Arrhenius constant for growth (13.5 to 15.0 k cal $mole^{-1}$; Epstein and Grossowicz, 1969 a) is similar to that for pyruvate kinase activity at saturating PEP levels (15.5 k cal $mole^{-1}$, Figure 4.14). It is therefore apparent from the data in Table 4.4. that the relationship between the potential enzyme activity at low PEP concentrations and the rate of growth is unaffected by temperature above 35°. It also seems most unlikely that the changes in this relationship at low temperatures are in any way sufficient to account for the cessation of growth at temperatures below 40°.

The effects of temperature on the values of $S_{0.5}$ and the Hill coefficient for ADP, presented in Table 4.5 are also slight. Such changes as do occur in the potential activity of the enzyme at low ADP concentrations in relation to the rate of growth of the organism (expressed arbitrarily as $v_{1.0}/V_{max}$) stem largely from changes in the $S_{0.5}$ value and are almost certainly too small to be of physiological significance.

The most significant effect of temperature on the regulation of the enzyme is that on the inhibition by ATP. The results recorded in Table 4.6 show that although the enzyme is most sensitive to inhibition by ATP at the lower end of the temperature range for growth the change in sensitivity to ATP is hardly sufficient to cause a cessation of growth.

Overall the effects of temperature on the regulation of the pyruvate kinase of the thermophile are not large. This finding may well be related to the wide range of temperatures which, as a free living organism, this thermophile might be expected to encounter. However it would be wise to await the availability of corresponding data for the regulatory enzymes from microorganisms normally associated with much narrower temperature ranges, e.g. parasitic microorganisms of homeothermic animals, before drawing firm conclusions on this point.

Although it has appeared from the discussion above that temperature effects on the regulation of the pyruvate kinase of the thermophile are hardly sufficient to influence the temperature range of the organism they may be involved in another feature of the physiology of the organism. The adverse effects of high temperatures on the efficient utilization of glucose (see Section 3.) may be explained by the reduced sensitivity of the pyruvate kinase to feed-back regulation by ATP at the upper end of the temperature range for growth. A failure of feed-back regulation would inevitably lead to an accumulation of pyruvate or the products of pyruvate degradation.

It has been necessary in this work to eliminate as far as possible questions of the interaction of one ligand with another. In this way particular effects of temperature could be identified. However any future work must take account of the multiplicity of interactions between the various substrates and effectors. One particularly satisfactory approach to this

situation is the application of Atkinson's "energy-charge" hypothesis (Atkinson 1968). Such an extension of this work must be accompanied by an examination of the effects of temperature on the intracellular concentrations of the ligands concerned. Inevitably it has been necessary to ignore this question in the preliminary investigation recorded here and the discussion above has been based on the assumption that temperature dependent changes in ligand concentrations are small. This is not such an extravagant assumption as it may at first appear. The response of the activity of pyruvate kinase to the "energy-charge" of the cell will be influenced by the equilibrium between ADP and ATP plus AMP mediated by the enzyme adenylate kinase. The equilibrium constant of the adenylate kinase reaction is sufficiently close to unity in the presence of typical intracellular concentrations of Mg^{++} (Rose 1968, Blair 1968) for the effects of temperature on the relative proportions of the adenine nucleotides (at any particular energy-charge value) to be small.

5. CONCLUDING REMARKS

The current view of the thermophilic micro-organisms must be that they are not the totally exceptional and bizarre organisms envisaged by the early workers in this field. The literature surveyed in the General Introduction and the experiments reported here leave little doubt that there are only two distinctive physiological features consistently associated with the thermophilic habit. The first of these is inevitably that which defines them as thermophiles, that is their optimal growth at elevated temperatures. The second feature is their possession of thermostable proteins. The results of the experiments on protein stability and molar growth yield described in this thesis definitely rule out the possibility that "rapid repair" of thermolabile macromolecules is a characteristic feature of the thermophilic Bacilli.

Studies of purified proteins from thermophiles have shown that this enhanced thermostability is a property of the proteins themselves and is not due to the activity of any unspecific "stabilizing factors". It should not of course be forgotten that many enzymes, from psychrophiles and mesophiles as well as thermophiles, are dependent for their thermostability on the maintenance of an environment similar to that provided by the cell or organelle in which they are normally active. Thus the thermostability of certain enzymes may appear to depend on such factors as, for example, Ca^{++} ions (Ljunger, 1970 and Ogasahara et al., 1970) when it may be more correct to regard such

factors as quite simply, essential components of particular thermostable systems.

In spite of the efforts of numerous investigators generally applicable explanations for the enhanced thermostability of thermophile proteins have remained elusive. The impression gained from the early literature of thermophily is that if the exceptional properties of thermophile proteins could not be accounted for by the activity of "stabilizing factors" then a study of the proteins themselves would reveal gross structural peculiarities from which the mechanism of thermostability could be deduced. Regrettably the studies so far completed have conspicuously failed to identify any such peculiarities. To some extent this failure is a reflection of the magnitude of the task of determining the complete structures of sufficient numbers of mesophile and thermophile proteins for valid comparisons to be made.

In the case of the ferredoxins of mesophilic and thermophilic Clostridia such a study has progressed to the determination of amino acid sequences (Tanaka et al. 1971). This study has provided a satisfactory explanation for the enhanced thermostability of the iron-apoprotein link (Devanthan et al. 1969) but since no loss of secondary structure is involved in the thermal inactivation of clostridial ferredoxins these results have little general significance.

A study that does provide a hint of what may prove to be a general observation is that made by Ohta and co-workers (Ohta et al. 1966 and Ohta 1967)

on the protease from B. thermoproteolyticus. Examination of the ultra-violet, fluorescence and optical rotatory dispersion spectra allied to a knowledge of the amino acid composition suggested that the enzyme owed its stability to a particular abundance of abnormally ionizing tyrosine residues which participate in hydrogen bonding.

It seems most likely that thermophile proteins differ from their mesophile counterparts, not in the possession of unusual structural features but rather in the possession of a greater abundance of those "usual" features to which a role in the maintenance of tertiary structure has been assigned from studies of mesophile proteins. If this view is correct then the proteins of thermophilic bacteria need not represent a special class of proteins but merely show an extreme degree of the same type of structural adaptation to the temperature of their environment as that demonstrated by Ushakov (1967) in poikilothermic animals living at low temperatures.

The evolution of protein structure in response to temperature stress implies that the loss of protein due to thermal inactivation will place an organism at a disadvantage. Thus it may be reasoned that the occurrence of protein turnover during normal vegetative growth implies that the organism is incompletely adapted to the temperature of its environment. However the studies of protein turnover reported in this thesis were conducted at the optimum temperature for growth rate of the thermophile, a temperature which may well be considerably higher than that of

the organism's natural environment. If this supposition is correct then the fairly low rates of protein turnover reported in this thesis may be of very minor significance to the organism in its natural environment.

The question of incomplete adaptation to a hot environment poses more difficult problems when the results of the molar growth yield experiments are considered. Amarasingham and Davis (1965) have suggested that the incomplete oxidation of glucose by E. coli provides for the rapid removal of glucose from the environment thus placing competing organisms, less well equipped to utilize organic acids, at a disadvantage. The possibility of the extension of this explanation must remain a subject for speculation at the present time.

The results of the molar growth yield experiments also indicated that high temperatures have an adverse effect on energy coupling in this organism. Experiments with a heterotrophic strain of B. stearothermophilus (NCA 1503-4R) (Wisdom and Welker, 1973) able to grow rapidly in the range 55-70°, have shown that this strain is able, through changes in both the membrane content of the cell and the lipid/protein ratio of the membrane, to increase the thermostability of certain membrane-bound enzymes in response to the growth temperature. The decreased efficiency of energy coupling at the upper end of the temperature range of the prototrophic strain of B. stearothermophilus may result from the absence from this organism of a mechanism for the thermal adaptation of the membrane such as that described by Wisdom and Welker

(1973). Certainly the results of the respiration experiments (Figure 3.13) give no indication that the stability of the respiratory enzymes is favourably influenced by the growth temperature.

The effects of temperature on the regulation of the thermophile pyruvate kinase were not as large as had been anticipated at the initiation of this work. While the changes in the regulation of the enzyme with temperature are probably involved in the temperature dependence of the molar growth yield it seems unlikely that they are involved in the establishment of the temperature range within which the growth rate obeys the Arrhenius equation. However the essence of Ingraham and Maaløe's explanation (1967) of the existence of a distinct lower temperature limit for growth is not that one particular enzyme activity is disrupted but rather that it is the metabolism of the organism as a whole that is disrupted by the cumulative effect of numerous derangements of control mechanisms at the levels of both enzyme synthesis and activity. Thus an examination of any one, isolated system, such as that reported in this thesis, describes only one facet of a composite and extremely complex situation. Work with many more enzymes, the effects of temperature on the coarse and fine control of their activities and on the effective in vivo concentrations of their substrates, cofactors and allosteric effectors, will be necessary before a more complete appreciation of this topic can be made.

To date by far the greatest research efforts in the field of thermophilic microorganisms have been

directed towards an understanding of how these organisms are able to proliferate at t emperatures which seem so unfavourable to normal life. However this is only one of many aspects of their physiology and biochemistry that are worthy of study. Recent advances in microbial technology, in particular the industrial application of microbial enzymes, have shown that the thermophiles will become increasingly important in the future. The commercial availability of thermostable enzymes, in particular extracellular proteases and amylases from thermophilic Bacilli, has already had far reaching effects. For instance for the production of glucose from starch the extracellular α-amylases from thermophilic strains of B. subtilis (used at temperatures as high as 85°) have completely replaced acid hydrolysis for the liquefaction of the starch pastes prior to their conversion to glucose by the fungal enzyme amyloglucoxidase (α-1,4-glucan glucohydrolase, EC 3.2.1.3) (Fleming, 1968).

As the possibilities for the use of thermophile enzymes as heat stable catalysts increase the culture of microorganisms at elevated temperatures will become an increasingly important aspect of microbial technology. The study of the general features of the physiology and biochemistry of these organisms will thus acquire far more than an academic significance. It is anticipated that the results reported in this thesis will provide a useful basis for these investigations.

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ERRATA

Page 7, line 9, insert '(1966)' after 'Holdsworth'

Page 16, line 6, for '(1947a)' read '(1947)'

Page 18, line 15, and page 20, line 29, for 'Stenesch' read 'Stenesh'

Page 31, line 25, for 'Io.5' read '$I_{0.5}$'

Page 31, line 28, for 'So.5' read '$S_{0.5}$'

Page 44, line 2, for '25000X g' read '25000 x g'

Page 46, line 2, for '250μg^{ml-1}' read '250$\mu g\ ml^{-1}$'

Page 34, line 17, for 'thermostat' read 'chemostat'

Page 59, line 29, insert 'the' after 'than'

Page 85, line 6, for 'that' read 'the'

Page 96, line 13, for 'at' read 'of'

Page 101, line 3 of legend, insert 'clarity' after 'For'

Page 121, line 16, insert 'was found' after 'temperature'

Page 128, line 24, the reference to Bock and Ling should stand in line 22 after the equation

Page 148, line 2 (of text), for 'date' read 'data'

Page 128, line 25, for 'c,' read 'c_I'

Page 152, line 20, insert 'with' after 'observed'

Page 163, line 10, omit 'of' after 'phase'

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ABSTRACT

Thermophilic microorganisms present two fundamental problems. (I) How are they able to grow at temperatures greatly above those normally considered suitable for life ? (2) Why do thermophiles not grow at more moderate temperatures ? This thesis is an account of experiments directed towards resolution of these problems using a prototrophic strain of the thermophile Bacillus stearothermophilus.

Although virtually all the thermophile enzymes isolated to date have proved to be thermostable compared with their mesophile counterparts the possibility has remained open that part of the mechanism of thermophily is a rapid resynthesis of denatured proteins. It is shown here that protein turnover does occur during growth but the rate is too low to be compatible with this "rapid repair" hypothesis. Studies of the stabilities of individual enzymes in intact cells confirm the results of the turnover experiments.

This organisms' ability to grow on minimal media facilitated an examination of the energetics of growth. The energy required for growth is shown to be comparable with that of related mesophiles with no evidence for a massive diversion of energy for resynthesis of denatured macromolecules. These growth yield studies also show that the efficiency of substrate utilization decreases as the temperature rises. This is largely the result of changes in the extent of oxidation of the substrate.

The effects of temperature on the regulation of a key enzyme, isolated from the thermophile, pyruvate kinase, have been studied to test the hypothesis that the temperature limits for growth of a particular microorganism are the result of an accumulation of derangements of metabolic regulation at temperature extremes. In most respects except thermostability the thermophile enzyme resembles those from mesophiles. Temperature effects on kinetic and regulatory parameters are too small to be implicated in the lower limit for growth but are probably involved in the temperature effects on growth yield.