AN INVESTIGATION OF ENDOGENOUS METABOLISM AND GLUCOSE UTILIZATION BY SARCINA LUTEA.

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William Henry Holms,

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PREFACE.

The rather unusual length of the introduction and bibliography to this thesis calls for some explanation. When I came to Glasgow in 1953 the field of microbiological biochemistry was completely new to me and the introductory sections represent the results of my reading in the realms of bacterial carbohydrate biochemistry. These sections are included as they were an integral part of the work and those who are familiar with these matters can turn immediately to page 71 or, if they are not interested in methods, to page 120. The bibliography requires no apology but it should be explained that, where a paper has actually been consulted, its full title is quoted; where a paper has only been read in abstract or review its title is omitted but reference is usually made to the appropriate volume of Chemical Abstracts (C.A.). As explained in the text, several aspects of the literature on Sarcina lutea and bacterial metabolism are classified in three appendices. The complete references to these are included in the general bibliography.

Several aspects of the work presented were carried out in collaboration with Dr. E.A. Dawes of this department. In particular, all of the isotopic work was done in this way and indeed it would have been impossible for one person to have attempted many of the experiments in this sphere.

I am very grateful to Miss A. Alston and Messrs. J. Smillie and W. Burns for technical assistance rendered at various stages of the work. Dr. C.D. Weir, of the Department of Mechanical Engineering of this University, kindly arranged for the stainless steel sleeve of our radioactive barium carbonate filtration apparatus to be polished. Mr. J.W. Leslie, of the University of Glasgow Chemistry Department, made the glass $^{14}CO_2$ alkali traps. Dr. G.E. Glock generously donated some 6-phosphogluconate and the strain of Sarcina lutea was obtained from Dr. E.F. Gale. Mrs. E.A. Dawes and Miss E. Burnett valiantly deciphered my writing to produce the first typed draft. Mr. D.R.S. Cameron and Miss M. Mathieson photographed and copied the figures, most of which were drawn by Mr. R. Callander. Miss A. Grabowski translated papers from the original Russian which would otherwise have been unavailable.

I am extremely grateful to Professor J.N. Davidson, not only for the opportunity to carry out this work, but also for the teaching experience I have gained as an assistant in his department. Dr. G. Crosbie made many helpful suggestions as to the handling and assay of radioactive materials. Above all my thanks are due to Dr. E.A. Dawes who, for the last three and a half years, has been my everyday companion, counsellor and friend.

W.H.H.

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INTRODUCTION

I. THE STUDY OF BACTERIA.

The purpose of this introductory chapter is to show the importance of microbiological studies in biochemistry and to illustrate the line of reasoning which has dictated the programme followed in our research.

(a) The unique character of bacteria.

Time and time again in the last twenty five years the guiding principles of the comparative biochemist (Kluyver, 1931), have been upheld and the basic biochemical reactions universally demonstrated. As Stanier (1954) points out, it is unfortunate that this has led to the assumption, by some workers, that the liver cell and Escherichia coli, the meristematic plant cell and purple bacterium, are sisters under the skin, their biochemical differences being principally ones of minor detail. The microscopist cannot fall into this error because he has seen the manifest differences between these cells and, in the last analysis, the unique character of any cell must depend on its biochemical repertoire. The unique character of bacteria, in particular, may be considered under the headings of distribution, shape, structure and size, chemical activities and energy production.

(1) Distribution of bacteria (Gale, 1947; Lamanna & Mallette, 1953). Bacterial ecology is virtually unexploited and the information that is available is confused by problems of

characterization and nomenclature. But wherever they have been sought, bacteria have been found from Arctic snows to hot mineral springs, from stagnant salt lakes to oil-saturated soil round oilwells, from the contaminated effluents of modern industry to the purest brook or stream. In short, bacteria are found wherever life exists and in a good many places where other forms of life do not survive. This ubiquitous distribution of bacteria as a class is one of their most unique characteristics.

(ii) Shape, structure and size of bacteria (Knaysi, 1951a, 1951b; Lamanna & Mallette, 1953; Stanier, 1954; Stephenson, 1946). The normal vegetative bacterial cell assumes one of three general forms, namely, spherical, rod-like or spiral. The cytoplasmic membrane of the cell is contained by a fairly rigid cell wall frequently surrounded by a slimy or capsular layer. Factors of size make the study of the internal organisation of the bacterial cell a difficult problem. Many inclusions have been noted in bacteria but despite strongly held opinions the occurrence of nuclei has not been proved. Nuclear material certainly exists in bacteria but even if "nuclei" exist they possess a different order of organization as compared with the nuclei of higher forms. In the swimming forms the organ of locomotion, although termed a flagellum, is not of the same nature as the flagella of protozoa, plants and

animals. In the higher forms the flagella consist of eleven fibrils, two of which are distinct from the rest while in bacteria the flagella are single fibrils without evidence of internal structure, and have been called "monomolecular hairs" by Astbury (1951). Incomplete though our knowledge of bacterial anatomy may be, it obviously presents singular features and it is at least possible that an increase of data will uncover even more differences between the structure of bacteria and higher forms.

Bacterial size can vary from the limit of visibility of the light microscope almost to the limit of the human eye. Thus, Bacterium pneumosintes varies from 0.15 to 0.3 μ in length while Begiatoa mirabilis is 16 to 45 μ in width and forms filaments which may be several centimetres in length. Despite this wide range, which overlaps the larger viruses and mammalian blood corpuscles, the great majority of bacteria have a width of from 0.5 to 2.0 μ. This small size has the important corollary of a very high surface/volume ratio. The implication of figures in this context is not so striking as the example quoted by Stephenson when she pointed out that a streptococcus enlarged to the volume of a mouse would have a surface area as large as a tennis court if its original surface/volume ratio were maintained. From this physical fact stems one of the greatest differences between bacterial and mammalian

systems. The mammal is protected from its environment by a series of regulatory mechanisms which control its internal temperature, oxygen and carbon dioxide tension, pH, and concentrations of nutrients and waste products. Because of this isolation the mammalian metabolism is somewhat rigid in operation but the bacterial cell, which is in such close contact with its environment, possesses a much more flexible and varied complement of chemical mechanisms. The processes of induced enzyme synthesis and mutation have been studied by many workers and it would be impossible to make a fair representative selection from their work, but even if many questions still remain unanswered, it is quite clearly established that the capabilities of bacteria in these directions are unique.

(iii) Chemical activities of bacteria (Gale, 1947; Kluyver, 1956). The chemical activities of bacteria are legion. The anaerobic mechanisms of glucose utilization are of special interest in this context because they distinguish bacterial from mammalian systems not only because they can support life but also by the varied nature of their products. Thus, while the mammalian anaerobic system has lactic acid as its end-product, bacteria can transform glucose to lactate, ethanol, acetate, propionate, 2,3-butanediol, glycerol, succinate, butanol, butyrate and acetone, and the same microorganism can yield different products under varied conditions. In other anaerobic

systems where methane and carbon dioxide are the products, the substrates can vary from formic to benzoic acids. The chemo-autotrophic bacteria also are distinct in their dissimilatory processes. Indeed, a study of the dissimilations carried out by bacteria shows that not only are they distinct from mammalian systems as a class but also that many are unique within the bacterial family itself. In the anaerobic reactions, also, many systems seem to be confined to bacteria (q.v.). It is of course true that the reactions described are all concerned with hydrogen and phosphate transfers, but the fact remains that bacteria as a class achieve these ends in a greater diversity of ways than the more limited mammalian systems. As Krebs (1954b) has pointed out, the actual synthesis of high energy phosphate bonds can occur in bacteria by the same seven types of reaction that are known in mammals. These are the two steps in glycolysis, three steps in oxidative phosphorylation, oxidative decarboxylation of α-ketonic acids and succinate oxidation. Bacteria utilise these reactions but possess in addition several unique methods. One of these is, in effect (but not necessarily in mechanism), a reversal of one of the reactions of urea synthesis in the animal:-

Citrulline + phosphate + ADP $\longrightarrow$ ornithine + NH_3 + CO_2 + ATP.

The actual methanism of the reaction is unknown although citrulline phosphate has been suggested as an intermediate. A

mechanism of high energy phosphate formation in fermentations is shown in the following sequence:

Pyruvate + Coenzyme A $\longrightarrow$ Acetyl-CoA + $2H + CO_2$ (i) + $H_2 + CO_2$ (ii) + $HCOOH$ (iii) Equation 1.

Acetyl-CoA $\xrightarrow[\text{transacetylase}]{\text{phospho -}}$ Acetyl-phosphate + CoA Equation 2.

Acetyl-phosphate + ADP $\xrightarrow[\text{phosphokinase}]{\text{aceto-}}$ acetate + ATP Equation 3.

Of these reactions only Equation 1 in the form of (i) occurs in animals when pyruvate is available as hydrogen acceptor. The other reactions are confined to bacteria.

(b) Conclusions.

The above very brief summary shows clearly that bacteria possess many unique features. It is these obvious qualities together with the relative ease of production which have led many workers to use bacteria as sources of material in the study of particular problems. This approach has been undoubtedly fruitful even since the days when Priestley obtained the first sample of oxygen gas from a suspension of unicellular photosynthetic organisms and thus proceeded to the experiments which overthrew the phlogiston theory (van Neil, 1956). And today we see members of the same group, such as Scenedesmus, being utilized in the study of the initial reactions of photo-

synthesis. The use of microorganisms in this way has illuminated many of the fundamental problems of biology but the basic questions of bacterial physiology should not be overlooked (Stephenson, 1946). Pasteur laid the foundation of microbiology because he considered fermentation not as a chemical problem of isolation and separation but as a chemical expression of a method of life - "la vie sans air". On the other hand, the cell-free systems of Buchner advanced our knowledge of biochemical principles while progress in microbiology marked time.

The writer feels strongly that the attitude of mind of the microbiological biochemist is of great importance for, coming to this subject some three and a half years ago as a carbohydrate chemist, he has been driven to the inescapable conclusion that bacteria are a unique expression of life which deserve to be studied as such and, while it may be necessary to investigate one particular aspect of their metabolism at a time, the position of the cell in the general economy of nature should never be overlooked.

II. CHOICE OF ORGANISM AND PURPOSE OF RESEARCH.

It was decided to study an obligate aerobe - a class of bacteria the metabolism of which is not so fully understood as the fermentative organisms. Sarcina lutea was chosen for several reasons:-

1. It has not been studied to any great extent in the past but has frequently been included in general surveys of particular reactions or cell contents.
2. Several strains are readily available from the National Collection of Type Cultures although, in the event, a strain supplied by Dr. H.F. Gale was used for the bulk of the work.
3. It is one of the relatively few bacteria which are sensitive to lysozyme.
4. It was known to possess a high endogenous rate of respiration and it was felt that this property might eventually be investigated.
5. It was thought that it could be cultured in simple synthetic media.

The long term aim is to characterize the organism. An attempt has been made in the first instance to establish the mechanism of carbohydrate metabolism (reported in this thesis; Holms & Dawes, 1955) fat metabolism (Murray & Dawes, 1956; Murray, 1956) and a start has been made with endogenous metabolism (reported in this thesis) and this aspect is at present being continued (Dawes, Dickson & Holms, 1956). The

work so far has been confined to non-proliferating washed cell suspensions and extracts made from them. Further work will deal with amino acid catabolism and the results of these studies should form a basis for an approach to the synthetic mechanisms of the organism, probably choosing as the point of departure those aspects of oxidative assimilation which are being considered in our work on endogenous respiration. At the same time it should be possible to relate the metabolism of other important biological compounds to the main streams of carbohydrate, fat and protein metabolism - for example the position of glucosamine in the general reactions of carbohydrate metabolism is of considerable interest.

III. THE LITERATURE OF SARCINA LUTEA.

There exists a considerable literature on the Sarcinae but not all is relevant to this thesis. In particular, much has been published on the position of Sarcinae in the brewing industry and these references and others are listed in Appendix I.

(a) Description in Bergey's manual.

This standard work (Breed et al., 1948) describes Sarcina lutea as follows:-

Spheres: 1.0 to 1.5 microns, showing packets in all media. Gram-positive.

Gelatin colonies: Circular up to 5 mm. in diameter, sulphur-yellow, sinking into the medium.

Gelatin stab: Slow infundibuliform liquefaction.

Agar colonies: Yellow, coarsely granular, circular, raised, moist, glistening, entire margin.

Agar slant: Sulphur to chrome yellow, smooth, soft.

Broth: Clear with abundant yellow sediment.

Indole: Slight indole formation.

Acids: No acid from glucose, lactose or sucrose.

H_2S: Hydrogen sulphide is formed.

Growth: Aerobic: optimum temperature 25°C.

Habitat: Air, soil and water, skin surfaces.

(b) The chemical composition of Sarcina lutea.

(i) The pigments. Chargaff & Dieryck (1932), Chargaff (1933) isolated two pigments. One of these acts like a hydrocarbon, is unaffected by 5% alcoholic alkali and has two strong absorption bands at 467 and 440 mμ with a weaker one at 415 mμ. This pigment is called "sarcinine" (Lederer, 1938). The other pigment is probably a xanthophyll but does not give a blue colour with 25% HCl, thus differentiating it from Tswett's β-xanthophyll. It has absorption bands at 469 and 440 mμ. Nakamura (1936) isolated a xanthophyll which has absorption bands at 490, 460 and 433 mμ. Imshenetski (1946) found that the resistance of pigmented organisms in general to the action of ultra-violet light was greater than that of non-pigmented organisms. This agrees with the finding of Auchincloss (1955) that Sarcina lutea was highly resistant to ultra-violet light.

(ii) Amino acids. Both Belozerskii & Kireenkova (1943) and Hoare (1955) have found a normal pattern of amino acids in Sarcina lutea. The second author also describes the acids in the free amino acid pool and this is found to vary with the composition of the growth medium. Of the several differences noted, the most striking is found with hydroxproline which is not a constituent of the cell protein but is accumulated when the organism is grown on nutrient agar but not on casein digest agar.

Work & Dewey (1953) report the absence of diaminopimelic acid.

(iii) The nucleic acids. The content of nucleic acids has been determined (Belozerskii & Kireenkova, 1943; Dutta, Jones & Stacey, 1953, 1956). The work of the latter group is of particular interest in that they determine the composition of deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) in cells of different history. They find no significant difference in composition in either DNA or PNA of cells harvested at different phases of the growth cycle. A comparison of a streptomycin-resistant strain with a wild type showed no difference in composition of DNA but an increase of guanine and cytosine at the expense of adenine and uracil in the PNA. The authors argue that the change in enzymatic complement associated with resistance is a reflexion of the observed changes in PNA composition.

(iv) The cell wall. Salton (1951, 1953) has isolated the cell wall of Sarcina lutea 'G' and gives the following composition (as % dry weight cell wall) N, 7.6; P, 0.22; reducing substance, 46.5; hexosamine, 16.3; lipid, 1.1. He found alanine, aspartate, glutamate, glycine and lysine but no evidence for nucleic acids. Cummins & Harris (1956) have found in cell walls of Sarcina lutea NCTC 611 glucose, glucosamine, an unknown hexosamine, alanine, glutamate, lysine and glycine.

(c) Growth.

Rubenstein (1933b) found that the growth of Sarcina lutea could be supported by several compounds such as alanine and ammonium lactate, pyruvate, glycerate and tartrate. Ammonium acetate would not support growth unless glucose was added. A substance or group of substances which abolished the initial lag in growth was found to be present in the medium of a 12-24 hour culture. The substance is thermostable, destroyed rapidly by weak alkali, dialysable and not fat soluble.

Den Dooren de Jong (1926) in his well known survey found that the growth of Sarcina lutea could be supported by:-

(i) Simple substances: glucosamine, ∝-alanine, tyrosine, cystine, ∝-aminovaleric acid, ∝-aminocaproic acid, aspartic acid and glutamic acid.

(ii) Inorganic nitrogen plus: acetate, propionate, butyrate, isobutyrate, valerate, caproate, heptylate, caprylate, nonylate, ∝-crotonate, lactate, malate, citrate, isobutanol, glycerol, xylose, rhamnose, mannitol, glucose, mannose, galactose and saccharate.

(iii) Glucose plus: methylamine, ethanolamine, glycine, hippurate, ∝-alanine, phenylalanine, tyrosine, histidine, isoleucine, aspartate, glutamate, propionamide, isobutyramide, valeramide, capronamide, lactamide, fumaramide, urea, assym. diethylurea,

arginine, parabonate, alloxan, allomanatine, allantoin and guanine.

(d) The intermediary metabolism of Sarcina lutea.

(i) Protein metabolism. Imaizumi (1938) showed that an unclassified Sarcina elaborated enzymes for the hydrolysis of peptide bonds although it did not hydrolyse casein or gelatin. Thus a glycerol extract of a dry powder and a maceration juice hydrolyse peptone, tripeptides and dipeptides and the maceration juice hydrolyses synthetic chloroacetyl-L-phenylalanine but not benzoyldiglycine. A bouillon filtrate hydrolyses peptone but not peptides. Stumpf & Green (1944) could not find L-amino acid oxidase.

It is of interest that Sarcina lutea is a member of that small class of aerobes which produce urease (Wohlfeill & Weiland, 1937) and the rise in pH following the addition of urea to a washed cell suspension of the micro-organism has been quantitatively measured (Stephan & Hemmens, 1947).

(ii) Fat metabolism. The fatty acids which serve as sources of carbon and energy for the growth of Sarcina lutea have been mentioned above (c ii). Although the organism is non-lypolytic it is fat oxidative (Mundt & Fabian, 1944) and Q_{O_2} values for three strains are to be found in the literature (Barron & Friedemann, 1941; Franke & Peris, 1937). This work has been extended in this laboratory (Auchincloss, 1955) and the Q_{O_2}

values of four strains on acetate, butyrate, valerate, caproate, heptoate, caprylate, pelargonate and caprate determined. Continuation of this work (Murray, 1956; Murray & Dawes, 1956) showed that lyophilized cells and protoplasts possess the same oxidative ability as whole cells. Some of the enzymes of fatty acid degradation have been detected in cell free extracts and the available evidence indicates that the classical fatty acid cycle operates in Sarcina lutea.

(iii) Endogenous and carbohydrate metabolism. Gerard & Falk (1931) examined a strain of Sarcina lutea, which no longer formed packets, manometrically as a washed cell suspension with the ultimate intention of investigating the dynamics of oxygen diffusion into the cell. They found that endogenous oxygen consumption diminished with time and suggested that the initial rate was increased by virtue of the "partial asphyxiation" of the cells in the harvesting procedure. Rubenstein (1931, 1932) found that endogenous oxygen uptake at 37°C was decreased by irradiation with visible light and that the uptake could be reduced to negligible proportions by aerating overnight in phosphate buffer at room temperature. Cyanide and carbon monoxide had no effect on endogenous respiration (Gerard, 1931). Oxygen uptake with glucose as substrate has been studied under a variety of conditions (Barron & Friedemann, 1941; Gerard, 1931; Gerard & Falk, 1931; Rubenstein, 1932). Rubenstein (1933a) obtained a cell-free filtrate which gave a

very slow oxygen uptake with glucose. The conflicting results of these studies may be due to the strain differences. Thus, cyanide has been reported as having no effect or a 98% inhibition. The most detailed paper is that of Barron & Friedemann (1941) and their results are summarized in Table 1.

Stephan & Hemmens (1947) showed that in a cell suspension metabolizing glucose an initial rapid drop in pH was followed by a gradual rise. This presumably corresponds to the formation and further metabolism of acid. Fosdick et al. (1937) claimed that Sarcina lutea shaken with pyruvate in a sealed tube yielded lactate but examination of their data shows that the observed changes are smaller than the experimental errors involved. Fosdick & Calandra (1945) by standard techniques using a dried powder or cell-free extract showed that Sarcina lutea phosphorylated glucose to hexosediphosphate, contained aldolase and isomerase, produced phosphoglycerate, produced pyruvate and acetaldehyde from phosphoglycerate, and produced small amounts of acetaldehyde and even smaller amounts of lactate from pyruvate. In the section of this paper headed "Conclusions" they say that the end product of glucose degradation is primarily acetic acid. Inspection of their results fails to reveal any mention of acetic acid and it is difficult to see on what basis they arrive at their conclusion.

Table 1.

(Barron & Friedemann, 1941)

Substrate	Q_{O_2}	% Inhibition by:- 0.02M NaF	0.005M IAA	0.005M HCN
None	0.4	-	51.0	41.0
Glucose	1.08	24.8	57.4	98.0
Hexosediphosphate	83.7	-	83.0	92.0
Hexosemonophosphate	90.5	-	60.0	95.0
Lactate	88.0	-	47.5	100.0
Pyruvate	44.8	-	73.4	90.7
α-Ketoglutarate	7.8	-	-	-
Formate	34.7	-	100.0	93.0
Acetate	55.0	-	97.1	99.3
Propionate	84.2	-	100.0	100.0
Butyrate	80.0	-	96.4	100.0
Succinate	100.1	-	96.7	98.4
Fumarate	83.7	-	94.2	94.8
Citrate	3.8	-	-	-
Ethanol	82.1	-	89.7	98.9
Glycerol	32.0	-	0.0	86.7
DL Alanine	39.0	-	84.3	96.1
L(+) Glutamate	72.0	-	58.8	94.7

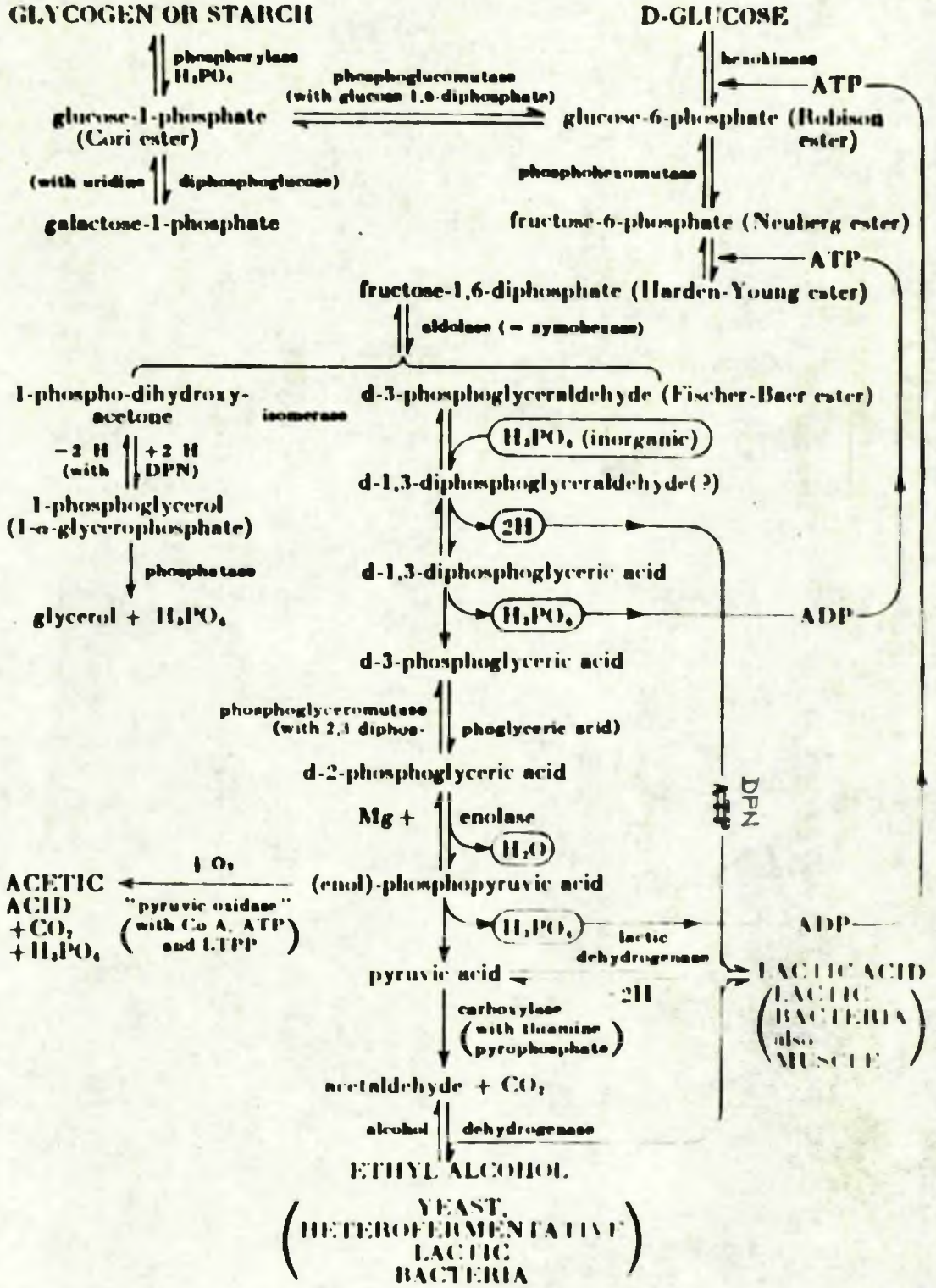
IV. GLYCOLYSIS.

(a) The reactions of glycolysis.

The most clearly documented pathway of carbohydrate metabolism is that of glycolysis. The history (Dickens, 1951; Nord & Weiss, 1951) of the elucidation of this sequence of enzymically controlled reactions is associated with many of the greatest names in biochemistry. Of these, Embden, Meyerhof and Parnas are preeminent and this route of metabolism is frequently designated by their names - Embden-Meyerhof-Parnas system (EMP). EMP has been discussed and reviewed from many different viewpoints (Gale, 1951; Elsden, 1952; Gunsalus, Horecker & Wood, 1955; Stumpf, 1954; Werkman & Schlenk, 1951). The generally accepted scheme is shown in Fig.1 (Thimann, 1955). From the point of view of the succeeding discussion the following aspects are of particular importance (Dickens, 1952):-

1. Adenosinetriphosphate (ATP) is required for the phosphorylation of glucose and of fructose-6-phosphate, but more ATP is generated in the latter stages than is used in the primary phosphorylations.
2. Inorganic phosphate is required for the phosphorylation of triose phosphates.
3. Diphosphopyridine nucleotide (DPN) acts generally as the hydrogen acceptor but can pass on this hydrogen to a variety

Figure 1.


GLYCOGEN OR STARCH
phosphorylase
H_3PO_4
glucose-1-phosphate
(Cori ester)
phosphoglucomutase
(with glucose 1,6-diphosphate)
D-GLUCOSE
hexokinase
ATP
glucose-6-phosphate (Robison ester)
(with uridine diphosphoglucose)
galactose-1-phosphate
phosphohexomutase
fructose-6-phosphate (Neuberg ester)
ATP
fructose-1,6-diphosphate (Harden-Young ester)
aldolase (= zymohexase)
1-phospho-dihydroxy-acetone
isomerase
d-3-phosphoglyceraldehyde (Fischer-Baer ester)
H_3PO_4 (inorganic)
−2 H (with DPN)
+2 H
1-phosphoglycerol
(l-α-glycerophosphate)
d-1,3-diphosphoglyceraldehyde(?)
2H
phosphatase
glycerol + H_3PO_4
d-1,3-diphosphoglyceric acid
H_3PO_4
ADP
d-3-phosphoglyceric acid
phosphoglyceromutase
(with 2,3 diphos-
phoglyceric acid)
d-2-phosphoglyceric acid
DPN
Mg +
enolase
H_2O
$\frac{1}{2}$ O_2
ACETIC ACID
$+CO_2$
$+H_3PO_4$
"pyruvic oxidase"
(with Co A, ATP and TPP)
(enol)-phosphopyruvic acid
H_3PO_4
lactic dehydrogenase
ADP
pyruvic acid
2H
LACTIC ACID
(LACTIC BACTERIA also MUSCLE)
carboxylase
(with thiamine pyrophosphate)
acetaldehyde + CO_2
alcohol
dehydrogenase
ETHYL ALCOHOL
(YEAST, HETEROFERMENTATIVE LACTIC BACTERIA)

of other acceptors (e.g., pyruvic acid ⟶ lactic acid, or acetaldehyde ⟶ ethanol) thus enabling the process to continue with only a very small pool of DPN.

4. Some of the enzymes of EMP are sensitive to inhibitors. Thus glyceraldehyde-3-phosphate dehydrogenase (Gly-3-Pdh) is inhibited by iodoacetate (IAA) and enolase is sensitive to fluoride.

(b) The occurrence of glycolysis in bacteria.

Until a short time ago it was considered that EMP and the Krebs' cycle (TCA) formed the main route of carbohydrate catabolism in bacteria. Wood* (1955) considers this to be mainly due to the results of three investigations. Firstly, Stone & Werkman (1937) surveyed a group of bacteria poisoned with fluoride and found phosphoglycerate (PGly) to be formed by every species except Clostridia. Following Embden's discovery of PGly the occurrence of this compound was taken as being synonymous with the operation of EMP, and these results naturally supported the conception of the universal distribution of this pathway. But it is now known that PGly is formed in the operation of other pathways (q.v.) and the mere isolation of this compound does nothing to indicate the route of its formation. Secondly, one representative (Escherichia coli) of the bacteria was extensively investigated and the tacit assumption was made that, unless other evidence was present, the reactions of this organism were representative of bacteria as a

*Harland G. Wood.

whole. It was found that Esch. coli contained all the enzymes of EMP (Still, 1940; Utter & Werkman, 1941). If any of a number of other organisms had been singled out for attention in this way the non-glycolytic pathways might have achieved such prominence that EMP could easily have become an alternative pathway as far as bacteria were concerned! It is, in any case, doubtful if the demonstration of the presence of a series of enzymes necessarily means that they have any particular quantitative significance in vivo. In this context it is interesting to note that only three enzymes are now thought to be unique to EMP. These are phosphohexokinase, aldolase specific for fructose-1,6-diphosphate (F1,6P) and triosephosphate isomerase.

Thirdly, when isotopes came into laboratory use, Wood, Lifson & Lorber (1945) chose Lactobacillus casei to degrade glucose in studies on glycogen synthesis in the rat. They found that Lb. casei split glucose exactly as predicted by EMP and checked these observations by chemical degradation of the glucose.

These coincidental observations, supported by many others, spread the belief that EMP was the primary mechanism of glucose utilization and this belief was nurtured by admiration of the very beauty of the studies on EMP where, for the first time, isolated and purified enzymes and coenzymes were recombined in vitro to operate as a complicated enzyme sequence.

(c) Occurrence of glycolysis in micro-organisms.

Although other pathways are now known to occur, glycolysis still remains a most important route in micro-organisms. Four main approaches have led to the assumption that EMP operates in any given material. These are:

(i) Production and utilization of EMP intermediates.

(ii) Extraction of the enzymes of EMP.

(iii) Sensitivity to inhibitors.

(iv) Fermentation of isotopically labelled substrates.

Although some of these findings are inconclusive, the widespread distribution of EMP in the microbiological world is beyond doubt. The above methods have been applied as shown in Appendix II.

That any given micro-organism does not initiate glycolysis, under given conditions, may be assumed to be due to lack of one or more glycolytic enzymes. De Moss, Bard & Gunsalus (1951) found that Leuconostoc mesenteroides lacked aldolase and triose phosphate isomerase. Subsequently, studies with isotopically labelled glucose indicated that the hexose molecule was fermented by another route (Gunsalus & Gibbs, 1952). Several micro-organisms, e.g., Pseudomonas fluorescens (W.A. Wood, 1955), are thought to be deficient in hexokinase but contain the subsequent enzymes of glycolysis. In these cases glycolysis could occur if phosphorylated

hexoses were formed by some other mechanism such as direct oxidation to gluconate and entry of this compound into the hexosemonophosphate oxidative pathway via gluconokinase. Of great interest, also, is the work of Hill & Mills (1954) with Bacterium tularense. This organism is unable to dissimilate glucose anaerobically but does not appear to utilize any method other than glycolysis for the aerobic utilization of sugar. Cell-free extracts exhibit a powerful glycolytic action if purified mammalian lactic dehydrogenase is added. This result is interpreted as indicating that the lack of an anaerobic mechanism for the reoxidation of DPNH in this organism prevents glycolysis under these conditions. Aerobically other mechanisms for this reoxidation must exist and the complete oxidation of glucose to carbon dioxide and water follows the initial glycolysis. A similar result was found with Agrobacterium tumefaciens but not with Alcaligenes faecalis, Pseudomonas aeruginosa or Sarcina lutea, which indicates that this particular enzymatic deficiency cannot be responsible for all cases where glycolysis does not occur anaerobically.

V. TERMINAL PATHWAYS OF BIOLOGICAL OXIDATION.

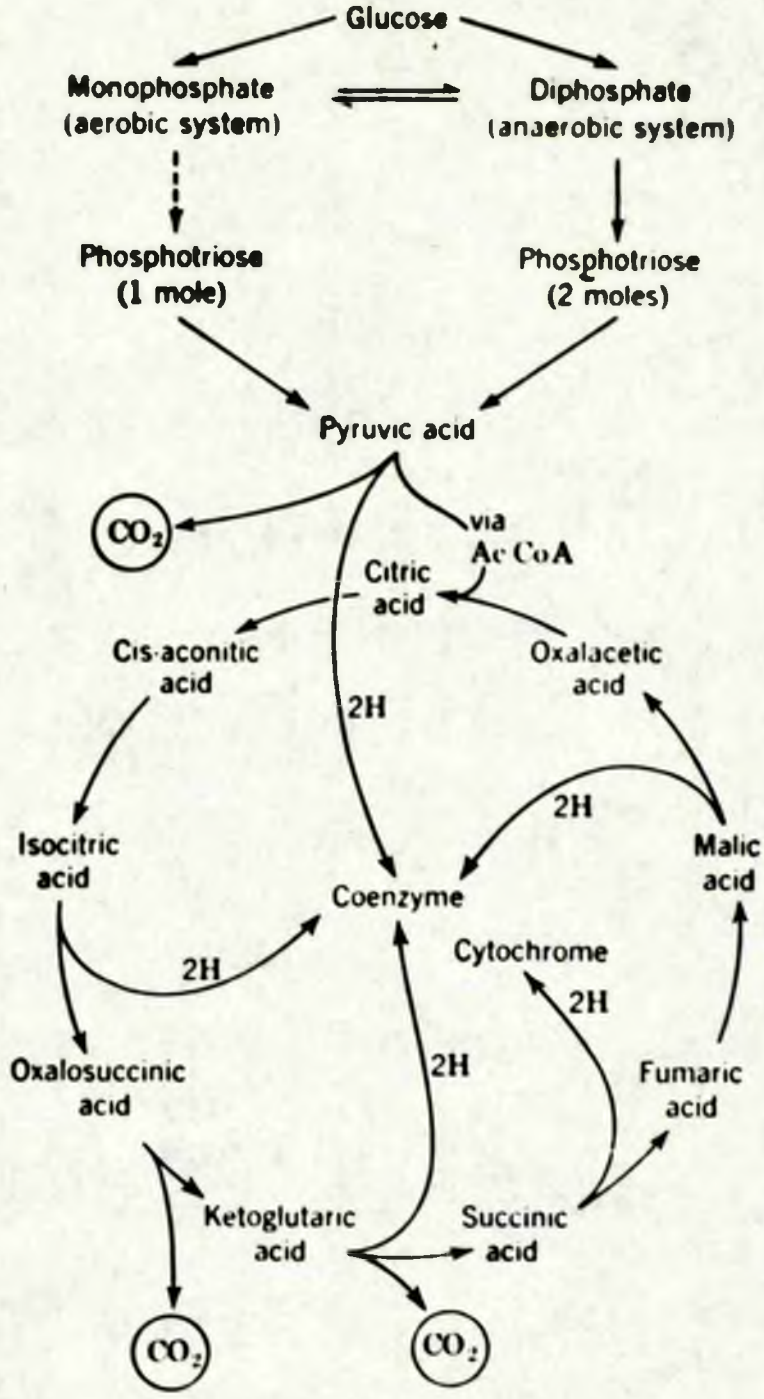
(a) The Krebs' or Tricarboxylic Acid Cycle (TCA).

The system of glycolysis (EMP) described in the preceding section results in the production of pyruvate from glucose. Under aerobic conditions the final products of glucose metabolism are carbon dioxide and water which are formed by the action of the TCA as shown in fig.2. The individual stages of this series of enzymic reactions have been reviewed recently (Krebs, 1954a, 1954b; Ochoa, 1954).

The significant features of TCA are:-

1. The complete sequence of events (including the reaction immediately prior to entry into the actual cycle) results in the complete oxidation of one molecule of pyruvate to carbon dioxide and water.
2. This is accomplished by a decarboxylation reaction followed by the condensing of the resulting 2-C fragment with oxaloacetate (OAA) to give citric acid. This tricarboxylic acid is then degraded by a series of enzyme-catalysed reactions to yield two further molecules of carbon dioxide.
3. The process is cyclic in that one of the substrates (OAA) of the initial condensing reaction is regenerated.
4. Reduced DPN is generated in the cycle.
5. Some of the reactions of the cycle are sensitive to inhibitors. This fluorocitrate (which may be synthesized in

Figure 2.



Summary of the conversion of glucose to pyruvic acid and the oxidation of pyruvic acid via the citric acid cycle.

situ from fluoroacetate) causes accumulation of citrate, arsenite prevents ∝-ketoglutarate (∝KGA) utilization, while malonate causes accumulation of succinic acid.

(b) The detection of TCA in micro-organisms.

Krebs (1954a) has pointed out the difficulties in assessing the importance of TCA as an energy-yielding process in micro-organisms. Thus in adult animal tissues, energy-yielding processes are more rapid than other kinds of metabolic systems involving oxidations and reductions. Conversely, if a rapid oxidoreduction system is discovered in these tissues, it may be assumed that the overall result is one of energy production. In micro-organisms, on the other hand, life is associated more with growth than with energy production and utilization. Indeed more than three-quarters of the organic substrates consumed can be used for the synthesis of cell material by some organisms (Clifton, 1946). It follows then, that a rapid utilization of substrate in a system involving oxidoreductions may not be primarily concerned with energy supply but rather with the production of intermediates required for the synthesis of cell material. The synthesis of citric acid and its subsequent conversion to ∝KGA may not be part of an energy-yielding chain of events but rather the synthesis of a precursor of glutamic acid required in the production of new cell material. Under these conditions, it must be borne in mind that once a reaction has been established

its function in the economy of the cell remains to be demonstrated. This can be done by comparing the rates of production and utilization of the intermediates with the overall respiration of the cell, by showing that the system is coupled with the synthesis of high-energy phosphate bonds (e.g., through reduced coenzymes), by the use of isotopically labelled substrates and measuring their incorporation into cell material, and by showing that the overall respiration of the cell is diminished when the reaction sequence under investigation is blocked (e.g., specific inhibitor). Besides difficulties of interpretation many practical obstacles are encountered. Of these the most important in studies of TCA are those effects of permeability of the cell walls of micro-organisms which prevent the penetration of added substrates to the site within the cell where they could be metabolized. These permeability barriers may be circumvented by carrying out the experiment at a pH above or below that which is considered physiologically normal or may in some cases be destroyed or diminished by lyophilization, exposure to low temperature or by disintegration of the cell.

(c) The occurrence of TCA in micro-organisms.

Despite the difficulties outlined above, many micro-organisms have been shown to utilize TCA. Four different types of approach indicate the presence of TCA in any given micro-organism. These are:-

(i) Utilization, accumulation and interconversion of TCA intermediates.

(ii) Extraction of enzymes of TCA.

(iii) Sensitivity to inhibitors.

(iv) Distribution of isotopes from labelled substrates.

In addition to these general methods, use of mutant strains indicates the presence of TCA in Escherichia coli (Gilvarg & Davis, 1956) and in Neurospora crassa (Strauss, 1955). Many workers have reported studies based on the four general methods outlined above and these are listed in Appendix III.

(d) Alternative routes for terminal pathways.

From time to time suggestions have been made that the Thunberg type of condensation of two C_2 units could initiate a chain of reactions allowing for the complete oxidation of acetate via the dicarboxylic acids. This possibility was mainly put forward by Ajl and has been reviewed by him (1951). Experiments with Escherichia coli utilising the technique of simultaneous adaptation (Ajl, 1950) first gave credence to the idea and later experiments measuring the incorporation of labelled acetate into carrier dicarboxylic acids supported the concept (Ajl & Kamen, 1951; Ajl, 1951). That these results were interpretated erroneously was due to lack of recognition of the fact that the activity of external added carriers may

not necessarily reflect the true activities of intracellular intermediates (Kaufman et al., 1951; Stadtman & Barker, 1949). This is probably accentuated in these experiments by the well known permeability effects associated with polycarboxylic acids. Further investigations (Saz & Krampitz, 1954; Swim & Krampitz, 1954; Ajl & Wong, 1955) showed that TCA does operate in Esch. coli and it seems unlikely that an alternative route is quantitatively significant. Experiments with mutants of Esch. coli and Aerobacter aerogenes also show that acetate cannot be oxidized via condensation to the dicarboxylic acids (Gilvarg & Davis, 1956). As Ajl himself has now said (Ajl & Wong, 1955), "Dried preparations incorporate significant amounts of acetate carbon into all Krebs cycle intermediates. These data and those of Krampitz and co-workers, plus the finding that this organism contains a potent isocitric dehydrogenase and an aconitase, strongly suggests that E. coli does indeed respire via the tricarboxylic acid cycle. The failure to date to demonstrate an acetate-to-acetate condensing enzyme in cell-free extracts leaves much doubt whether this bacterium oxidizes acetate via an abridged type of cyclic mechanism, as the results with non-proliferating, resting cells previously suggested."

VI. THE HEXOSEMONOPHOSPHATE OXIDATIVE PATHWAY.

The very rapid increase of our knowledge of intermediary carbohydrate metabolism in the last two decades has rested on two main supports: glycolysis, springing from Embden's discovery of phosphoglyceric acid in 1933, and the oxidative cycle of tricarboxylic acids postulated by Krebs & Johnson in 1937 (H.G. Wood, 1955). An alternative to glycolysis was discovered as long ago as 1931 by Warburg when oxidation of hexosemonophosphate by haemolysed red blood cells was noted (Warburg & Christian, 1931). These observations were elaborated and extended to yeast by Warburg & Christian (1936, 1937) and by Dickens (1936, 1938). The enzyme responsible for these oxidations was shown to reduce specifically a new coenzyme (Warburg et al., 1935) triphosphopyridine nucleotide (TPN) and thus from the beginning this new oxidative pathway was differentiated from glycolysis which is linked to diphosphopyridine nucleotide (DPN). In this way a new metabolic sequence was discovered and its unravelling, which is not yet complete, makes one of the fascinating stories of biochemistry. As this chapter is planned on the basis of the enzymes of the sequence rather than on their chronological discovery, the reader is referred to the many excellent reviews which cover the historical approach to the Warburg-Dickens-Lipmann pathway (WDL) or, as it is now called, the hexosemono-

phosphate oxidative pathway (HMP). These reviews are by Dickens (1952, 1953), Horecker (1953), Cohen (1954) and Racker (1955). The pathway is also reviewed by Dickens (1955), Gunsalus, Horecker & Wood (1955), De Ley (1952, 1953, 1955) and by W.A. Wood (1955a, 1955b).

(a) The enzymes of the hexosemonophosphate oxidative pathway.

(i) Kinases. The activating step in HMP is the phosphorylation of glucose by a kinase using adenosine triphosphate (ATP) as phosphate donor. In this case hexokinase acts on several sugars (e.g., glucose, fructose, mannose) and has been found in yeasts (Bailey & Webb, 1948; Berger et al., 1946; Kunitz & McDonald, 1946). What is termed "hexokinase activity" has been demonstrated by various methods in Clostridium butyricum (Gavard, 1954), in Lactobacillus bulgaricus (Rutter & Hansen, 1953), Leuconostoc mesenteroides (De Moss, 1953), in Pseudomonas aeruginosa and Ps. putrefaciens (Claridge & Werkman, 1954a; Klein & Doudoroff, 1950; Klein, 1953) and in Streptococcus faecalis (Sokatch & Gunsalus, 1954). A specific glucokinase has been shown in Escherichia coli (Cardini, 1951). In addition to these particular studies, many micro-organisms possess kinases; thus the fermentative micro-organisms phosphorylate glucose and other carbohydrates prior to their anaerobic dissimilation. Gluconokinase is the other enzyme which allows the entry of 6-C fragments into HMP and is of particular

interest as its presence is taken as being synonymous with a non-glycolysing pathway (see below). Gluconokinase has been demonstrated in Aerobacter cloacae (De Ley, 1953a), Escherichia coli (Cohen, 1951a), Pseudomonas aeruginosa and fluorescens (Claridge & Werkman, 1954a; Harrod & Wood, 1954), Streptococcus faecalis (Sokatch & Gunsalus, 1954) and in yeast (Sable & Guarino, 1952).

The general type of reaction catalysed by kinases is

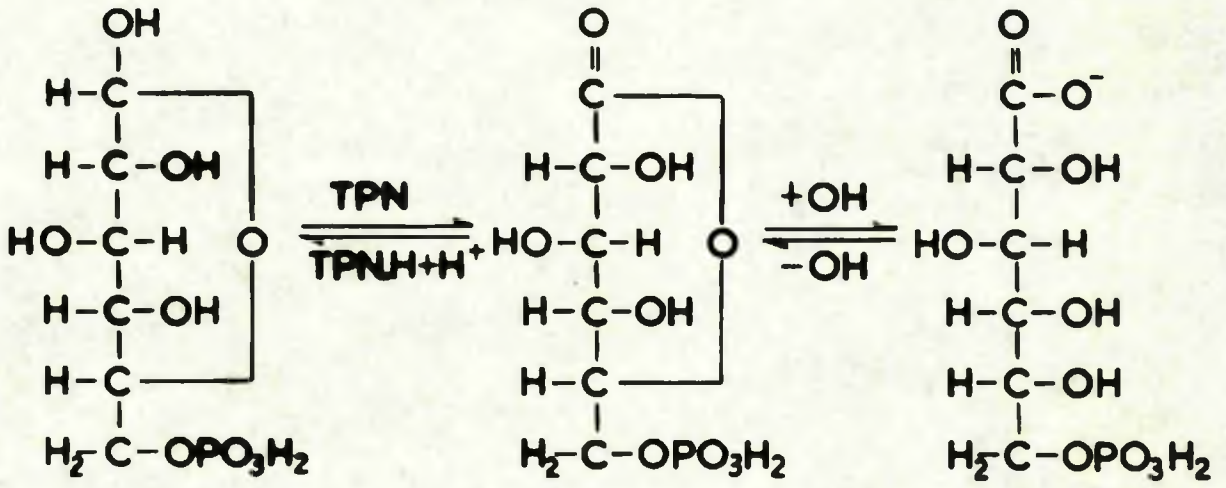
$$\text{Substrate} + \text{ATP} \xrightarrow{\text{kinase}} \text{Substrate} - \text{phosphate} + \text{ADP}$$

and it is this reaction which is presumed to take place in bacteria but it should be emphasised that not many studies on purified bacterial kinases have been attempted. Despite this lack of data, it is generally assumed that kinases are both widespread and active in micro-organisms.

(ii) Glucose-6-phosphate dehydrogenase (G-6-P dh). The first step of HMP is the dehydrogenation of G-6-P. The earlier history of this enzyme has been mentioned above; the former name of "Zwischenferment" has now been replaced by the systematic term "glucose-6-phosphate dehydrogenase". The hydrogen acceptor is TPN and Cori & Lipmann (1952) have shown that it occurs in two stages. These are the dehydrogenation step which yields 6-phospho-5-gluconolactone which is subsequently hydrolysed to 6-phosphogluconate (6-PGA) as shown in fig.3. The hydrolysis occurs slowly but may be catalysed by a specific

Figure 3.

Oxidation of Glucose-6-phosphate



lactonase which has been demonstrated in Azotobacter vinelandii for δ-gluconolactone (Brodie & Lipmann, 1954, 1955). G-6-P dh has been shown to have a widespread distribution and has been found in many micro-organisms including several algae (Cohn, 1960), Aerobacter cloacae (De Ley, 1955), Aspergillus niger (Jagannathan & Singh, 1954), Azotobacter vinelandii (Mortenson & Wilson, 1954a, 1954b), Bacillus brevis, megatherium and subtilis (Dedonder & Noblesse, 1953; De Ley, 1955; Marquet & Dedonder, 1955), Corynebacterium creatinovorans (Ghiretti & Barron, 1954), Escherichia coli (Scott & Cohen, 1951a, 1951b, 1953), Leuconostoc mesenteroides (De Moss et al., 1953; De Moss, 1953, 1954), Neurospora crassa (Tissières et al., 1953; Strauss & Pierog, 1954), Pseudomonas aeruginosa, fluorescens, lindneri and saccharophila (Claridge & Workman, 1954; De Moss & Gibbs, 1952; Entner & Doudoroff, 1952; Wood & Schwerdt, 1954), Penicillium chrysogenum (Koffler, 1953), Saccharomyces cerevisiae (Dickens, 1938; Glaser & Brown, 1955; Warburg et al., 1935), Streptomyces coelicalor and scabies (Cochrane et al., 1953) and Streptococcus faecalis (Schatch & Gunsalus, 1954). All these systems can use TPN as hydrogen acceptor but several can also use DPN. Of these, some are non-specific in their coenzyme requirements (e.g., Leuconostoc mesenteroides), while others (e.g., Pseudomonas fluorescens) are specific for TPN but contain pyridine nucleotide transhydrogenase which allows the system to be linked to DPN as long as some TPN is present (Colowick et al., 1952).

(iii) 6-Phosphogluconate dehydrogenase (6-PGA dh). The second dehydrogenation in HMP is of 6-PGA. The hydrogen acceptor is TPN and, in 1936, Lipmann showed the reaction to be an oxidative decarboxylation and suggested that the product might be arabinose-5-phosphate. Dickens (1938a, 1938b) argued that ribose-5-phosphate (R-5-P) was the product in yeast as this compound is further metabolized. Scott & Cohen (1951a,b) supported the role of R-5-P and Horecker & Smyrniotis (1950) isolated this compound after the enzymic degradation of 6-PGA. Theories of enol or enediol formation to account for the epimerization of C-atom 3 of glucose were abandoned when Horecker et al. (1951) isolated the primary product of 6-PGA dh action as the keto pentose, ribulose-5-phosphate (Ru-5-P). The appearance of R-5-P is due to the presence of pentose phosphate isomerase (R-5-P iso). In addition, many extracts are thought to contain phosphoketopentoepimerase (Ru-5-P epi) which catalyses the interconversion of Ru-5-P and xylulose-5-phosphate (Xu-5-P) so that the 5-C units produced from 6-PGA can be an equilibrium mixture of at least three pentoses (Dickens & Williamson, 1955; Horecker & Smyrniotis, 1956; Hochester, 1955; Hurwitz, 1956; Srere et al., 1955; Stumpf & Horecker, 1956). The equilibria seem to favour R-5-P with Xu-5-P being present in the least amount. The position of Ru-5-P as the primary product of 6-PGA dh has led to the suggestion that oxidation might yield 3-keto-6-phosphogluconate (3-K-6-PGA),

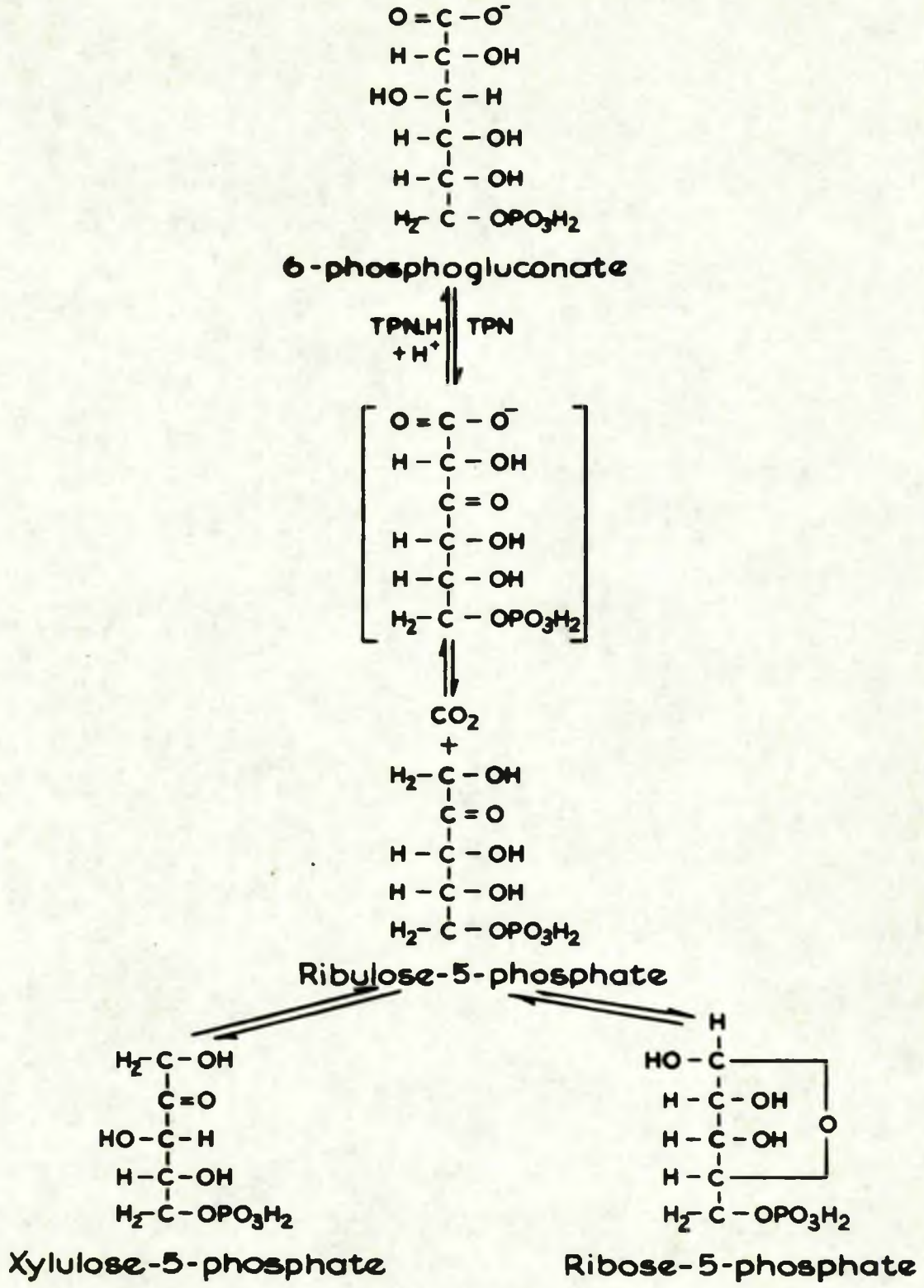
but this intermediate has never been isolated. Consequently, it may be assumed as a working hypothesis that 6-PGA dh has the dual functions of dehydrogenation and decarboxylation. The action of this enzyme, including the hypothetical intermediate, and the subsequent isomerization and epimerization are shown in fig.4.

The equilibrium of 6-PGA is in favour of decarboxylation but the reaction has been shown to be reversible by $^{14}CO_2$ fixation into C-1 of 6-PGA and by the reductive carboxylation of Ru-5-P with TPNH and CO_2 (Horecker & Smyrniotis, 1952). On the other hand, the equilibrium of hydrolysis of 6-phospho-5-gluconolactone favours the free acid and it does not seem possible that hexoses could be formed from pentoses by reversal of this pathway. Any evidence for reversal which has been brought forward depends on the use of unphysiological values of pH which favour the stability of the lactone.

Not much is known regarding the distribution of pentose phosphate isomerases and epimerases but they are assumed to be widespread. 6-PGA dh has been found in several micro-organisms including several algae (Cohen, 1950), Aerobacter cloacae (De Ley, 1955), Azotobacter vinelandii (Mortenson & Wilson, 1954), Bacillus brevis, megatherium and subtilis (Dedonder, 1953; De Ley, 1955), Corynebacterium creatinovorans (Ghiretti & Barron, 1954), Escherichia coli (Scott & Cohen, 1951a, 1951b, 1953), Leuconostoc mesenteroides (De Moss, 1953, 1954), Neurospora

Figure 4.

Oxidation and Decarboxylation of 6-phosphogluconate



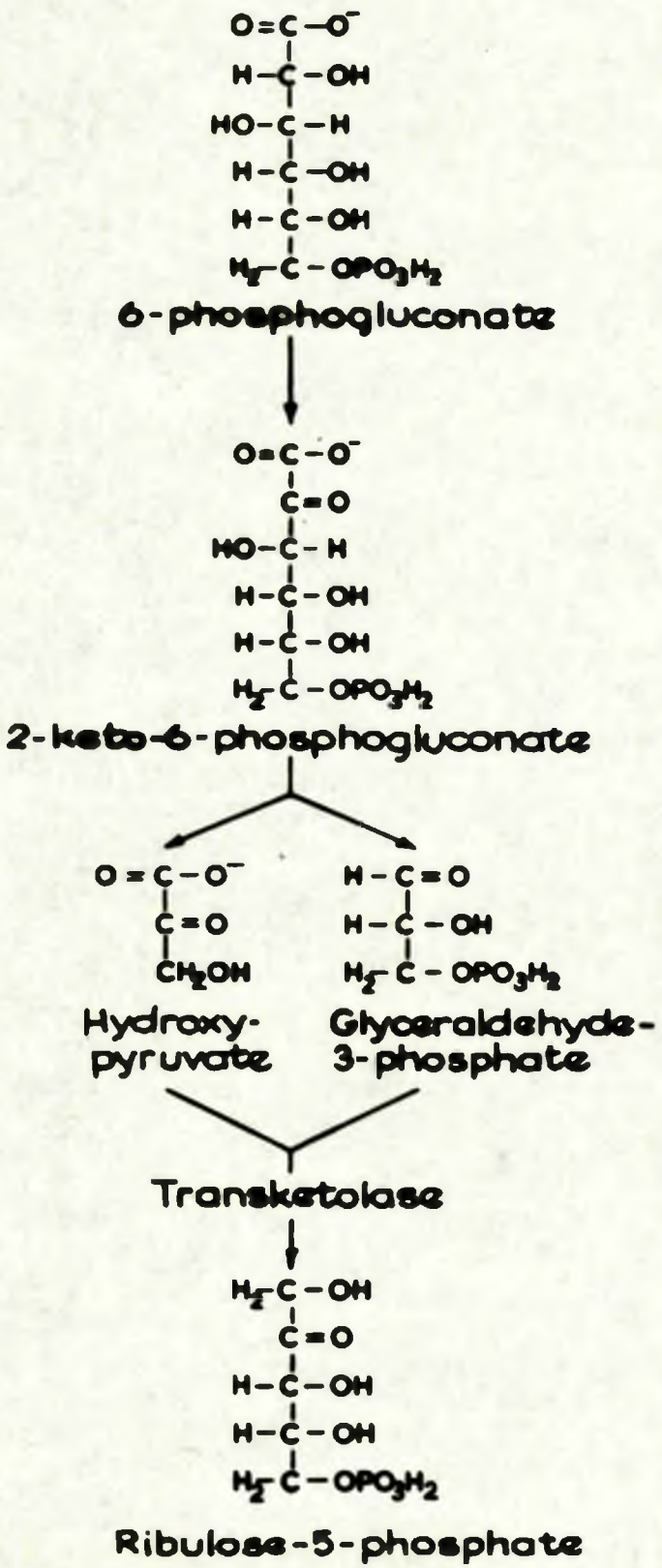
crassa (Tissières et al., 1953), Pseudomonas fluorescens (Wood & Schwerdt, 1954), Penicillium chrysogenum (Koffler, 1953), Streptomyces coelicolor and scabies (Cochrane et al., 1953).

Many workers have been inspired to suggest keto-derivatives as intermediates in 6-PGA decarboxylation. The greatest departure from the accepted views was made by Uehara (1952) whose suggestions are shown in fig.5. He postulated the formation of 2 keto-6-phosphogluconate (2-K-6-PGA) which could then be split to hydroxypyruvate and 3-phosphoglyceraldehyde. As it is known that in the operation of the enzyme transketolase (TK) hydroxypyruvate can act as a 2-C donor and 3-phosphoglyceraldehyde as a 2-C acceptor, it is possible that Ru-5-P may be synthesized by this route. These postulates are considered unlikely because 2-K-6-PGA has not been isolated from systems synthesizing Ru-5-P and also because hydroxypyruvate and glyceraldehyde-3-phosphate cannot act as Ru-5-P in yeast preparations (Horecker, 1954).

(iv) Transketolase (TK). The further metabolism of the five carbon fragments produced by 6-PGA dh and pentose isomerases has been noted in yeast (Dickens, 1938a; Sable, 1955) and in micro-organisms (Bergman et al., 1954a, 1954b; Marmur & Schlenk, 1951; Waldvogel & Schlenk, 1947). Although a TPN requirement for R-5-P oxidation has been reported (Sable, 1952) and although Dickens (1938) suggested the oxidative formation of phosphopentonic acid, it is now accepted that the pentose

Figure 5.

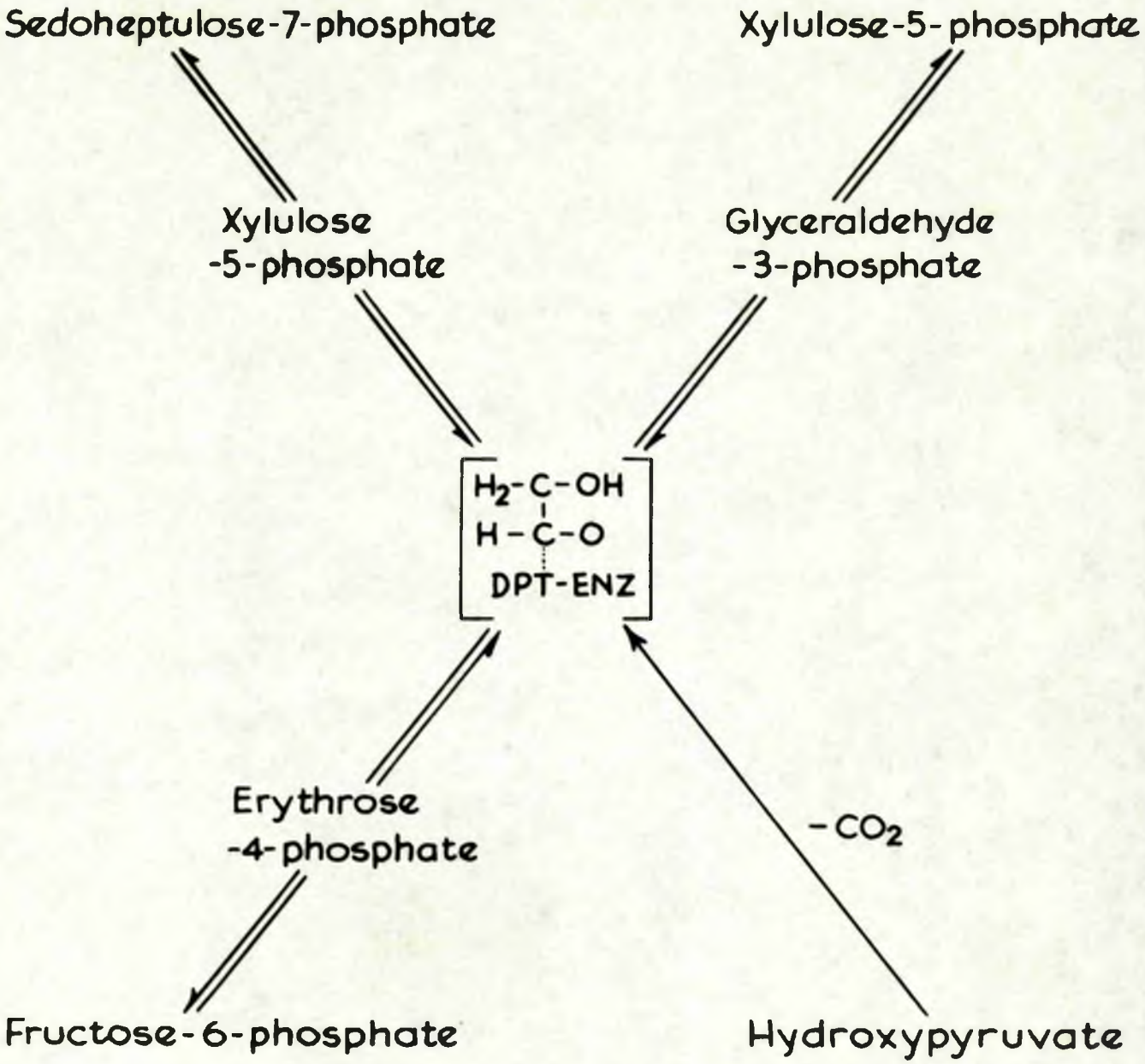
Oxidation of 6-phosphogluconate according to Uehara (1952)



phosphates are only oxidized after non-oxidative intra-molecular rearrangements have taken place (Glock, 1952). Triosephosphate was detected as a product of pentosephosphate metabolism by bacteria and yeast (De La Haba & Racker, 1952; Marmur & Schlenk, 1951; Sable, 1952). The enzyme responsible for the initial pentosephosphate cleavage is transketolase (TK). This enzyme has been found in yeast (Racker et al., 1953) and bacterial systems (Gunsalus et al., 1955) and is presumed to be the cause of sedoheptulose-7-phosphate formation in Acetobacter suboxydans (Hauge et al., 1954), Corynebacterium creatinoverans (Ghiretti & Barron, 1954), Microbacterium lacticum (Vandemark & Wood, 1956), Pseudomonas hydrophila (Hochester, 1955), Pseudomonas fluorescens (Wood & Schwerdt, 1954) and Rhodospirillum rubrum (Benson et al., 1952). The enzyme has been obtained in a highly purified state from both liver and spinach and was found to contain diphosphothiamine (DPT) as a tightly bound prosthetic group (Horecker & Smyrniotis, 1953; Horecker et al., 1953; Racker et al., 1953). Several reactions are catalysed by TK but all involve the transfer of a two-carbon fragment which acts as an activated glycolaldehyde. The principal transferase actions of TK are shown in fig.6. TK, obtained from yeast in a crystalline state by De La Haba et al. (1955), appeared to use Ru-5-P as a source of "active glycolaldehyde" which could be transferred to a number of acceptor aldehydes. DPT and Mg^{++} functioned as cofactors.

Figure 6.

Reactions of Transketolase



It is now known (Srere et al., 1955) that xylulose-5-phosphate (Xu-5-P) rather than Ru-5-P takes part in TK action and the earlier suggestion resulted from the lack of knowledge concerning the pentose phosphate isomerases and epimerases which are listed above. The action of these enzymes meant that the "Ru-5-P" used in the earlier work was a mixture of R-5-P, Ru-5-P and Xu-5-P. In passing, it is interesting to note that the discovery of TK solves the long outstanding problem of the origin of the seven-carbon sugar sedoheptulose-7-phosphate (S-7-P) (fig.7).

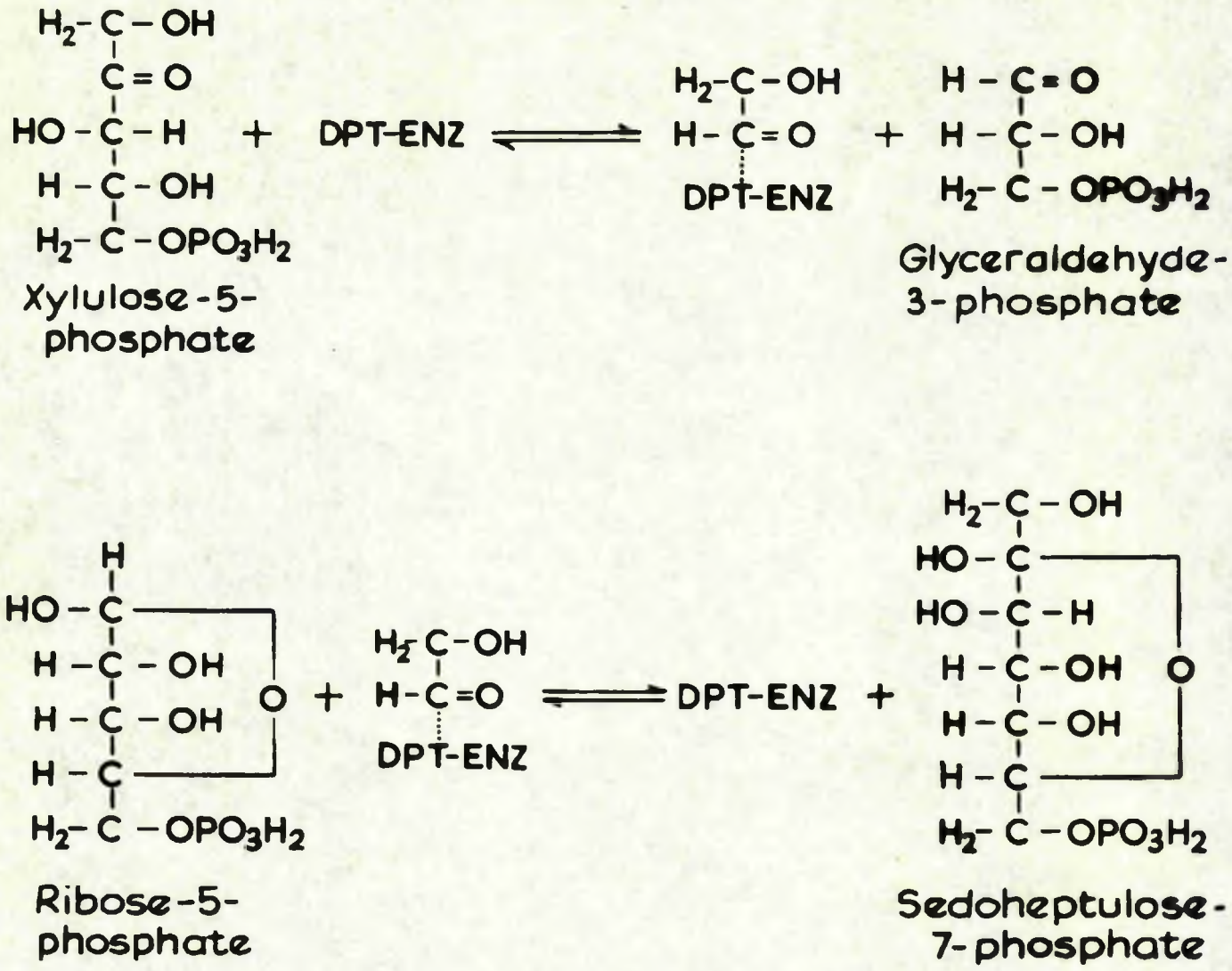
(v) Transaldolase (TA). The S-7-P formed by the action of TK acts as a substrate for another enzyme, transaldolase (TA), which produces six-carbon fragments. A 400-fold purification of TA from yeast has been achieved by Horecker & Smyrniotis (1955) who have not been able to show any cofactor requirements. For the reaction:-

Sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate $\rightleftharpoons$ erythrose-4-phosphate + fructose-6-phosphate

Horecker et al. (1955) suggested that the 4-C fragment was erythrose-4-phosphate (E-4-P) because of its reaction with "active glycolaldehyde" from the TK system to yield fructose-6-phosphate (F-6-P). The above reaction is supported by evidence obtained regarding the labelling of F-6-P formed by yeast preparations from S-7-P and labelled glyceraldehyde-3-phosphate.

Figure 7.

Formation of Sedoheptulose-7-phosphate



Little is known regarding the distribution of TA in micro-organisms, but it is generally considered to accompany TK, especially in those systems where S-7-P is produced and then metabolized further.

(b) The hexosemonophosphate oxidative reactions as a cycle.

The various reactions described in the preceding section may form a cycle which allows for the complete oxidation of glucose (as G-6-P) to carbon dioxide and water. It is perhaps convenient to divide these reactions into two phases - the first being the oxidative reactions which lead to the formation of pentose phosphates (fig.8) and the second being the rearrangement of the carbon chain of these molecules (fig.9). In the diagrams the enzymes are numbered:-

1. Hexokinase
2. G-6-P dh
3. 6-PGA lactonase
4. 6-PGA dh
5. R-5-P iso
6. Ru-5-P epi
7. TK
8. TA.

The products of these enzymatic reactions may be converted to G-6-P by known routes. In this case the overall reaction is:-

Figure 8.

Formation of Pentose Phosphates by Hexosemonophosphate oxidative route

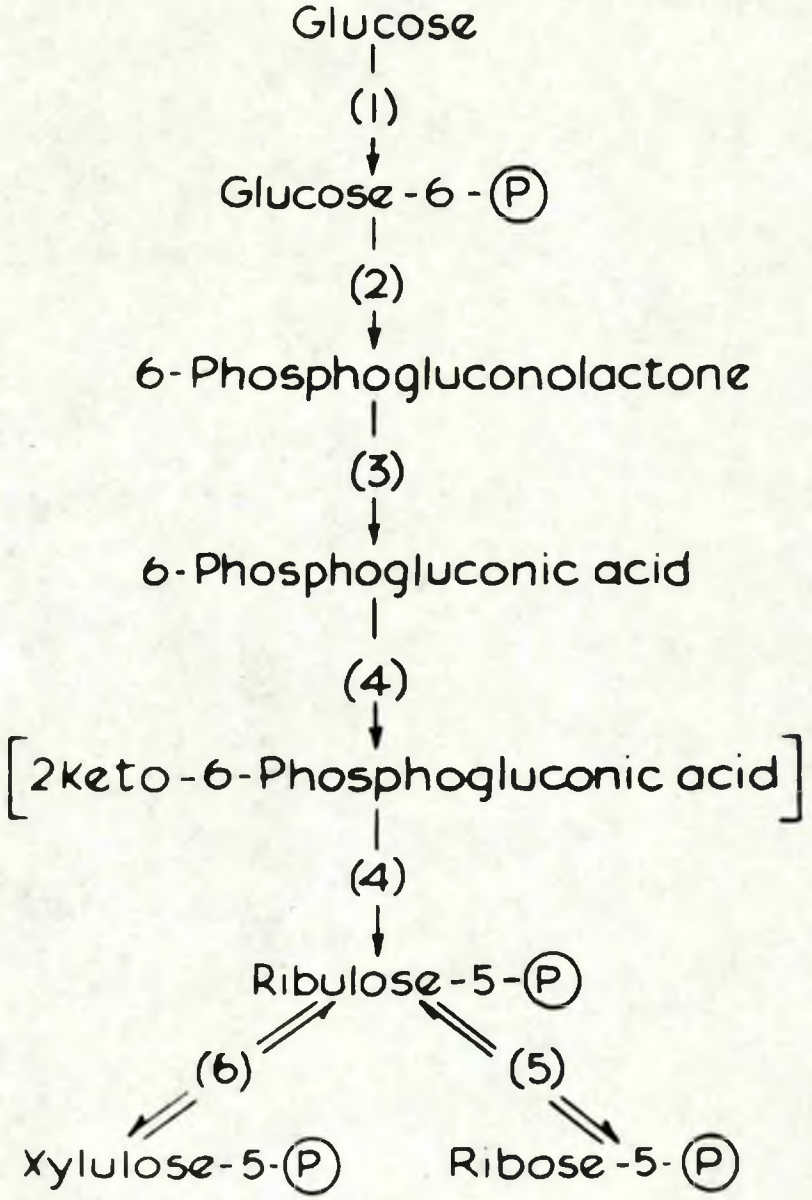
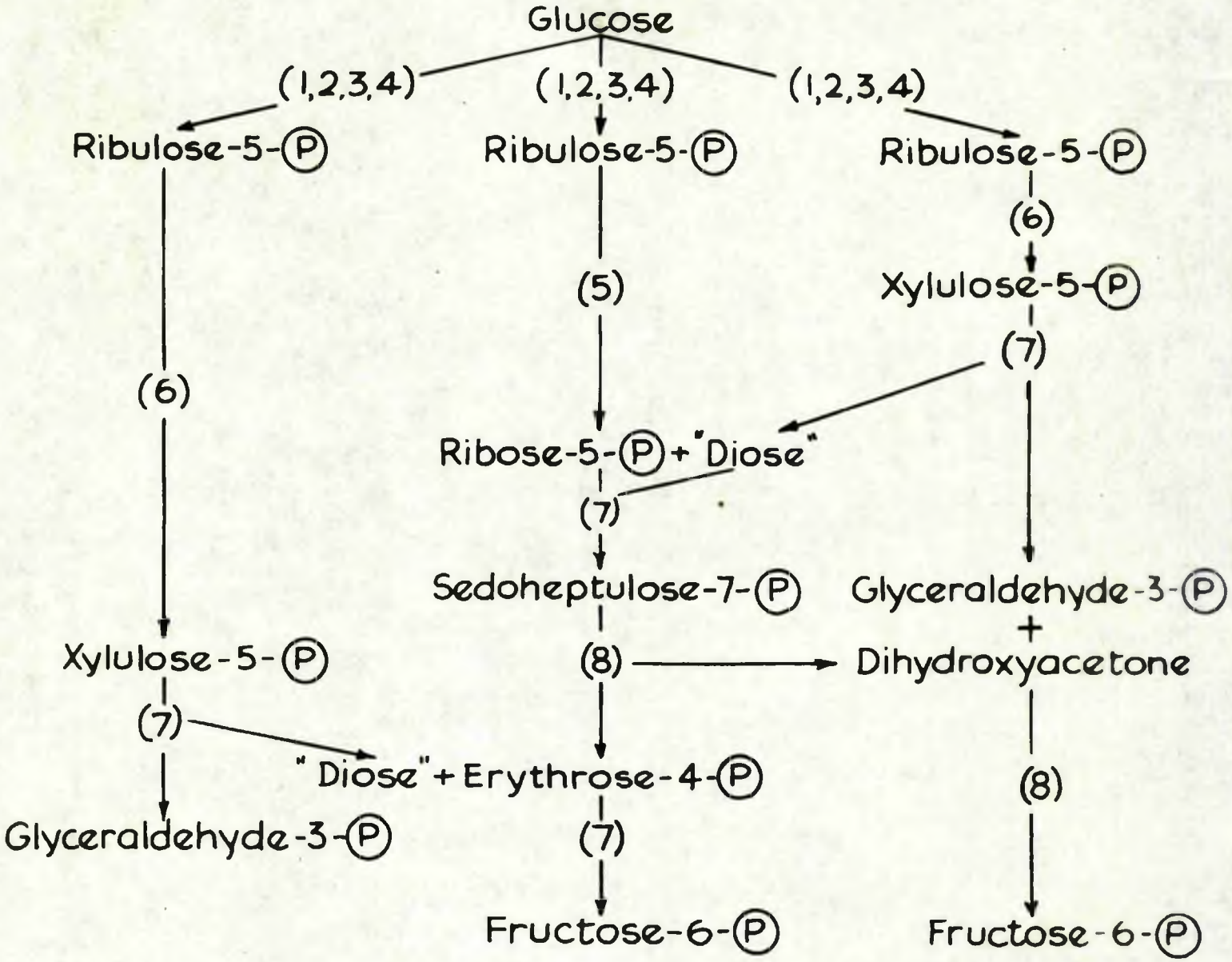


Figure 9.

Utilization of Pentose Phosphates by Hexosemonophosphate Oxidative Route.



$$3 \text{ glucose} \longrightarrow 2\tfrac{1}{2}\ \text{G-6-P} + 3CO_2 + 3H_2O.$$

The G-6-P is then available for a further turn of the cycle. It must be emphasized that it is not at all clear if this cycle ever actually operates as such and many variations of this basic scheme can be visualised. For example, the triose portion could be converted to pyruvate and thence to carbon dioxide and water via the Krebs' Cycle. In addition to possible variations in the further metabolism of the fructose-6-phosphate and glyceraldehyde-3-phosphate, the coupling of the enzymic reactions can be achieved in different ways, but each step of the sequence shown has experimental support. To summarize, then, the main features of HMP are as follows:-

1. Dehydrogenations are linked to TPN.
2. Carbon dioxide is evolved by fission of the C-6 chain to yield C-5 units.
3. The C-5 units are catabolized by non-oxidative methods and are converted to C-6 units by a series of steps involving C-3, 4 and 7 units.
4. The C-6 units formed in this way are then available for a further turn of the cycle.

VII. THE ENTNER-DOUDOROFF SYSTEM.

Entner & Doudoroff (1952) noted a preferential release of $^{14}CO_2$ from glucose-1-^{14}C by a washed cell suspension of *Pseudomonas saccharophila* ~~fluorescens~~. They had previously discovered that:-

1. intact cells assimilated almost two-thirds of the carbon of glucose, pyruvate or lactate,
2. the α and β-carbon atoms of pyruvate or lactate were assimilated, while the carboxyl group appeared almost entirely as CO_2, and
3. when assimilation was inhibited with dinitrophenol (DNP), pyruvic acid accumulated as glucose was oxidised.

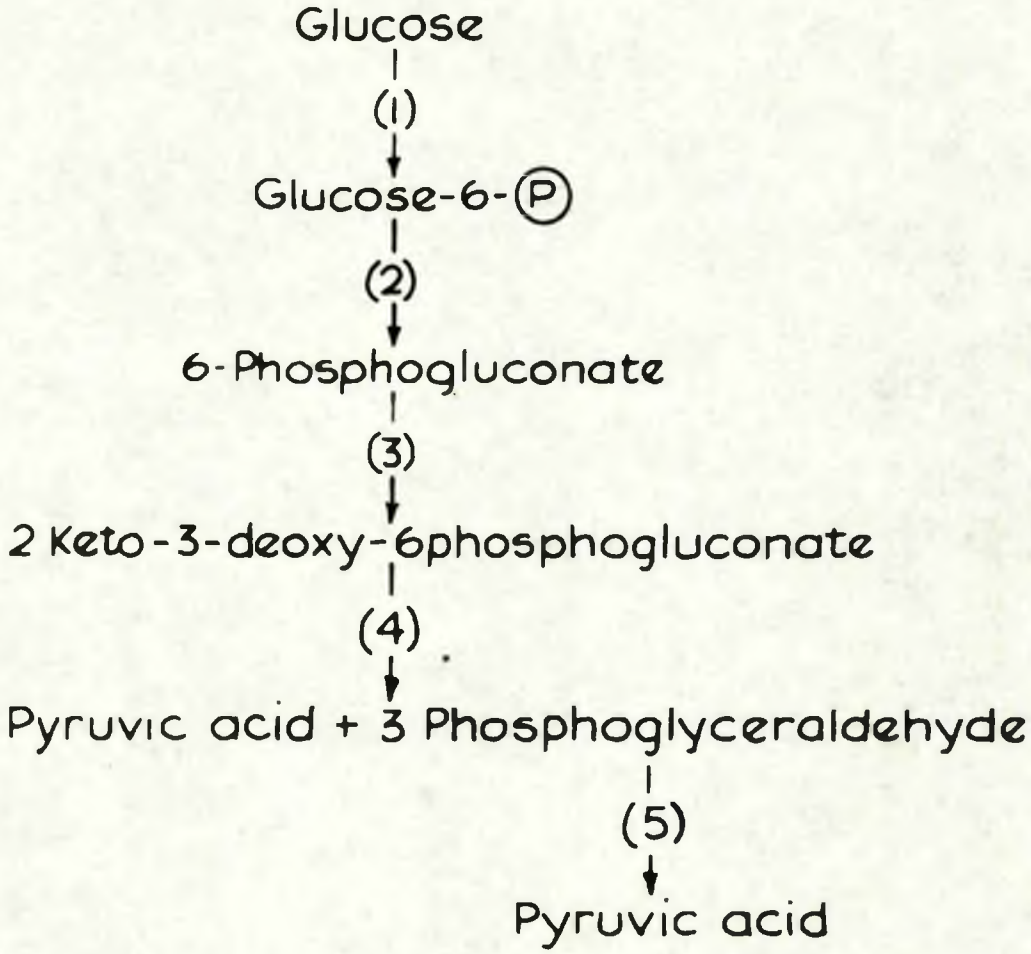
From these data they suggested that the glucose is split to two 3-carbon fragments before oxidative assimilation takes place, and on the basis of the labelling of pyruvate isolated from systems metabolizing glucose-1-^{14}C they suggested the scheme shown in fig.10. The enzymes are numbered as follows:-

1. Hexokinase
2. G-6-P dh and 6-PGA lactonase
3. 6-PGA dehydrase
4. 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase)
5. Enzymes as in EMP.

They were later able to isolate (MacGee & Doudoroff, 1954) the new intermediate, 2-keto-3-deoxy-6-phosphogluconate (KDPG) and were also able to demonstrate the new enzymes, 6-PGA dehydrase

Figure 10.

Dissimilation of Glucose by the Entner-Doudoroff Scheme



and KDPG aldolase, and show that KDPG was highly specific for KDPG aldolase. Kovachevich & Wood (1955a, 1955b) have purified the enzymes from bacterial extracts. 6-PGA dehydrase requires ferrous ions (4×10^{-3}M) and glutathione (10^{-2}M) for maximal activity and these requirements can be partially met by Mn^{++} or Mg^{++}, and cysteine or thioglycollate respectively. No cofactors could be found for KDPG aldolase. In a limited survey these authors detected 6-PGA dehydrase and KDPG aldolase in Aerobacter melanogenum and suberydans, Escherichia coli and Proteus vulgaris as well as in Pseudomonas aeruginosa, fluorescens and fragi. It is interesting to note that these enzymes have not been shown in Gram-positive bacteria, but, where they occur, their distribution indicates that they could be quantitatively significant for glucose utilization in that there is usually sufficient KDPG aldolase to utilize all the output from the concomitant 6-PGA dehydrase.

VIII. THE DIRECT OXIDATION OF SUGARS.

(a) Non-phosphorylated oxidation.

(1) Substrates and products. Many organisms of the Pseudomonas and Acetobacter genera oxidize glucose without prior phosphorylation. Katznelson, Tanenbaum & Tatum (1953) found that aged cells and extracts of Acetobacter melanogenum oxidize glucose to give, in succession, gluconate, 2-ketogluconate and 2,5-diketogluconate. Other systems do not carry the oxidation so far but yield gluconate, 2-ketogluconate or 5-ketogluconate, or a mixture of these compounds (Bernhauer & Knobloch, 1938; Bernhauer & Riedl-Tumova, 1950; Butlin 1938; Claridge & Workman, 1953; Kluyver & Boezaardt, 1938; Koepsell et al., 1952; Kulka & Walker, 1954; Lockwood et al., 1941; Norris & Campbell, 1949; Ramakrishnan & Campbell, 1955; Stokes & Campbell, 1951; Stubbs et al., 1940).

In a similar way pentoses can be oxidized to pentonic acids (Bertrand, 1898a, 1898b; Lockwood & Nelson, 1946) and disaccharides to bionic acids (Kluyver et al., 1950; Stodola & Lockwood, 1947). From glucose the Pseudomonads generally produce 2-ketogluconate but Acetobacter sp. yield 2-ketogluconate, 5-ketogluconate or both. The further metabolism of the acids can be minimized by adjusting the nitrogen content (Koepsell, 1950; Kondo & Takeda, 1952) or by reducing the iron content of the medium (Koepsell, 1950). Where further

metabolism has been observed 2-ketogluconate yields, besides carbon dioxide and water, acetate (Campbell et al., 1949) or pyruvate and ∝-ketoglutarate (Koepsell et al., 1952; Lockwood & Stodola, 1946; Warburton et al., 1951). Fractionation following the metabolism of labelled 2-ketogluconate has yielded labelled amino acids, TCA intermediates, lactate, pyruvate, dihydroxyacetone, G-6-P and a nucleic acid (Claridge & Werkman, 1954). Less is known of the oxidation of 5-ketogluconate but a Congress abstract of 7 years' standing lists tartrate, glyoxalate, oxalate and formate as products (Jackson et al., 1949).

(ii) Evidence against participation of phosphate. Despite the evidence of the isolation of gluconate and 2-ketogluconate it is possible to imagine the oxidations occurring on phosphorylated derivatives with a subsequent dephosphorylation. Evidence which makes this a most unlikely supposition has been put forward by several authors. Campbell & Morris (1950) could not extract any hexosephosphates from Pseudomonas aeruginosa metabolising glucose. With the same organism Stokes & Campbell (1951) found insensitivity of oxygen uptake to M/18 sodium fluoride and no stimulation by ATP, which result was extended to the purified gluconic dehydrogenase by Ramakrishnan & Campbell (1955). Claridge & Werkman (1953) have separated the system for oxidation via gluconate from phosphorylated pathways in Pseudomonas aeruginosa as have Wood &

Schwerdt (1954) in Pseudomonas fluorescens.

There seems to be little doubt that oxidations of glucose can occur via gluconate without phosphorylation.

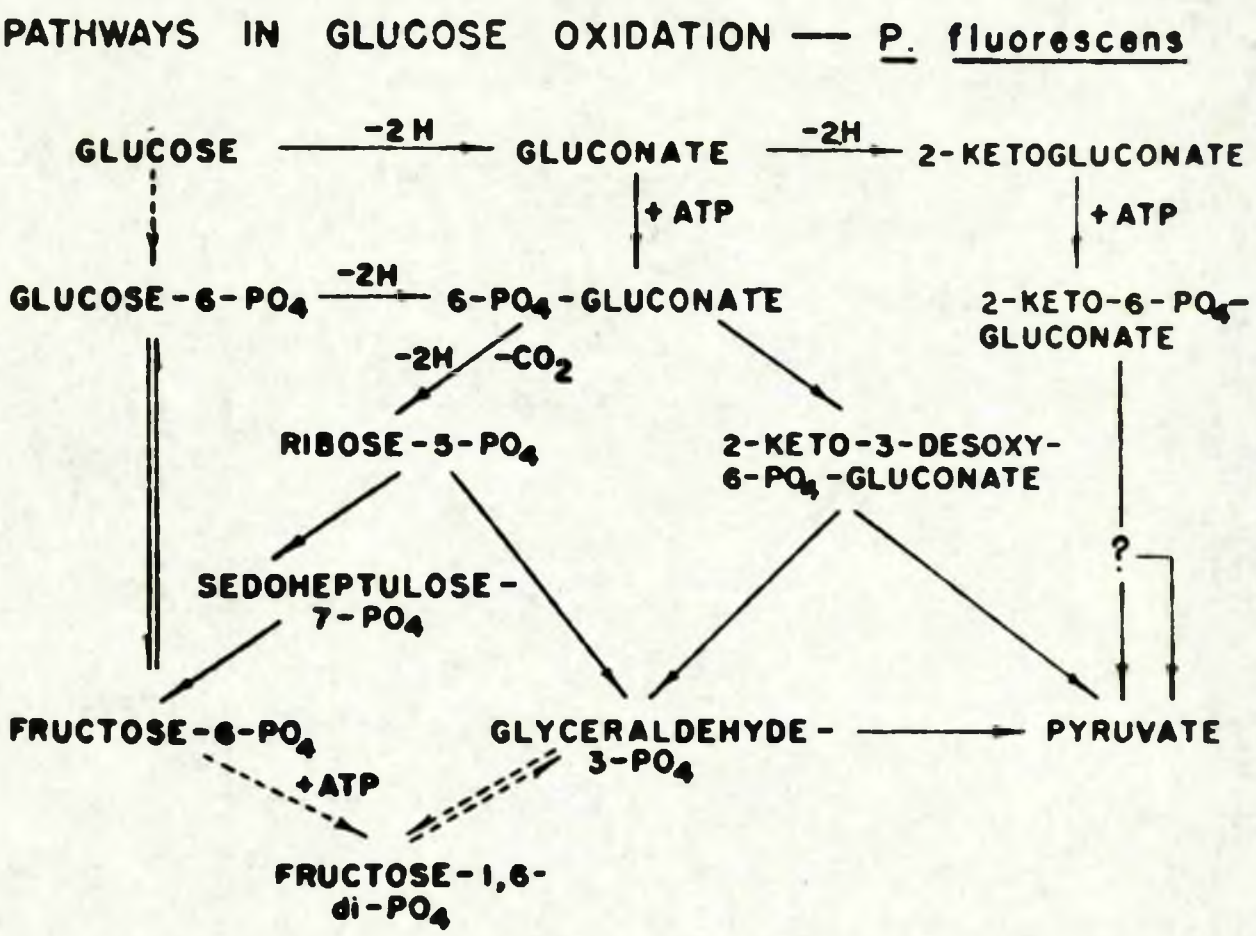
(iii) The position of 2-ketogluconate. By using the techniques of simultaneous adaption, including the blocking of synthesis of adaptive enzymes by ultra-violet irradiation, Entner & Stanier (1951) working with Pseudomonas fluorescens suggested that 2-ketogluconate was formed by an adaptive enzyme but that the glucose dehydrogenase was constitutive. They also suggested that 2-ketogluconate was not on the main pathway of glucose oxidation. Against this may be placed the many examples of cell-free systems oxidizing glucose to 2-ketogluconate and the observations of Claridge & Workman (1953) with Pseudomonas aeruginosa which possesses a constitutive gluconic dehydrogenase. This point is not yet clearly settled.

(iv) Energetics. The direct oxidative system does not furnish useful energy at the substrate level and there is no evidence that the enzymes are of the pyridine nucleotide-linked glucose dehydrogenase type as found in mammalian systems or of the flavoprotein type of glucose oxidase found in moulds. However, a cytochrome system mediates in hydrogen transport which could yield useful energy by the processes of oxidative phosphorylation and this system has been studied (Wood & Schwerdt, 1953) and is associated with particles obtained on cell disruption.

(b) The relation of the direct to phosphorylated pathways.

The discovery of specific kinases for gluconate and 2-ketogluconate (De Ley, 1953; Marrod & Wood, 1954) has indicated the linkage of the direct route to phosphorylated routes. The position in extracts of Pseudomonas fluorescens as seen by W.A. Wood (1955) is shown in fig.11. Phosphorylation of gluconate yields entry into both HMP and EDS (Ch. VI & VII) but the mechanism of degradation of 2-keto-6-phosphogluconate is more obscure. It is interesting to note that, in this particular organism, all of the glucose is metabolised via the direct pathway because of the lack of hexokinase.

Figure 11.



Pathways of glucose oxidation in *Pseudomonas fluorescens*.

IX. QUANTITATIVE EVALUATION OF THE PATHWAYS OF GLUCOSE METABOLISM.

(a) Carbon atom distribution by different pathways.

Figs. 12, 13 and 14 illustrate the contribution of the individual carbon atoms of glucose to the carbon chains of the products formed by glycolysis (or Embden-Meyerhof-Parnas pathway, EMP) by the hexosemonophosphate oxidative pathway (or hexosemonophosphate shunt, HMPS) and by the 2-keto-3-deoxy-6-phosphogluconate pathway (or Entner-Doudoroff system, EDS). These systems differ in several respects:-

(i) Oxygen requirement. The EMP will dissimilate glucose anaerobically to glyceraldehyde-3-phosphate (G-3-P). Further catabolism by this pathway yields reduced diphosphopyridine nucleotide (DPNH) which may be reoxidized at a later stage during the formation of ethanol or lactic acid. The HMPS and the EDS on the other hand are primarily activated (subsequent to the phosphorylative step) by the triphosphopyridine nucleotide (TPN) linked oxidation to 6-phosphogluconate (6PGA). If the later products of these pathways undergo reactions which regenerate oxidized coenzymes the systems can operate anaerobically, e.g., as in Leuconostoc mesenteroides (Bernstein et al., 1955; Gunsalus & Gibbs, 1952; Horecker et al., 1954) and in Pseudomonas lindneri (Gibbs & DeMoss, 1954). In general, however, both the HMPS and the EDS are considered to be aerobic

Figure 12.

Distribution of carbon atoms of glucose in glycolysis.

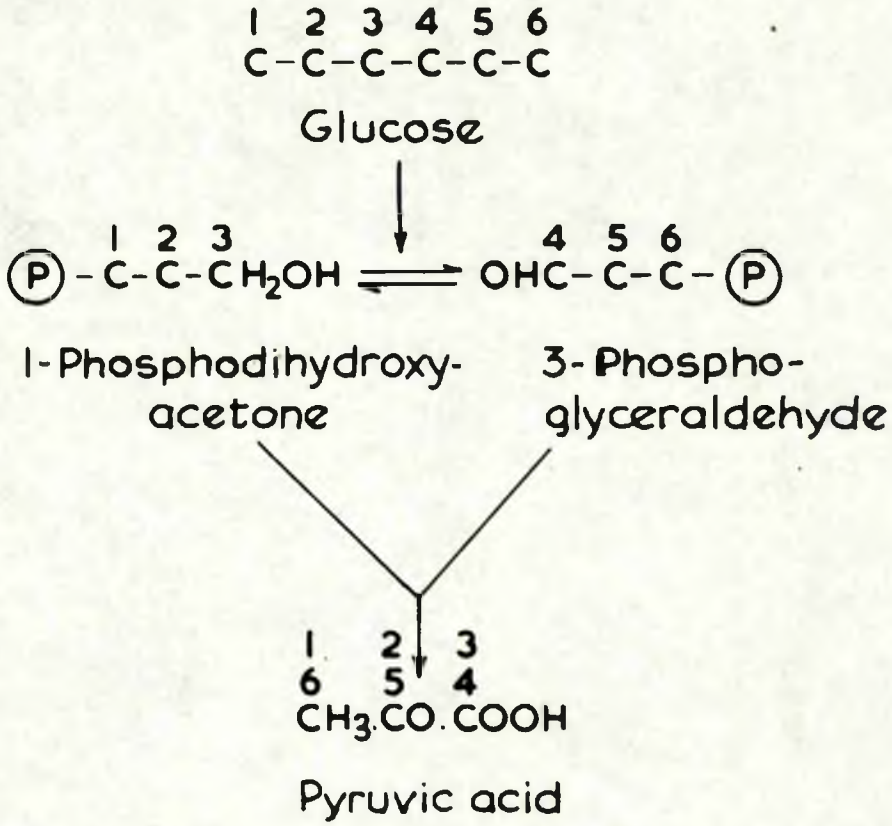
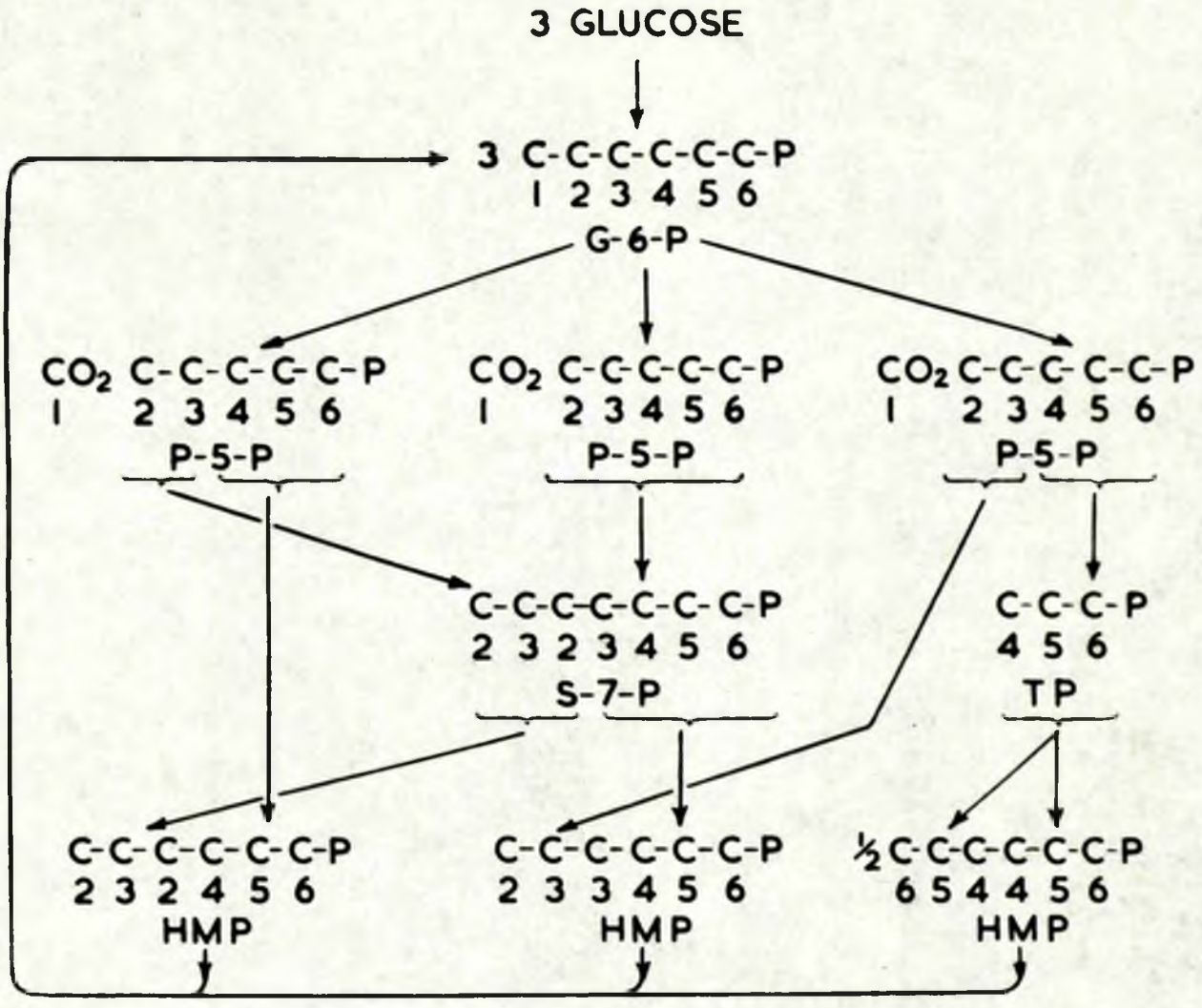


Figure 13.

Distribution of glucose carbon atoms in the products of the hexosemonophosphate oxidative cycle. [*After H.G. Wood (1956)*]

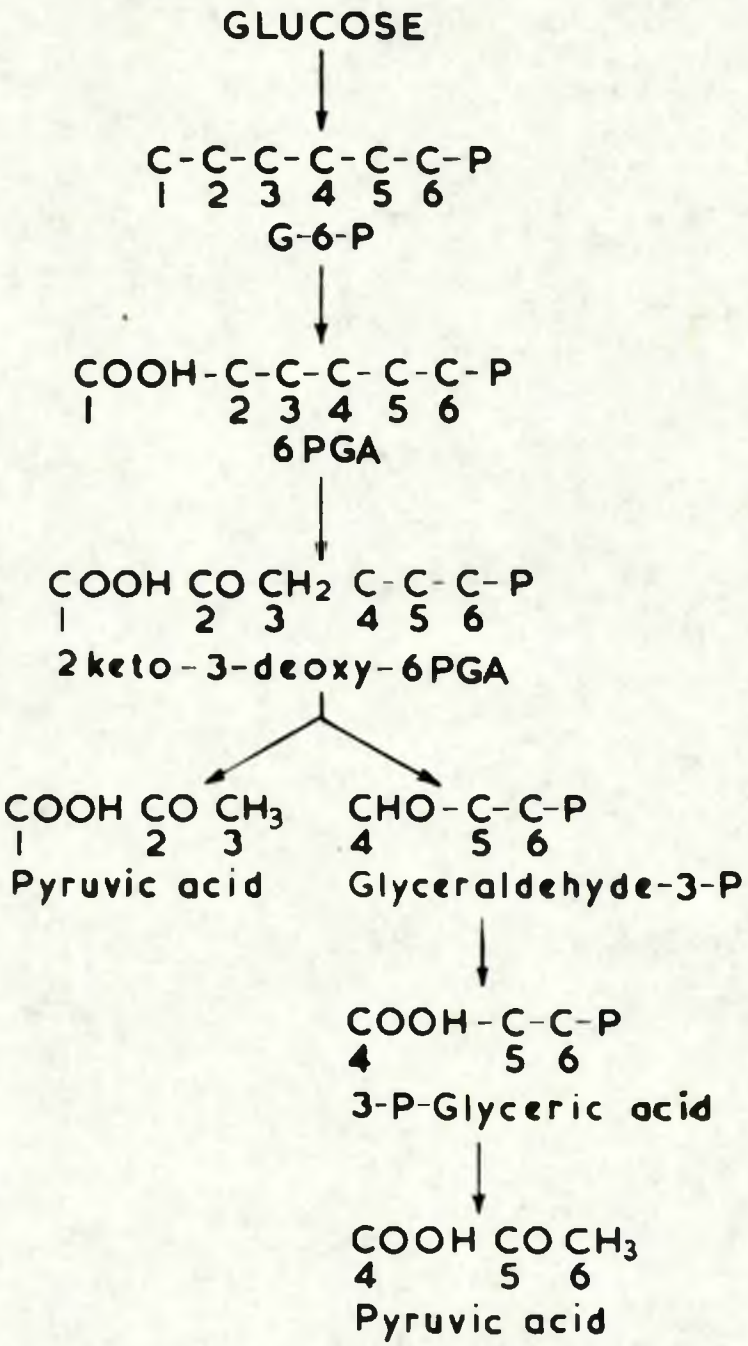


Overall Reaction:-

$$3\ \text{GLUCOSE} \longrightarrow 3CO_2 + 2\tfrac{1}{2}\ \text{HMP}$$

Figure 14.

Distribution of glucose carbon atoms in the products of the phosphogluconate fermentation.



pathways.

(ii) Carbon dioxide production. Following the EMP route carbon dioxide is liberated in the first place from the carboxyl groups of pyruvate by decarboxylation. These carbon atoms represent C-3 and 4 of the original glucose and may be followed by the liberation of C-2 and 5 and C-1 and 6 if the Krebs' cycle (TCA) is operating. In the HMPS the C-1 of glucose is first liberated as CO_2 and then the other C-atoms are freed by recyclisation of the system as shown in fig.13, or some may be evolved by the metabolism of C-4, 5 and 6 of the third glucose molecule via pyruvate and the TCA. The EDS also shows a preferential release of CO_2 from the C-1 of glucose (as compared to the EMP) because this C-atom appears in the carboxyl group of one of the pyruvate molecules formed and is therefore evolved together with C-4 but before C-atoms 2, 3, 5 and 6.

(iii) The contribution of glucose C-atoms to products other than carbon dioxide. Inspection of figs. 12, 13 and 14 shows that while the various pathways may lead to the same product, the C-atoms of this product are derived from different C-atoms of the substrate glucose. For example, the methyl group of pyruvic acid may rise from C-1 and 6 (EMP), from C-3 and 6 (EDS), or from C-6 (HMPS) of glucose. The constitution of many other products with reference to the six carbon atoms of the substrate glucose is equally characteristic of the

pathway involved.

(b) Carbon dioxide production as a guide to the relative importance of EMP-TCA and alternative pathways.

(i) The use of glucose-U-^{14}C and glucose-1-^{14}C. Both carbon dioxide production and the isolation of other products may be used to evaluate the incidence of the various pathways. Bloom, Stetten & Stetten (1953) attempted to evaluate the HMPS in rat liver slices by measuring the yield of $^{14}CO_2$ from glucose-U-^{14}C, glucose-1-^{14}C, lactate-1-^{14}C, lactate-2-^{14}C and lactate-3-^{14}C. Consider first the two labelled glucoses:- In the case of the glucose-U-^{14}C,

let a = % of added counts evolved as CO_2

b = % of added counts evolved by HMPS as CO_2

c = % of added counts evolved by EMP-TCA as CO_2

and for the glucose-1-^{14}C,

let d = % of added counts evolved as CO_2

e = % of added counts evolved by HMPS as CO_2

f = % of added counts evolved by EMP-TCA as CO_2,

then,

$$a = b + c \qquad (1)$$

$$d = e + f \qquad (2)$$

Let m be the quantity of CO_2 produced from the C-1 position of glucose and E be the fraction of this CO_2 produced by HMPS. In glucose-U-^{14}C each C atom represents $\frac{1}{6}$ of the activity of

the molecule and in glucose-1-^{14}C, atom C-1 represents all the activity. Assume that CO_2 is formed only from glucose-C-1 by the action of HMPS. Equations (1) and (2) may now be written

$$a = \frac{1}{6}m(1 - E) + X \qquad (3)$$

$$d = m(1 - E) + mE \qquad (4)$$

where X is the % CO_2 production from all the C-atoms of glucose by EMP-TCA. X is not measured experimentally but is eliminated on results obtained by the use of labelled lactate.

$$\frac{X}{mE} = \frac{\%^{14}CO_2 \text{ from glucose-U-}^{14}C \text{ by EMP-TCA}}{\%^{14}CO_2 \text{ from glucose-1-}^{14}C \text{ by EMP-TCA}}$$

$$= \frac{\%^{14}CO_2 \text{ from lactate-U-}^{14}C}{\%^{14}CO_2 \text{ from lactate-3-}^{14}C} \qquad (5)$$

This ratio is based on the assumption that CO_2 production subsequent to glycolysis occurs via pyruvate and the Krebs' cycle and that lactate is also metabolized via pyruvate in this way. In practice, lactate-U-^{14}C is not used but lactate-1-^{14}C and 3-^{14}C used separately are preferred. Let

g = % of added counts evolved as CO_2 from lactate-1-^{14}C

h = % of added counts evolved as CO_2 from lactate-2-^{14}C

i = % of added counts evolved as CO_2 from lactate-3-^{14}C.

The % CO_2 evolved from lactate-U-^{14}C must equal $\frac{g + h + i}{3}$.

Therefore from equation (5):-

$$\frac{X}{mE} = \frac{g + h + i}{3i}$$

and $$X = m\left[\frac{g + h + i}{31}\right] E \qquad (6)$$

Using the value of X from (6) and taking the ratio of equations (3) and (4) -

$$\frac{a}{d} = \frac{\frac{m(1 - E)}{6} + m\left[\frac{g + h + i}{31}\right] E}{m(1 - E) + mE}$$

$$= \frac{1 - E}{6} + \left[\frac{g + h + i}{31}\right] E \qquad (7)$$

As a, d, g, h and i are experimentally observed figures, E may be calculated

$$\frac{a}{d} = \frac{1}{6} - \frac{E}{6} + \left[\frac{g + h + i}{31}\right] E$$

$$= \frac{1}{6} + E\left[\frac{g + h + i}{31} - \frac{1}{6}\right]$$

$$E_{max.} = \frac{\frac{a}{d} - \frac{1}{6}}{\frac{g + h + i}{31} - \frac{1}{6}} \qquad (8)$$

This value of E is the maximum value ($E_{max.}$) because it is assumed that only C-1 is liberated from glucose as CO_2 by HMPS. This means that any CO_2 from C-2, 3, 4, 5 and 6 by HMPS is attributed to EMP-TCA. If it is assumed that these five C-atoms are liberated as CO_2 by HMPS, the maximum contribution of HMPS is assumed and E then represents a minimum value ($E_{min.}$). Under these conditions equation (3) becomes

$$a = m(1 - E) + X \qquad (3a)$$

and equation (8) becomes

$$E_{min.} = \frac{\frac{a}{d} - 1}{\frac{g + h + i}{3i} - 1} \qquad (8a)$$

Bloom, Stetten & Stetten (1953) derived the above equations in a slightly different way by using ratios of radiochemical yields so that

$$\frac{a}{d} = U \qquad \frac{g}{i} = N \qquad \frac{h}{i} = T$$

Substituting these values in (8)

$$E_{max.} = \frac{6U - 1}{2N + 2T + 1}$$

which is the form in which these authors present the derivation (equation (4) of their paper). Wood* (1955) has also derived this equation but on the basis of specific activities. This seems to be a rather unfortunate choice of method as he assumes that all substrates used have the same specific activity and this circumstance rarely obtains in the laboratory.

The significance of $E_{max.}$ and $E_{min.}$ is difficult to assess and it must always be borne in mind that they refer to certain ratios, the factors of which are yields of CO_2 from the C-1 position of glucose. As Wood (1955) points out, "the comparison therefore is between the 1-position via EMP-TCA and the 1-position via the alternate pathway. This almost certainly weights the calculation in favour of the alternate

*Harland G. Wood.

pathway since the 1-position probably is converted exclusively to CO_2 in the alternate pathway whereas via the EMP-TCA it is converted to the methyl group of pyruvate and then must pass through all the reactions of the tricarboxylic acid cycle, and in fact must make the circuit twice, before <u>any</u> of the C-1 becomes $^{14}CO_2$. The alternate pathway probably involves two intermediate compounds (more likely three - W.H.H.) before the 1-position becomes CO_2 and the EMP-TCA approximately twenty intermediate compounds, many of which are interconvertible with fatty acids or amino acids. It is clear that there is great opportunity for loss of ^{14}C other than as CO_2 via the EMP-TCA and, moreover, it is important to note that any labelled glucose that passes into breakdown products other than CO_2 is not included as glucose processed via this pathway." While what this author states carries the weight of his great experience, it must also be borne in mind that a basic assumption in the calculation of $E_{max.}$ was that C-2, 3, 4, 5 and 6 of a glucose molecule metabolized via HMPS do not contribute to CO_2 production. This means that <u>if</u> some of these C-atoms <u>are</u> released as CO_2 their contribution is counted towards the EMP-TCA. As there is no evidence for an accumulation of the subsequent intermediates of HMPS it must be assumed that these five C-atoms are largely released as CO_2. This is bound to raise $E_{max.}$ to a value above that which actually occurs. The

calculation of $E_{min.}$, on the other hand, allows for CO_2 production from these five C-atoms and the value $(1 - E_{min.})$ must, therefore, represent the maximum participation of the alternative pathway. The values obtained in this way are undoubtedly biased too much in favour of the alternative pathway, as was shown in the original paper by Bloom, Stetten & Stetten (1953). When using this equation they found that in liver slices $E_{min.}$ yielded a negative value. It would seem, therefore, that the true value of E lies somewhere between $E_{max.}$ and $E_{min.}$ and that its final evaluation cannot be achieved by this approach.

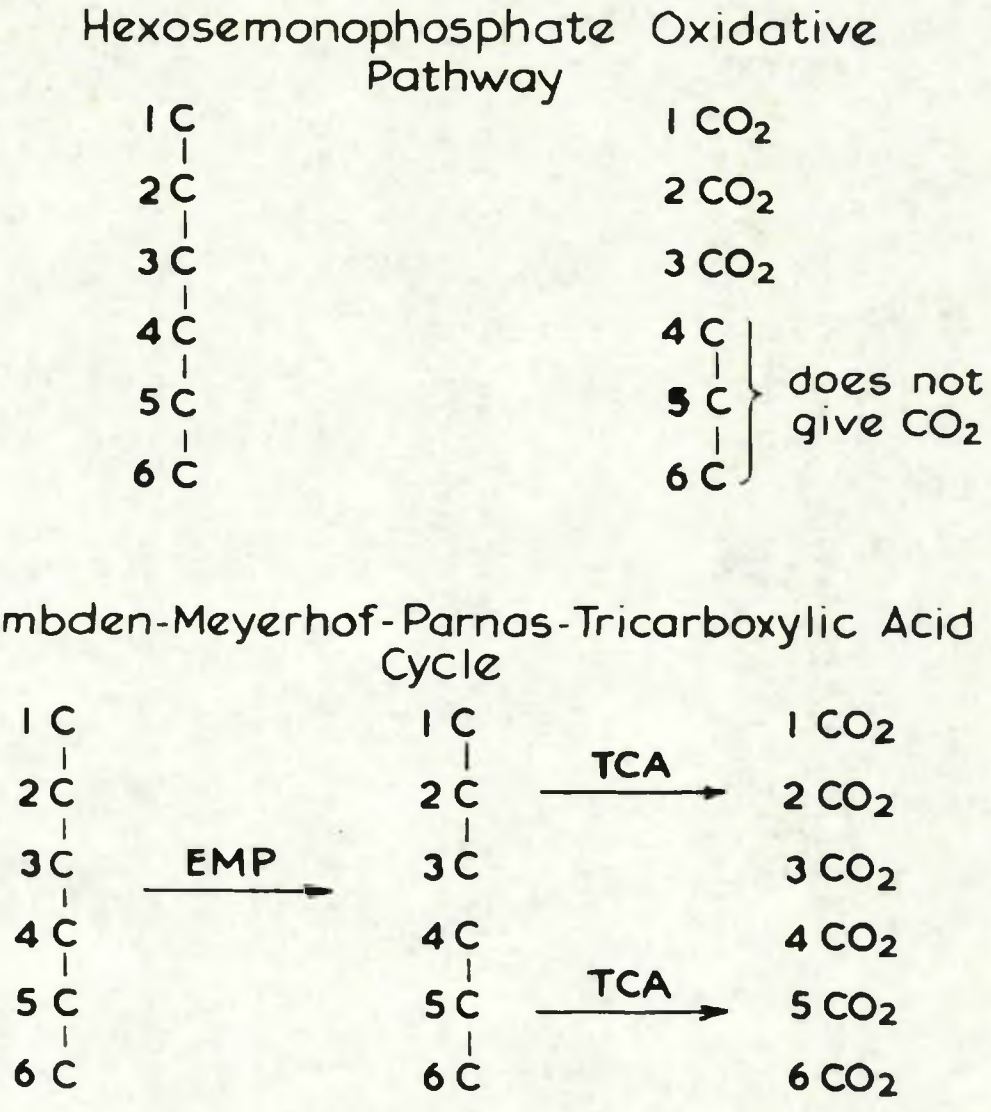
In addition to the above criticisms it must be remembered that alternative pathways other than HMPS may occur, that EMP-TCA and HMPS have intermediates in common which might allow metabolism to occur by a combination of the two routes and, finally, there is the possibility of randomization of the ^{14}C in glucose-1-^{14}C both in EMP-TCA and in HMPS. All these factors cannot be allowed for in the calculation of E and the experimentally obtained values must therefore be regarded as provisional. A somewhat different interpretation of results obtained with glucose-U-^{14}C and glucose-1-^{14}C is included in the discussion.

(ii) <u>The use of glucose-1-^{14}C and glucose-6-^{14}C.</u> Since the publication of the results described in the above section, many authors have described experiments with glucose-1-^{14}C,

glucose-6-^{14}C and with glucose labelled in other positions (Bloom & Stetten, 1953; Katz, Abraham, Hill & Chaikoff, 1954, 1955; Abraham, Hirsch & Chaikoff, 1954, Agranoff, Brady & Colodzin, 1954). The type of calculation employed in these experiments has been described by Korkes (1956). In this review it is pointed out that the carbon atoms of glucose may be randomized by the various steps of HMPS taken separately. This may not be a valid point because the scheme shown in fig.13 may well operate without randomization by virtue of the products of the enzymic reactions being formed in the correct loci required for their metabolism by the subsequent enzyme of the cycle. In addition, Korkes' argument does not allow for the relative rates of the various reactions, but it is nevertheless of value in showing the considerable possibilities for randomization inherent in HMPS. Randomization may be further increased if triosephosphate isomerase, aldolase and hexose diphosphatase were regenerating hexose from the three-carbon fragment. Assuming that no randomization of this type is affecting carbon atoms 4, 5 and 6, and assuming an equal rate of formation of CO_2 from C-atoms 1, 2 and 3 (these assumptions are summarized in fig.15), then the following equations may be derived:

Figure 15.

Assumptions inherent in the evaluation of pathways with glucose -1- and -6-^{14}C.



Let A = specific activity of glucose (counts/min./mole)

S = fraction of CO_2 via HMPS

G = fraction of CO_2 via EMP-TCA

X-1 = specific activity of CO_2 from glucose-1-^{14}C

X-6 = specific activity of CO_2 from glucose-6-^{14}C

Then G = 1 - S

$$X\text{-}1 = \frac{AS}{3} + \frac{AG}{6}$$

$$= \frac{A}{6}(S + 1) \qquad (1)$$

$$X\text{-}6 = \frac{AG}{6}$$

$$= \frac{A}{6}(1 - S) \qquad (2)$$

Let $\frac{X\text{-}6}{X\text{-}1} = R$

$$\text{Then } R = \frac{\frac{A}{6}(1 - S)}{\frac{A}{6}(1 + S)}$$

$$= \frac{1 - S}{1 + S} \qquad (3)$$

$$\text{Whence } S = \frac{1 - R}{1 + R} \qquad (4)$$

$$\text{and } G = \frac{2R}{1 + R} \qquad (5)$$

The validity of the fractions S and G can be no greater than that of the assumption on which the calculations are based.

As there seems to be no reason to assume that C-atoms 4, 5 and 6 do not give CO_2 in the HMPS the results may be in error. This system does not even allow of the calculation of maximum and minimum contributions of the HMPS for in the former case equations (1) and (2) become

$$X\text{-}1 = \frac{AS}{6} + \frac{AG}{6}$$

$$= \frac{A}{6}(S + G)$$

$$= \frac{A}{6}(S + 1 - S)$$

$$= \frac{A}{6} \qquad (1a)$$

and similarly

$$X\text{-}6 = \frac{A}{6} \qquad (2a)$$

and while both (1a) and (2a) are true under the conditions defined they do not yield information as to S and G.

(c) Products of radioglucose dissimilation as an index of the pathway of metabolism.

(1) Basic assumptions and expression of results. Isolation of radioactive products of variously labelled glucose has been used to evaluate the evidence of alternative pathways in microbiological systems (Blumenthal, Lewis & Weinhouse, 1954; Lewis, Blumenthal, Wenner & Weinhouse, 1954; Lewis, Blumenthal, Weinroch & Weinhouse, 1955). The products isolated contain three carbon atoms or less and the basic assumption made is

that glucose yields two three-carbon fragments via EMP and one three-carbon fragment via HMPS. Glucose-U-^{14}C is used to correct for endogenous metabolism. This can be done because all products of metabolism of glucose-U-^{14}C should have the same specific activity, on a C-atom basis, as the substrate. Any diminution in this activity indicates a dilution by endogenous metabolism which can be allowed for in the mathematical treatment of the data from experiments with the singly labelled sugars. In calculating results the ^{14}C content of the isolated products may be expressed in terms of relative specific activity (RSA). In this context, RSA is the number of radioactive C-atoms per C-atom in the product divided by the number of radioactive C-atoms per C-atom in the substrate multiplied by 100. Thus if a product has x radioactive C-atoms in its molecule of y C-atoms and it was formed from glucose-1-^{14}C

$$RSA = \frac{\frac{x}{y}}{\frac{1}{6}} \times 100$$

Observation of the RSA of a product derived from glucose-U-^{14}C gives a quick method of correcting for endogenous dilution of product. Thus the RSA of <u>all</u> products from glucose-U-^{14}C should be 100 and if the observed value is z, the correction factor of dilution for the RSA of the same product formed from, say, glucose-1-^{14}C is $\frac{100}{z}$.

(ii) Calculation based on labelling of ethanol. Fig.16 shows ethanol production from glucose-1-^{14}C by EMP and HMPS. Thus the RSA of ethanol produced by EMP would be 150 (i.e., $\frac{1}{\frac{1}{4}}\frac{1}{6}$ x 100) and by HMPS would be zero. Experimentally, the RSA of the ethanol is corrected by reference to the RSA of the ethanol isolated from the glucose-U-^{14}C reaction as described above. Then:-

$$\frac{\text{RSA ethanol (corrected)}}{150} \times 100 = \% \text{ of total ethanol formed via EMP} \qquad (1)$$

The percentage of ethanol formed via the EMP is not, however, the same as the percentage of glucose molecules degraded via EMP. If one assumes that the conditions of fig.16 obtain (i.e., each glucose molecule metabolized via HMPS yields only one ethanol molecule), the following relationship holds:-

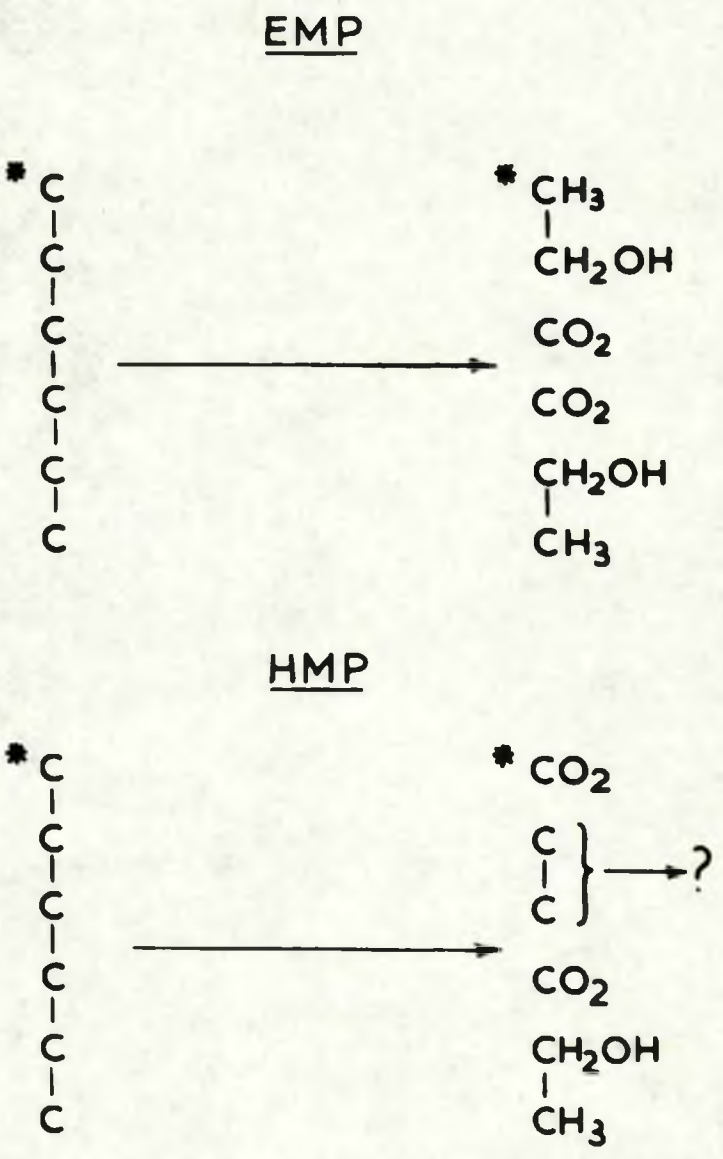
$$\frac{\text{no. of glucose molecules via EMP per 100 ethanol molecules} \times 100}{\text{no. of glucose molecules via EMP per 100 ethanol molecules + no. of glucose molecules via HMPS per 100 ethanol molecules}}$$

$$= \frac{\frac{1}{2}(\% \text{ ethanol via EMP}) \times 100}{\frac{1}{2}(\% \text{ ethanol via EMP}) + (100 - \% \text{ ethanol via EMP})} \qquad (2)$$

Equation (1) is probably more accurate than equation (2) but both may be in error. The first equation depends for its derivation on the assumption that the RSA of ethanol via EMP is 150. This is equivalent to assuming that dihydroxyacetone phosphate and glyceraldehyde phosphate are completely

Figure 16.

Ethanol production from glucose-1-^{14}C



interchangeable in the EMP. However, Schambye, Wood & Popjak (1954) found glycogen isolated from rats fed glycerol-1-^{14}C is more heavily labelled in the 3-position than the 4-position. They suggest that the glycerol is converted to dihydroxyacetone phosphate which is not brought into complete isotopic equivalence with glyceraldehyde phosphate formed endogenously and the condensation of the two 3-carbon fragments thus yields glucose more heavily labelled in that half of the glucose molecule derived from dihydroxyacetone phosphate. The equivalence of the two triose phosphates is also implicit in experiments with glucose-1-^{14}C and glucose-6-^{14}C where C-atoms 1 and 6 are assumed to be equivalent in the EMP. But Blumenthal, Lewis & Weinhouse (1954) found in Torula utilis that glucose-1-^{14}C data indicated a 25% participation of HMPS while glucose-6-^{14}C data indicated a 13% participation of HMPS. These data indicate that the triose phosphates may not always be in isotopic equilibrium but errors implicit in these observations are minimized if endogenous metabolism is at a minimum and if the three carbon fragments are isolated as such before further metabolism to smaller or larger molecules has taken place.

Equation (1) also assumes that the RSA of ethanol via HMPS is zero, but if some of the glucose-1-^{14}C is randomised by going "down" and "up" EMP, a glucose molecule could be made which would give labelled ethanol via HMPS.

Equation (2) is uncertain in its derivation to the extent that the fate of C-atoms 2 and 3 of glucose is uncertain as shown in fig.16. Indeed the 2-C fragment of the HMPS may be a better precursor of ethanol than the 3-C fragments of EMP. This would mean that the assumption of twice as many moles of ethanol from each mole of glucose via EMP as by HMPS would not hold and equation (2) would be invalid. Additionally it is assumed either that the two pathways feed into a common triose-phosphate pool or that if the pools do not mix they yield ethanol at equivalent rates. This remains to be proved. The above criticisms show that many of the difficulties of this approach rest on the fate of 3-C fragments in their further metabolism. Many of these difficulties are overcome if 3-C rather than 2-C fragments are isolated and examined.

The above derivations and criticisms apply equally to the other 2-C fragment which has been isolated in this connexion (acetate).

(iii) <u>Calculation based on labelling of three carbon fragments.</u> The arguments and derivations of the above section apply in a general way to 3-C fragments. Thus, in the case of lactate, the $RSA_{lactate}$ via EMP is $100(\frac{1}{3}/\frac{1}{6} \times 100)$ and via HMPS is zero when glucose-1-^{14}C is the substrate. Thus:-

$$\frac{\text{RSA}_{\text{lactate}}\,(\text{corrected})}{100} \times 100 = \% \text{ of lactate formed via EMP} \qquad (1)$$

and:-

% of glucose molecules via EMP =

$$\frac{\text{no. of glucose molecules via EMP per 100 moles lactate} \times 100}{\text{no. of glucose molecules via EMP per 100 moles lactate} + \text{no. of glucose molecules via HMPS per 100 moles lactate}}$$

$$= \frac{\frac{1}{2}(\% \text{ lactate via EMP}) \times 100}{\frac{1}{2}(\% \text{ lactate via EMP}) + (100 - \% \text{ lactate via EMP})} \qquad (2)$$

As noted in the preceding section, this equation may be more accurate than the similar one based on 2-C fragments because of the possibly different routes followed by the two triose-phosphates of EMP. In addition, as will be shown in the experimental section, it is possible to assess more than one alternative pathway by examining the disposition of labelling within the 3-C fragments. More detailed discussion of this point and its application to pyruvic acid is given then.

X. ENDOGENOUS METABOLISM AND OXIDATIVE ASSIMILATION.

Some of the experimental results which are described later in this thesis are affected in their interpretation by considerations of endogenous respiration and assimilation of substrates. Only the briefest of summaries is given here: extensive reviews of oxidative assimilation have been published by Clifton (1946, 1951, 1952), and although there is no modern review available which covers endogenous metabolism, at least one textbook gives a good summary of this field (Lamanna & Mallette, 1953).

(a) Endogenous metabolism.

The activities of higher forms of life do not cease immediately the supply of nutrients is cut off and, indeed, muscular activity can continue for many days under these circumstances. The energy required to sustain this effort comes from the oxidation of compounds previously accumulated in the body and their utilization is consequently called endogenous metabolism. When bacteria are denied a source of food, endogenous metabolism goes on for some time and is frequently, and most conveniently, measured by oxygen uptake or endogenous respiration (ER). Examination of this phenomenon soon raises three fundamental questions - Is ER necessary for the maintenance of life? Does ER continue in the presence of a plentiful supply of oxidisable substrates? What is the intracellular

substrate of ER? It is the purpose of this section to indicate possible answers to these questions and also briefly to describe a method of measuring endogenous metabolism which does not depend on determining respiration (oxygen uptake) and the methods available for lowering ER.

(i) ER and maintenance of life. Washed bacteria continue to respire in a non-nutrient medium and as their ER falls off so does the viable count decrease until all the bacteria are dead. However, it is dangerous to assume that an active ER is obligatory for viability because it is possible to treat bacteria, e.g., with mercury salts, so that their ER disappears and then by reversal of the treatment, i.e., removal of the mercury salts, restore the ER without complete loss of viability. If then ER is not essential for the maintenance of life as such, one is driven to one of several assumptions which do not have experimental support and which by their very nature are difficult to test. These include the suggestions that energy maintenance is required to prevent the spontaneous disruption (e.g., by hydrolysis) of essential cellular components, that the normal synthetic reactions of the cells working in reverse cause ER, or that ER reflects a valueless series of spontaneous reactions continually occurring. There are few data to help in the choice of explanation, but one thing stands out and that is whatever theory is adopted, it must explain the reactions of

ER in the presence of a supply of external oxidizable substrate.

(ii) Suppression of ER by external substrates. Many workers have satisfied themselves that addition of an external substrate inhibits ER. This conclusion has been reached with Escherichia coli (Clifton & Logan, 1939), Prototheca zopfii (Barker, 1936), Pseudomonas calcoacetica (Clifton, 1937), Pseudomonas saccharophila (Bernstein, 1943; Doudoroff, 1940) and with baker's yeast (Stier & Stannard, 1936). Other workers have found that not only does added substrate not depress but may even stimulate the ER of Achromobacter fischeri (McElroy, 1944), Chorella vulgaris (Moses & Syrett, 1955), Pseudomonas aeruginosa (Morris et al., 1949) and yeast (Reiner et al., 1949). Two of the general approaches used deserve special mention. Moses & Syrett (1955) used cells which had been labelled with ^{14}C and metabolised unlabelled substrates when the production of $^{14}CO_2$ indicated the extent of ER. Morris et al. (1949) used a constant amount of cells and a varying amount of substrate or a varying amount of cells with a constant substrate concentration. In the first method, if the ER is suppressed, the observed oxygen uptake is directly proportional to the substrate concentration; if ER is not suppressed this condition does not apply unless the ER of the cells is subtracted. The same type of reasoning may be applied to the interpretation of data obtained by the second approach.

(iii) The substrate for ER. It might be thought that an examination of its breakdown products would indicate the nature of the endogenous reserve (E.Res) but usually the only products formed in detectable amounts are carbon dioxide and water. The evolution of CO_2 is not without diagnostic value in that calculation of the respiratory quotient (R.Q., CO_2 evolved/O_2 consumed) yields information as to the general nature of the compounds being oxidised. Oxidation of carbohydrate gives an R.Q. of 1.0 (although R.Q. of acetate is 1.0), of protein an R.Q. of 0.9 and of fat an R.Q. of 0.8 (although aromatic compounds and others containing little oxygen also have low respiratory quotients). Application of manometric data to the study of bacterial R.Q. values shows that some organisms may well have carbohydrate and others fat E.Res. A complication of interpretation arises when it is found that the R.Q. gradually falls during starvation. Thus Mycobacterium tuberculosis as harvested has an endogenous R.Q. of 0.85 which falls to 0.78 in 24 hours and 0.72 in seven days. Presumably the ER of this organism depends on the oxidation of a fatty E.Res with an initial and preferential utilisation of certain of the fatty acid residues. The R.Q. of Thiobacillus thio-oxidans is close to 1.0 suggesting a carbohydrate E.Res. Norris et al. (1949) have consistently found an R.Q. of 1.12 with Pseudomonas aeruginosa. Presumably the E.Res is at least partly

carbohydrate in nature, perhaps containing carboxyl or keto groups in order to account for the R.Q. being slightly higher than 1.0. Stickland (1956) has published an interesting paper on the endogenous metabolism of yeast. He notes an endogenous Q_{O_2} of 0.85 (although other strains are quoted from the literature at an R.Q. of 1.0) and shows by direct analysis that the processes of ER do not lower the cellular carbohydrate content of the cell. Thus polysaccharide (measured as total material yielding reducing sugars on acid hydrolysis) is shown not to be the endogenous reserve although this material is used up if the cells are suspended in 3 mM 2,4-dinitrophenol (DNP).

In conclusion, it may be said that the true natures of endogenous reserve are as obscure as the enzymatic mechanisms of their degradation, but it is certain that wide differences exist within the family of organisms.

(iv) Lowering of ER. A high level of ER can be undesirable in practice either because it masks a quantitatively small phenomenon or because it may be difficult to decide whether the phenomenon under observation is partially suppressing the ER. Because of this, methods have been sought for producing cells of low ER or of lowering the ER where it is undesirably high. In the first approach it is usual to devise media yielding cells of high metabolic activity but with practically

no storage materials (Wood & Gunsalus, 1941) but it is more usual to attempt the second approach by briskly aerating a suspension of micro-organisms in a non-nutrient medium. Quastel & Whetham (1924) working with Escherichia coli first suggested this technique and it was applied to Sarcina lutea by Rubenstein (1932). The method does not have universal application as Morris et al. (1949) have shown that it does not reduce the ER of Pseudomonas aeruginosa.

(v) Reduction of dyes. The reduction of several dyes, such as methylene blue, has been used as an index of metabolic activity. The disadvantage of reoxidation of methylene blue is not met with in the use of 2,3,5-triphenyltetrazolium chloride (TTC) which may be reduced to the red 2,3,5-triphenyl-formazan (TPF). This method has been applied to bacterial studies by Bielig et al. (1949) and to succinic dehydrogenase assays by Kun & Abood (1949). The former workers found that reduction of TTC took place in well defined polar loci and showed by extraction and colorimetric estimation of TPF the optimum pH for TTC reduction in Escherichia coli is 8.4 and that the reaction is inactivated at 70° C. Kopper (1952) confirmed the optimum pH of 8.4 but showed that a lower value (7.5) obtained if the cells were harvested in the stage of exponential growth. His paper also shows the effect of salts, antibiotics and various inhibitors on TTC reduction and compares

this effect with methylene blue reduction, but it is most unfortunate that no attempt was made to correlate the observations on TTC with any of the other indices of metabolic activity such as RR. This paper also claims that the amount of TPF extracted was estimated colorimetrically by comparison "with standards prepared with known amounts of reduced 2,3,5-triphenyltetrazolium chloride", but no experimental details are given. A personal communication from the author indicated that the method of calibration used was that of Kun & Abood (1949) which consists of reducing known amounts of TTC with "a few crystals" of sodium hydrosulphite. On attempting this procedure in this laboratory, it was found that a precipitate was formed, presumably of colloidal sulphur, and it proved impossible to construct a calibration curve by this method (q.v.).

(b) Oxidative assimilation.

(i) Manometric studies. Early manometric work showed that washed cell suspensions of bacteria did not quantitatively oxidise added substrates. It gradually became apparent that part of the substrate was assimilated into the cell material and Barker (1936) showed that the manometric data were consistent with the synthesis of carbohydrate material in the colourless alga Prototheca zopfii and he developed equations as follows:-

glycerol
$$2C_3H_8O_3 + 2O_2 \longrightarrow 5(CH_2O) + CO_2 + 3H_2O$$

ethanol
$$2CH_3CH_2OH + 3O_2 \longrightarrow 3(CH_2O) + CO_2 + 3H_2O$$

acetate
$$CH_3COOH + O_2 \longrightarrow (CH_2O) + CO_2 + H_2O$$

propionate
$$2C_2H_5COOH + 4O_2 \longrightarrow 3(CH_2O) + 3CO_2 + 3H_2O$$

butyrate
$$2C_3H_7COOH + 5O_2 \longrightarrow 5(CH_2O) + 3CO_2 + 3H_2O$$

valerate
$$2C_4H_9COOH + 6O_2 \longrightarrow 7(CH_2O) + 3CO_2 + 3H_2O$$

Similar results have been obtained with other substrates and with different organisms although it should be noted that different organisms may assimilate different proportions of the same substrate. The results obtained for the oxidative assimilation of substrates more reduced than carbohydrate are of particular interest in that two phases of oxidation, characterized by different R.Q. values, were observed (Clifton, 1937). The R.Q. for the oxidation to completion of butyric acid is 0.8 while the observed value was 0.68 during the period of rapid oxygen consumption. After the butyrate had been utilized the R.Q. shifted to 0.94, a value characteristic of the respiration of control cells. It must not, however, be assumed that the product of assimilation is identical in all cases with the substrate of ER, as Stickland (1956) has shown in yeast that the polysaccharide laid down by oxidative

assimilation of glucose is not metabolized endogenously.

In some cases oxidative assimilation of certain substrates may be inhibited by sodium azide or 2,4-dinitrophenol. Clifton (1937) found that either would block the assimilation of butyrate and it is significant that under these conditions the theoretical R.Q. of 0.8 was achieved.

(ii) Carbon balances. A more direct measure of assimilation is to estimate the amount of new material laid down in the cells. Pickett & Clifton (1943) and Clifton (1947) established carbon balances for the oxidative assimilation of glucose by suspensions of Saccharomyces cerevisiae. Manometric studies suggested

$$C_6H_{12}O_6 + 2O_2 \longrightarrow 4(CH_2O) + 2CO_2 + 2H_2O$$

but the observed increase in C-content of the cells was only about half that postulated in the equation. This may indicate that a more complicated series of events than that covered by the equation actually exists. However, other systems have shown, by C-balances, up to 94% of the assimilation predicted on the basis of manometric data.

(iii) TTC reduction. Kopper (1954) has used TTC reduction as an index of assimilation of various substrates by Escherichia coli. The general procedure was to incubate cells and substrate in phosphate buffer for twenty minutes, centrifuge, take up in water, centrifuge again, take up in a small volume of water, add buffer and TTC solution, incubate at 37° and

determine the TPF produced. Of the substrates tested, only those which would support growth of the organism in a synthetic ammonium salt medium gave a reduction of TTC. Certain criticisms may be made of the results as presented:-

1. No details of the centrifuge procedure are given and, in particular, no indication was given as to whether or not further metabolism is taking place during this process.

2. The amount of TTC reduced is not related either to the amount of cells used or to the times of the two incubation periods involved.

3. The figures given for the effect of various substrates, as presented, are meaningless in that, as they are not related to time of incubation, one substrate may have reached its maximum level of reduction while another may still be increasing at the time arbitrarily chosen.

4. The results are attributed to substrate assimilation and the laying down of "reserve food" and no consideration is given to the possibility that the elevated levels of TTC reduction are due to accumulation of pools of intermediates within the cell.

METHODS

"Blessed is he who maketh due proofe.
With due proofe and with discreet assaye
Wise men may learn new things every day."
Thomas Norton (b. 1433) in
Ordinall of Alkimy.

XI. GENERAL METHODS AND PROCEDURES.

(a) Estimation, growth and production of Sarcina lutea.

The growth characteristics of the strain have not been completely determined, but sufficient data were obtained to rationalise the approach to the bulk production of the organism.

(i) Estimation of bacterial density. It was decided to utilise turbidimetric measurements as an index of the bacterial density of suspensions but, when calibration was attempted, considerable deviation from smooth curves was found. The deviation is greatest when cells are grown without aeration and it was noted that, under these conditions, the degree of clumping (as distinct from packet-formation) is considerable. Direct microscopic examination shows that the clumps are rapidly dispersed by very low concentrations of a detergent marketed by Irano Products Ltd. under the name Comprox A. When various dilutions of bacterial suspensions are added to equal volumes of a solution containing 3.8% formalin and 4% Comprox A it is found that smooth curves are obtained from the readings of a Hilger Spekker absorptiometer using filters H 508 and OG 2. By this technique calibration curves were constructed of optical density plotted against both bacterial dry weight and bacterial nitrogen. Formalin is added to the dispersing solution since it is convenient to kill the cells

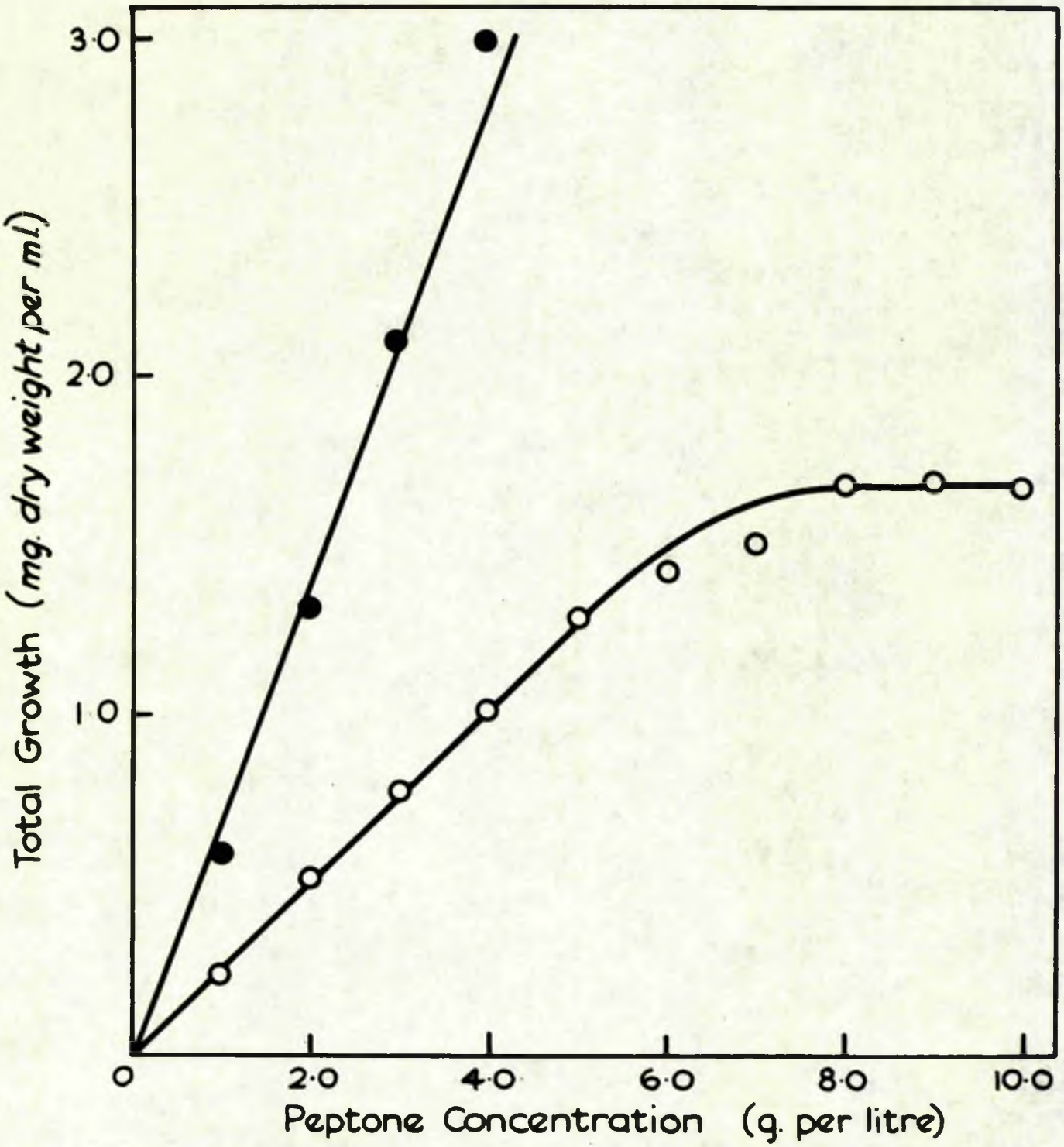
at this stage. In later work when lyophilized cells were used, these were weighed out directly on a balance. It is essential that the weighing procedure be effected rapidly as the cells quickly absorb moisture.

(ii) Defined media. The strain of Sarcina lutea grows well on peptone, luxuriantly on peptone-glucose but not at all on glucose-ammonium sulphate-phosphate medium. This last observation distinguished the strain from that described in Bergey's Manual. In an effort to obtain a defined medium which could support growth a synthetic medium containing glucose, ammonium sulphate, phosphate and Mg^{++} was supplemented with single amino acids to a final concentration of 0.01 M. Under these conditions alanine, aspartate, glycine, histidine, leucine, phenylalanine, serine, tyrosine and tryptophan do not support growth, nor does supplementation with yeast extract. The only amino acid tested which does support growth is glutamate, but the amount of growth was not sufficiently great to enable this medium to be used for the production of cells for metabolic studies.

(iii) Media containing peptone. Growth occurs only in media containing peptone with or without the addition of glucose. In all cases the amount of growth increases with aeration and the response to variation in peptone concentration was determined (with and without glucose) and is shown in fig.17. As

Figure 17.

Relation between total growth and peptone concentration for *Sarcina lutea* in aerated culture.



O—O KH_2PO_4, 0·6; NaCl, 0·06; $MgSO_4$, 0·033 g. per 100 ml. NaOH to pH 7·4; peptone as indicated.

●—● Above medium, glucose (1 g. per 100 ml.) added.

would be expected, growth is greatly stimulated by the presence of glucose.

(iv) Bulk production. In choosing a medium for the bulk production of cells for metabolic studies, it was decided to omit glucose from the medium in the hope that this might diminish the observed high rate of endogenous respiration. In addition, a concentration of peptone is used which is limiting for growth. The medium has the composition:-

Difco Bacto-peptone	5.0 g.
A.R. KH_2PO_4	6.0 g.
A.R. NaCl	0.6 g.
5N NaOH to pH 7.1	approx. 6.6 ml.
Water to 1 litre	

At the time of inoculation, sterile $MgSO_4$ solution is added to a final concentration of 0.04% (w/v). Cells have been grown in this medium in various volumes up to 6 l. per flask; brisk aeration is accomplished by sintered glass disks which are fed from an air compressor after suitable sterilization. After inoculation, growth is allowed to take place at 37° for 24 hours when each flask is checked for homogeneity by direct microscopic examination, and the cells are then harvested by centrifugation in a Sharples supercentrifuge using a stainless steel bowl. After one washing the cells are suspended in

water or in 0.2% (w/v) phosphate buffer, pH 7.1, and briskly aerated for anything up to 7 hours at 37° to reduce the endogenous respiration (ER). The cells are then washed twice in water, lyophilized and stored at -10°. This describes the routine procedure, but for special purposes certain steps such as ER reduction or freeze-drying are either omitted or curtailed.

(b) Estimation and removal of inorganic ions.

(i) Phosphorus. This was generally estimated as orthophosphate by the method of Fiske & Subbarow (1925) as amended by Allen (1940) or, less often, by the modification of the method of Berenblum & Chain (1938) due to Ennor & Stocken (1950). The second method is of use when it is desired to estimate inorganic phosphorus in the presence of labile organophosphorus compounds. The method of Allen (1940) was applied as follows - the phosphorus containing solution (0-20 ml.) was pipetted into a 25 ml. volumetric flask and 1 ml. 12 N H_2SO_4, 1 ml. 8.3% (w/v) ammonium molybdate and 2 ml. 1% (w/v) amidol in 20% (w/v) sodium metabisulphite added before the contents are made up to 25 ml. with water. The resulting blue colour is unstable and is read within 10 - 30 min. in a Spekker absorptiometer using neutral filter H 503 and Ilford spectral red 608. Under these conditions the optical density is directly proportional to the phosphorus added over the

range 0 to 8.0 μmoles. Total organic phosphorus and acid-labile phosphorus are determined by the same procedure after a preliminary digestion in conc. sulphuric acid and in N HCl for 10 min. respectively. Small traces of inorganic phosphorus on the glassware introduce errors and this is eliminated by soaking in conc. nitric acid overnight, followed by washing with distilled water.

(ii) Removal of barium and calcium. Many of the substrates used in this work are obtained in the form of barium or calcium salts, e.g. barium glucose-6-phosphate heptahydrate and calcium 2-ketogluconate. Some of these are insoluble in water and, in any case, it is usually desirable to remove such ions before the substrate is used in metabolic experiments. This is accomplished by dissolving a weighed amount of the salt in some dilute hydrochloric acid in a centrifuge tube and adding the calculated amount of aqueous sodium sulphate solution. The precipitate of insoluble sulphate is spun down, washed twice with distilled water and the washings added to the original supernatant. An amount of sodium hydroxide, calculated to effect complete neutralization, is now added and the whole made up to volume.

(c) Estimation of nitrogen in protein.

(i) Cell-free extracts. The protein in cell-free extracts was determined by the method of Stickland (1951). Sodium hydroxide

is added to the extract, followed by copper sulphate. The precipitate of copper hydroxide is broken up with a fine glass rod and removed by centrifugation. The supernatant is purple, the intensity of the colour being linearly proportional to the protein content of the extract within the calibrated range of 0 - 0.65 mg. protein.

(ii) Total nitrogen. The total nitrogen content of cells was determined by standard micro-Kjeldahl technique. After digestion with conc. sulphuric acid, catalysed by selenium, the solution is made alkaline and steam distilled in a Markham still. The distillate is trapped in boric acid solution and the ammonia titrated with dilute acid.

(d) Estimation of carbohydrates.

(i) Glucose was estimated by Nelson's (1944) colorimetric modification of Somogyi's (1937) copper reductimetric procedure. The quantities taken are 1 ml. glucose solution plus 1 ml. alkaline copper reagent in a Folin-Wu tube which is heated in a boiling water bath for exactly 20 min. After cooling, 1 ml. of the arsenomolybdate reagent is added and the whole made up to 25 ml. The blue colour is read in a Spekker absorptiometer, using neutral filter H 508 and spectral red 608, and under these conditions the optical density measured is directly proportional to the glucose content up to 1.25 mM

glucose. The interference of ribose was estimated by preparing a calibration curve for ribose in the range 0 - 1.0 mM. An attempt was also made to estimate glucose by the commercial enzyme preparation Dee0 (Takamine Laboratory, Inc., Clifton New Jersey, U.S.A.) Dee0 catalyses the reactions:-

$$\text{glucose} \xrightarrow{O_2} \text{gluconate} + H_2O_2$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2$$

It contains both glucose aerodehydrogenase, which catalyses the first reaction, and catalase which degrades the hydrogen peroxide formed in the oxidative step. The net result is the oxidation of one mole of glucose to one mole of gluconate with the uptake of 0.5 mole of oxygen. Measurement of the oxygen uptake by manometry gives a direct measure of the amount of glucose present. This procedure has the advantage of being unaffected by the presence of other reducing sugars, but its overall accuracy is not so great as the copper reductimetric method and it is only used if that method cannot be applied.

(11) Pentose. Pentose was estimated by the Mejbaum reaction (1939) modified as follows:-

2 ml. unknown or xylose standard,

1 ml. 3% (w/v) orcinol in 50% (v/v) ethanol, and

3 ml. 0.099% (w/v) $Fe(NH_4)(SO_4)_2.12\ H_2O$ in conc. HCl,

are heated in a boiling water bath for exactly 35 min., cooled,

and the optical density determined at 500 and 675 mμ in a Unicam SP 350 diffraction grating spectrophotometer. Glucose contributes to the colour production but this interference is noticed by the ratio of the two readings obtained.

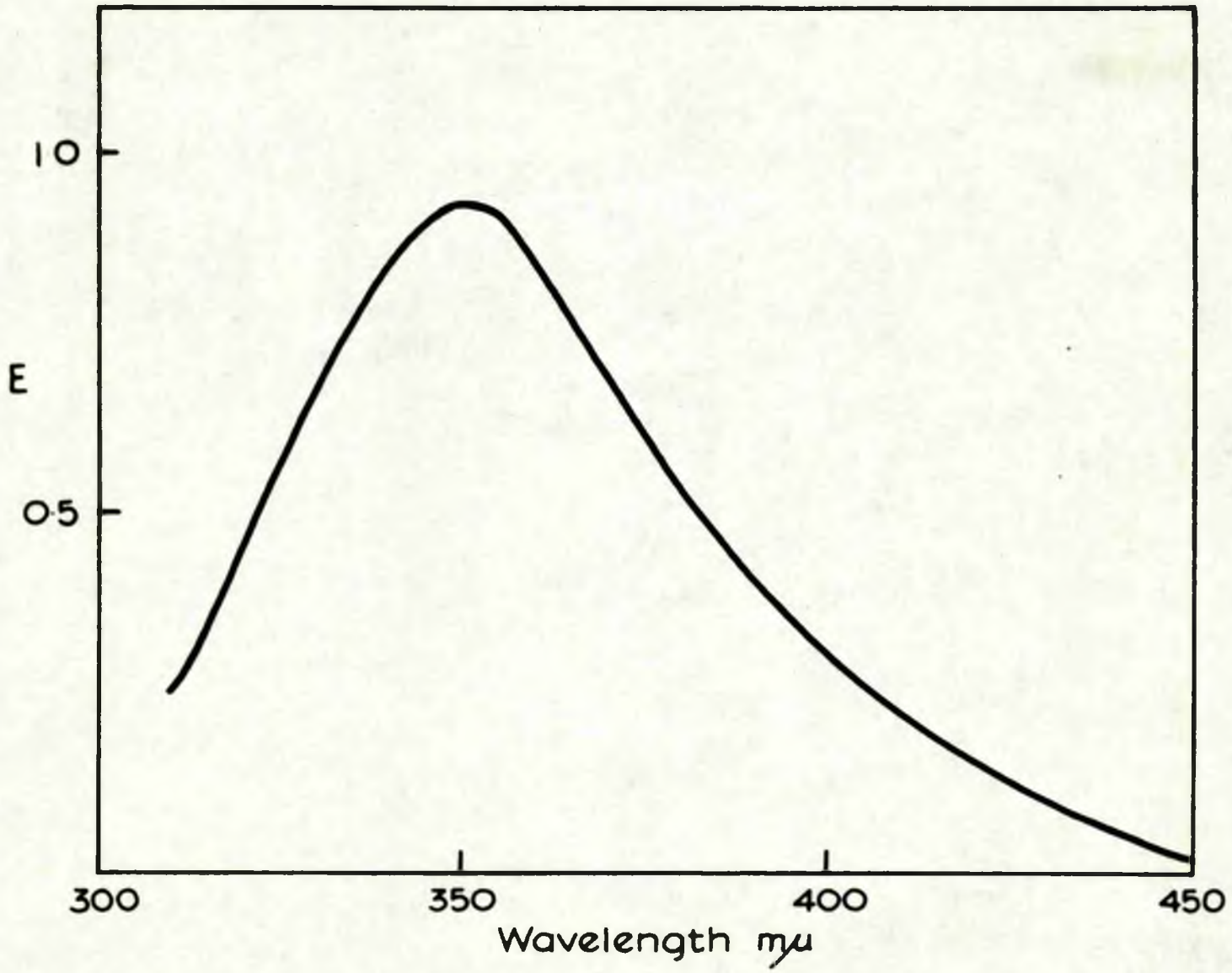
(iii) Polysaccharide was determined by the method of Trevelyan & Harrison (1952) and as reducing sugar after preliminary hydrolysis (Dagley & Dawes, 1949). In the first method the polysaccharide solution is mixed with anthrone in sulphuric acid under controlled conditions, heated in a boiling water bath for exactly 10 min. and the intensity of colour produced is read at 620 mμ in a Unicam SP 350 diffraction grating spectrophotometer. A glucose standard is used and linearity of response is good between 25 and 100 μg. of glucose. In the second method bacteria, in suspension in 2 N sulphuric acid, are sealed in glass ampoules and placed in an oven at 110° overnight. After cooling, the contents are transferred with filtration to volumetric flasks, neutralised and made to volume. Portions of neutralised hydrolysate are then assayed for reducing sugar by the method of Nelson (1944) already described.

(e) Estimation of keto-acids.

(i) Pyruvic acid was determined by the methods of Friedemann & Haugen (1943), using the direct method when interfering substances were absent and also by the toluene extraction procedure.

Figure 18.

Absorption Spectrum of Pyruvic acid 2:4-Dinitrophenyl-hydrazone in Ethyl acetate



(ii) Pyruvic acid was separated from reaction mixtures by steam distillation (see XII, d, i) and the distillate evaporated to dryness under reduced pressure after neutralization. The pyruvate obtained in this way was separated from other steam-volatile acids and estimated by chromatography on a Celite/sulphuric acid column by the method of Phares et al. (1952). This method was used to obtain the specific activity of ^{14}C-labelled pyruvate (XII, d, ii).

(iii) Pyruvic acid was estimated by measurement of the optical density of the solution of its 2,4-dinitrophenylhydrazone in ethyl acetate solution. The pyruvic-2,4-dinitrophenylhydrazone (PyDPH) was prepared by reaction with 2,4-dinitrophenylhydrazine (DPH) in acid solution, extracted into ethyl acetate, re-extracted into 10% sodium carbonate which was then acidified (3 ml. 10% Na_2CO_3 + 1 ml. 7.7 N HCl) and re-extracted with ethyl acetate. This procedure frees the PyDPH of unreacted DPH. If other keto acids were present these were removed by separating precipitated PyDPH from the original reaction (if the concentration were sufficiently high) or by substituting toluene for ethyl acetate in the first extraction as this solvent preferentially extracts PyDPH. All measurements of optical density of these solutions were made in a Uvispek spectrophotometer using quartz cuvettes of 1 cm. light path. The absorption spectrum of PyDPH is shown in fig.18

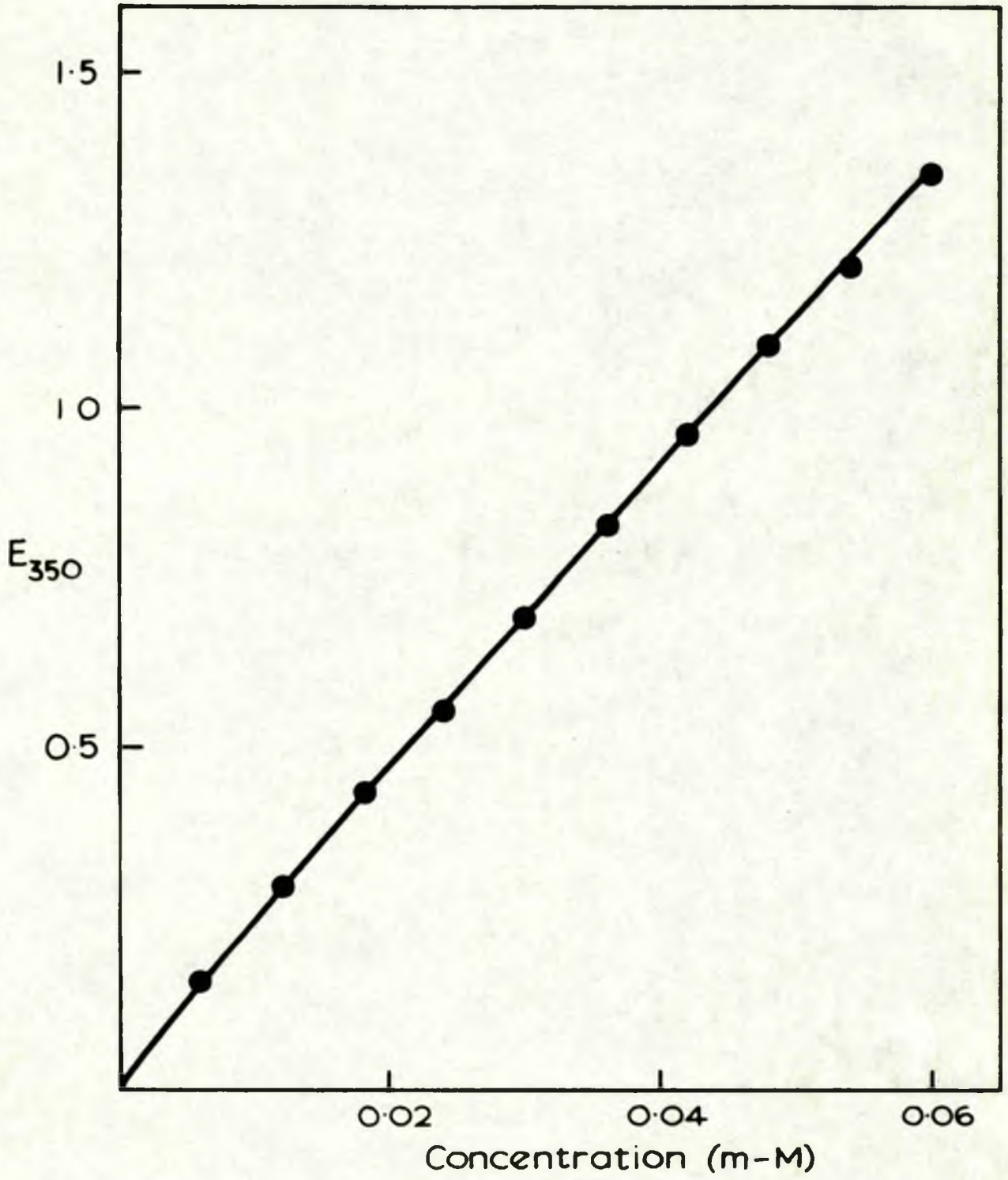
and it will be seen that the maximum absorption occurs at 350 mμ. The calibration curve at this wavelength is shown in fig.19 but as the sensitivity was so great the method was calibrated at 400 and 420 mμ and this extended the range up to 0.4 mM with respect to PyDPH.

This method was used to obtain the specific activity of ^{14}C-labelled pyruvate and gave results identical to those obtained by the steam distillation/chromatographic procedure.

(iv) Pyruvic and α-ketoglutaric acids were determined simultaneously by a modification of the single solvent extraction procedure of Friedemann & Haugen (1943). The available methods have been reviewed by Koepsell & Sharpe (1952) who improved and extended the existing techniques. These methods are somewhat cumbersome both in application and in the calculation of results. Accordingly simplifying modifications were worked out based on the observation of Friedemann & Haugen (1943) that alkaline solutions of the two acids in the form of their 2,4-dinitrophenylhydrazones and give values for the ratio of optical density at 420 mμ to that at 520 mμ which were almost linearly proportional to the relative molar concentrations of keto acids. After a suitable procedure had been elaborated it was subsequently found that Goodwin & Williams (1952) had published a similar method based on the same principle in one of their papers on Vitamin A.

Figure 19.

Relation of Optical Density at 350 mμ to Concentration of Pyruvic acid 2:4-Dinitrophenylhydrazine in Ethyl acetate.



In this method optical densities were measured in glass cells of 1 cm. light-path in a Unicam S.P. 500 spectrophotometer. Pyruvic acid was purified by redistillation at 18 mm. Hg and 68-69° while ∝-ketoglutaric acid was recrystallized from acetone/benzene to yield a product of m.p. 112.8-113.3° (uncorr.). Standard solutions of the two acids were made up by weighing and then neutralised by the calculated amount of anhydrous Na_2CO_3. DPH (0.1% in 2N HCl), Na_2CO_3 (10% w/v) and NaOH (1.5N) were as used by Friedemann & Haugen (1943). Ethyl acetate of British Drug Houses Ltd. laboratory reagent quality was used.

General Principle. The method used is essentially the single solvent extraction of Friedemann & Haugen (1943), using ethyl acetate as the non-specific extraction solvent and 10% (w/v) Na_2CO_3 as the specific hydrazone extractant. Briefly, a 3 ml. sample is treated with 1 ml. DPH reagent and incubated until the reaction is complete. The hydrazones (and excess DPH) are extracted into 8 ml. ethyl acetate and the lower aqueous layer removed by a Pasteur pipette and discarded. The acidic hydrazones are then re-extracted into 8 ml. Na_2CO_3 reagent, 5 ml. of which are transferred to a test tube and the colour of the hydrazones developed by the addition of 5 ml. of 1.5N NaOH. The optical density is then read at two wavelengths, the first reading giving the total molar concentration of keto-

acids and the ratio of the two values giving the molar proportions of the two keto-acids by reference to two calibration curves.

Extraction procedure. The extraction procedure of Friedemann & Haugen (1943), involving passage of a rapid stream of air or nitrogen, is replaced by mechanical shaking. This permits more reproducible conditions when many simultaneous determinations are carried out and, in this connexion, offers advantages over the method of Goodwin & Williams (1952) where extractions are carried out singly in separating funnels. A rack was constructed to hold 32 6 x ¾ in. test tubes in four rows of eight, and when placed on its side fitted a Griffin & Tatlock mechanical shaker. The tubes, closed by rubber stoppers, were held in position by a 1 in. rubber pad and the whole shaken with a 1½ in. traverse 300 times per min. for an extraction period of two minutes. It was noted, however, that the clear aqueous layer obtained in the initial ethyl acetate extraction became slightly yellow on standing for a few seconds. It would appear that this "over-extraction" is due to the marked increase in surface area between the two phases caused by the break up into small droplets. Accordingly, all extractions were allowed to stand for 5 min. after cessation of shaking to permit equilibration of hydrazones between the two phases.

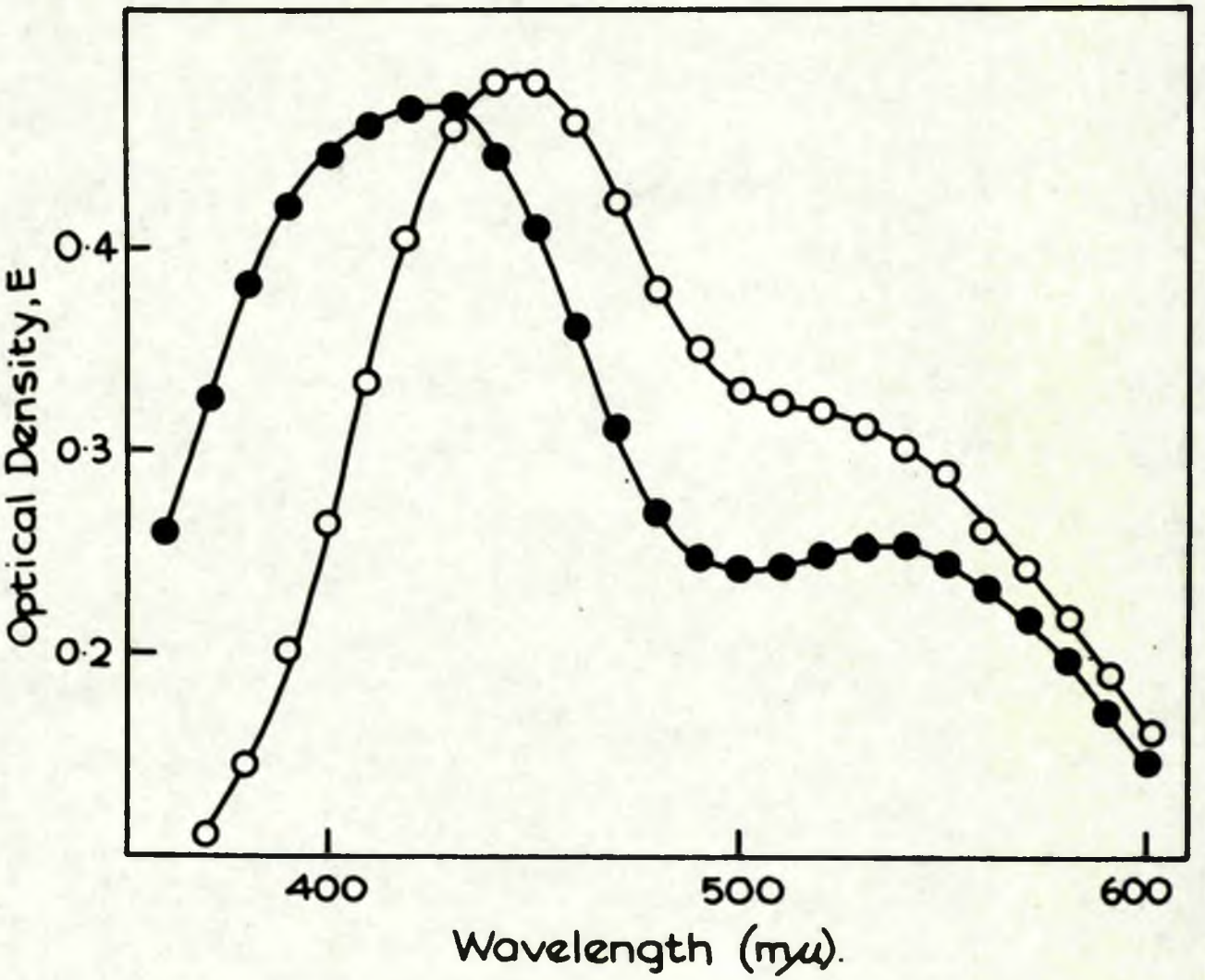
Rate of reaction. The results of experiments on the rates of the reaction agree substantially with the data of Friedemann & Haugen (1943) and incubation at 37° for 10 min. followed by 20 min. at 28° was adopted to ensure complete reaction of both acids.

Colour production. The intensity of colour produced is proportional to the final concentration of alkali but higher concentrations cause fading. As a compromise between these two observations a final concentration of 0.75N-NaOH is used.

Absorption spectra. The absorption spectra shown in fig.20 were obtained from 3 ml. portions of 133.3 µM solutions of the two keto acids treated as described above. Although equivalent amounts of starting material were used, it cannot be said that the spectra represent those of equimolar solutions. The curves obtained differ from those of Friedemann & Haugen (1943) which were not obtained in a spectrophotometer but agree substantially with those of Le Page (1950), Koepsell & Sharpe (1952) and Humphrey & Robertson (1953). The spectra do not agree with those of Goodwin & Williams (1952) but inspection of their curves shows that they are very similar except that their spectrum for α-ketoglutarate is lower. This might indicate that their sample of this compound is not pure, especially as they do not make any mention of its purification.

Figure 20.

Absorption spectra of the 2,4-dinitrophenyl-hydrazones of Pyruvic acid (o–o) and α-keto-glutaric acid (●–●) in 0·75 N-NaOH.



Calibration curves. From the spectra it can be seen that equivalent amounts of starting material give solutions of identical optical density at 431 mμ but that at 390 mμ there is a significant difference. These data indicate that it should be possible to obtain a calibration curve of optical density at 431 mμ which would be directly proportional to the molar concentration of either pyruvic or α-ketoglutaric acids or both. This was verified and is shown in fig.21. Furthermore, the reading obtained at 390 mμ depends on the total molar concentration and the relative proportions of the two acids. But as the reading at 431 mμ depends only on one of these factors the ratio of the reading should be related to the other factor (i.e., to the relative proportions of the two acids). This was confirmed and the 390/431 mμ ratio shown to be linear over the range 0.2-0.8 μmole per 3 ml. (fig.22).

Extension of range. As will be seen in fig.21, breakdown of linearity occurs at about 1.5 μmoles per determination (i.e., per 3 ml.). It was shown that dilution of the hydrazones in alkali did not follow the Beer-Lambert law but dilution of the Na_2CO_3 solution of hydrazones was satisfactory and by this means the range could be extended to 5.0 μmoles per determination.

Figure 21.

Optical density at 431 mμ of Pyruvic (o–o) and α-Keto-glutaric (●–●) 2,4-dinitrophenylhydrazones in 0·75N-NaOH.

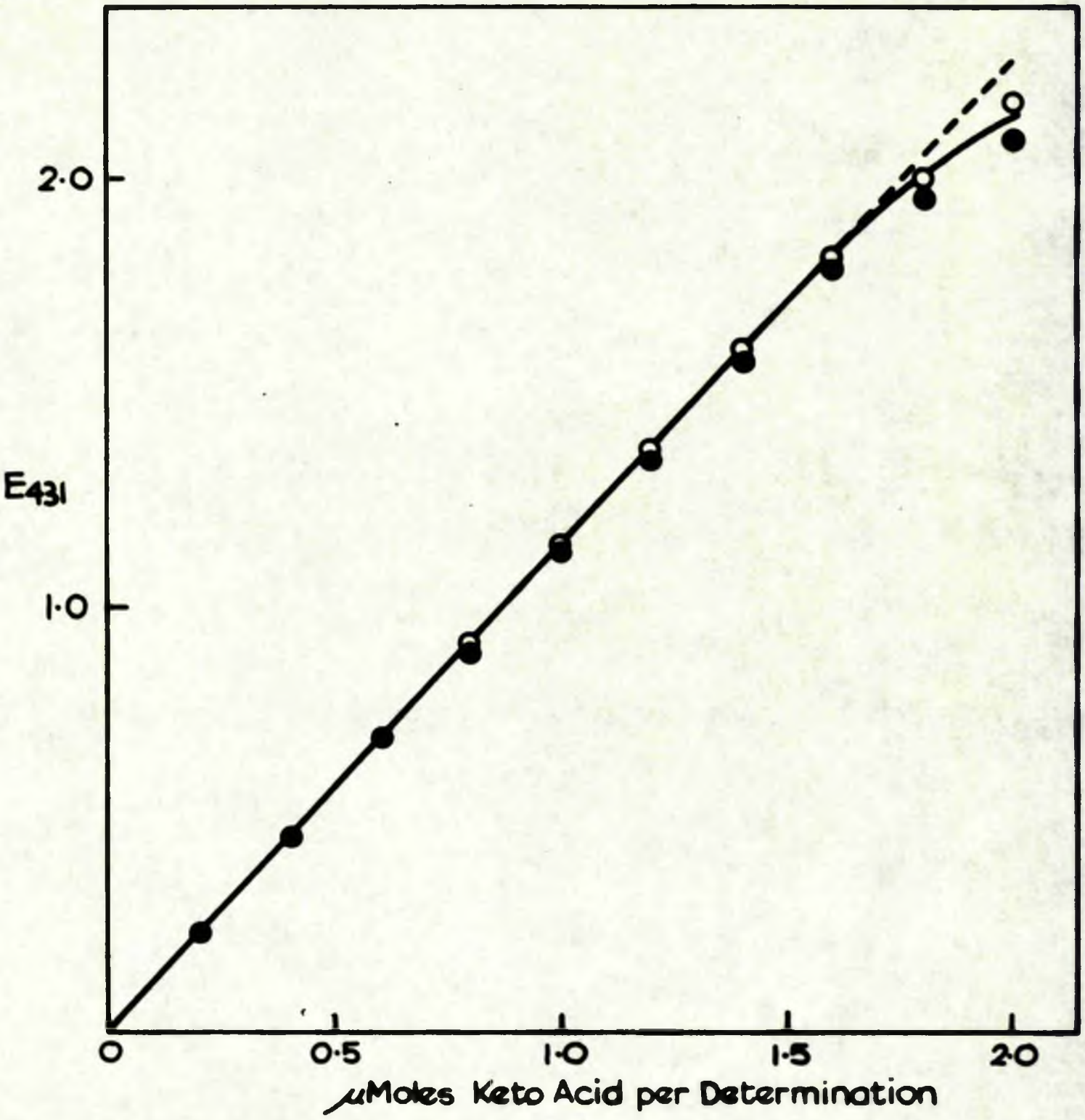
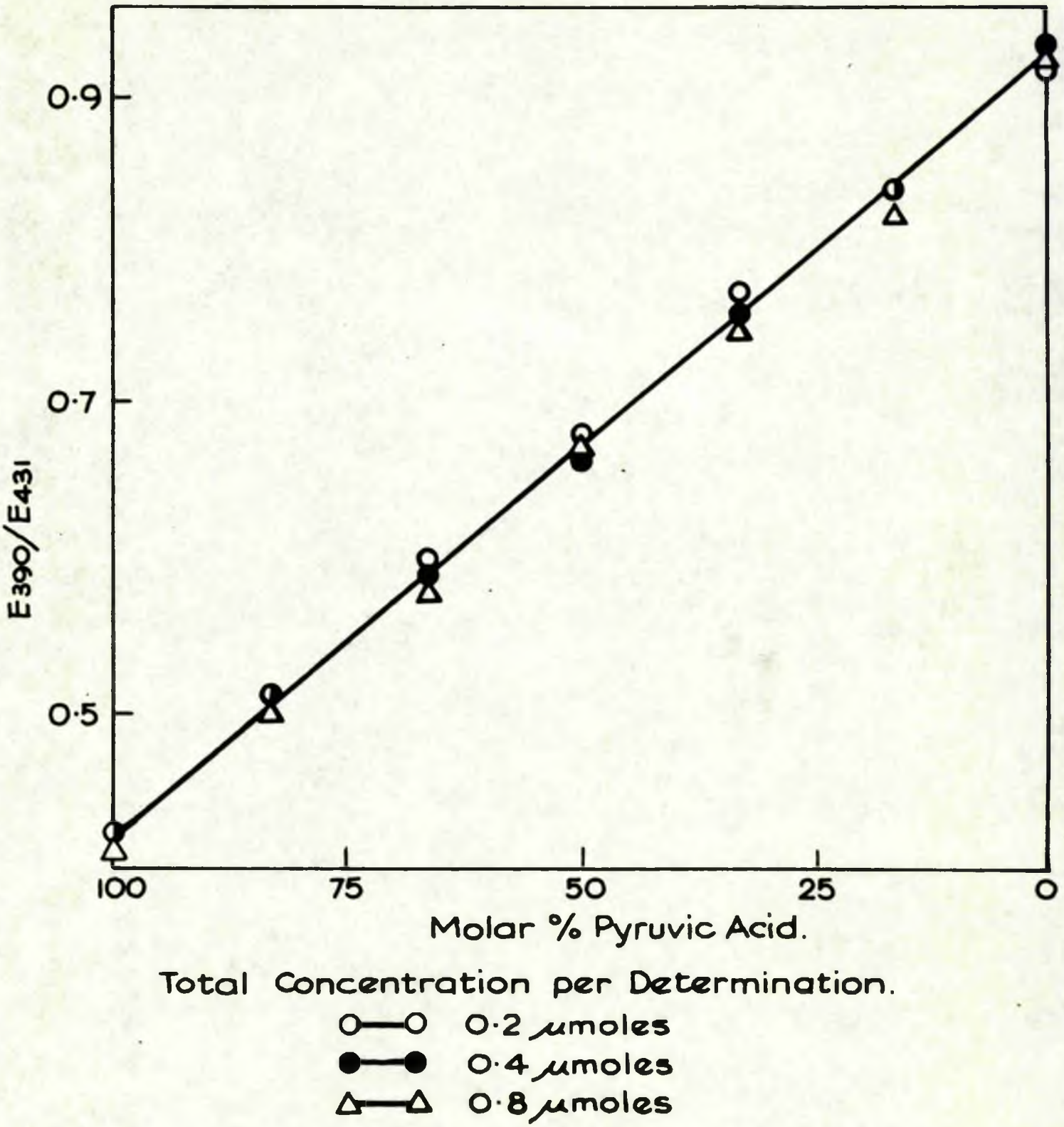


Figure 22.

E390/E431 as a Function of the Molar Proportions of Pyruvic and α-ketoglutaric Acids in the Original Reaction Mixture



Summary. The modification described above allows the determination of total keto acids to an accuracy of ± 1% and the determination of the relative proportions of pyruvic and ∝-ketoglutaric acids to ± 5%. The method is quick and simple and 31 determinations can be carried out simultaneously. The results are easily obtained from the experimental data by reference to two calibration curves.

(v) 2-Ketogluconate. This was estimated by the procedure of Lanning & Cohen (1951) in which 2 ml. of neutral solution containing 2-ketogluconate is heated with 1 ml. 2.5% (w/v) o-phenylenediamine dihydrochloride in a boiling water bath for 30 min. and the colour produced read at 330 mμ in a spectrophotometer.

(f) Cleaning of glassware.

In general, glassware was cleaned with one or more of the following agents: conc. nitric acid, 10% (w/v) nitric acid, 0.5% (v/v) Stergene (Domestos Ltd.), 40% (w/v) methanolic KOH or a solution of metasilicate (C & M). C & M is prepared by dissolving 40 g. of Calgon (Albright & Wilson Ltd.) and 360 g. sodium metasilicate in 1 gallon of tap water to give a stock solution which is diluted 1/100 before use. The solutions are used as follows:-

(i) Pipettes, when new or greasy are soaked in methanolic KOH for 4-6 hours, washed in the pipette washer, washed with

dilute HCl (approx. N/100), washed in the pipette washer and finally washed in glass-distilled water. Routinely, pipettes when used are placed in C & N overnight, washed in the pipette washer, washed with dilute HCl (approx. N/100), washed in the pipette washer and finally washed with glass distilled water. Pipettes for rough work are dried in the oven at 105° but those used in more accurate work are dried with acetone.

(ii) Growth tubes, test tubes, etc., are washed in tap water, brushed clean if necessary, and boiled in 10% HNO_3 or C & N. They are then rinsed with glass-distilled water and dried in the oven.

(iii) Volumetric flasks and apparatus for phosphorus estimations are soaked in conc. HNO_3 for at least 24 hr. and then washed in glass-distilled water. It is not usually necessary to dry this apparatus.

(iv) Warburg flasks are first washed with warm water and ethyl acetate to remove both the flask contents and the lanolin used to seal the joints. They are then boiled in 0.5% Stergene, washed in water and boiled in 10% HNO_3, or, alternatively, they are soaked in conc. HNO_3 overnight. In either event they are then washed with glass-distilled water and dried in the oven at 105°.

(g) Manometric methods.

Conventional manometric methods were used (Umbreit, Burris & Stauffer, 1949). The "direct" method was used for estimation of carbon dioxide production; this method is based on the assumption that oxygen uptake in nitrogen/oxygen is the same as in nitrogen/oxygen/carbon dioxide. In general, flask contents totalled 3 ml. including 0.2 ml. 40% KOH in the centre well where applicable. Incubation was carried out in a Hoslab Warburg apparatus at 37° and with a shaking rate of 140 cycles per min..

(h) Non-proliferating cell suspension methods.

(i) Apparatus. The apparatus used depended on the amount of material used and the conditions of aerobiosis required. For anaerobic experiments 6 x 1 in. or 8 x 1½ in. tubes fitted with tight-fitting rubber bungs carrying capillary aerators and glass exit tubes were used held in a bath at 37°; anaerobic conditions were attempted by passing a stream of nitrogen which was bubbled through alkaline pyrogallol and passed over heated copper foil. In one series of experiments anaerobic conditions were achieved in evacuated Thunberg tubes which were shaken in a bath at 37° at 140 cycles per min.. For aerobic experiments aeration was achieved by passing air or oxygen through capillary or sintered glass disks in the following apparatus:-

<u>for less than 5.0 ml.</u> small 5 x ¾ in. tubes fitted with side arms as gas exits,

<u>for 10 - 20 ml.</u> 6 x 1 in. glass tubes,

<u>for 20 - 40 ml.</u> 8 x 1½ in. glass tubes.

The above were all immersed in a bath at 37°. For larger volumes up to 600 ml. the special apparatus designed by Lawes & Holms (1956) was used in an incubator or hot-room at 37°. The mouths of these vessels were plugged with cotton wool which also held the aerators in place except as stated below (iv).

(ii) <u>Washed cell suspensions</u> were obtained from the cells as grown by washing them several times with water (or buffer) and suspending them in water (or buffer). The ER of the cells was usually reduced as described above. The freeze-dried cells were weighed out and taken up in water (or buffer), centrifuged down and made up to a predetermined volume with water (or buffer).

(iii) <u>General procedure.</u> In general, the cell suspension with all additions less substrate (i.e., buffer, inhibitors, etc.) were pipetted into the reaction vessel and equilibrated to temperature. The time of addition of substrate (or water in control experiments) was taken as zero and samples removed at suitable time intervals into chilled centrifuge tubes containing dilute sulphuric or trichloroacetic acid. The

tubes were then centrifuged and the separated cells and supernatant collected.

(iv) Special precautions. It was found that the passage of dry gas through the reaction medium caused concentration by removal of water as vapour. Accordingly a Dreschel bottle of water held at the same temperature as the experiment was always included in the aeration train.

In comparative experiments, where several reactions were being compared, the rates of aeration were equalised either by reference to a flow-meter or by collecting the gases bubbled through each tube.

In experiments with ^{14}C-labelled substrates the vessels were closed with tightly fitting stoppers and the gases issuing from the exit tube were passed through sodium hydroxide solution to trap any $^{14}CO_2$ that was formed.

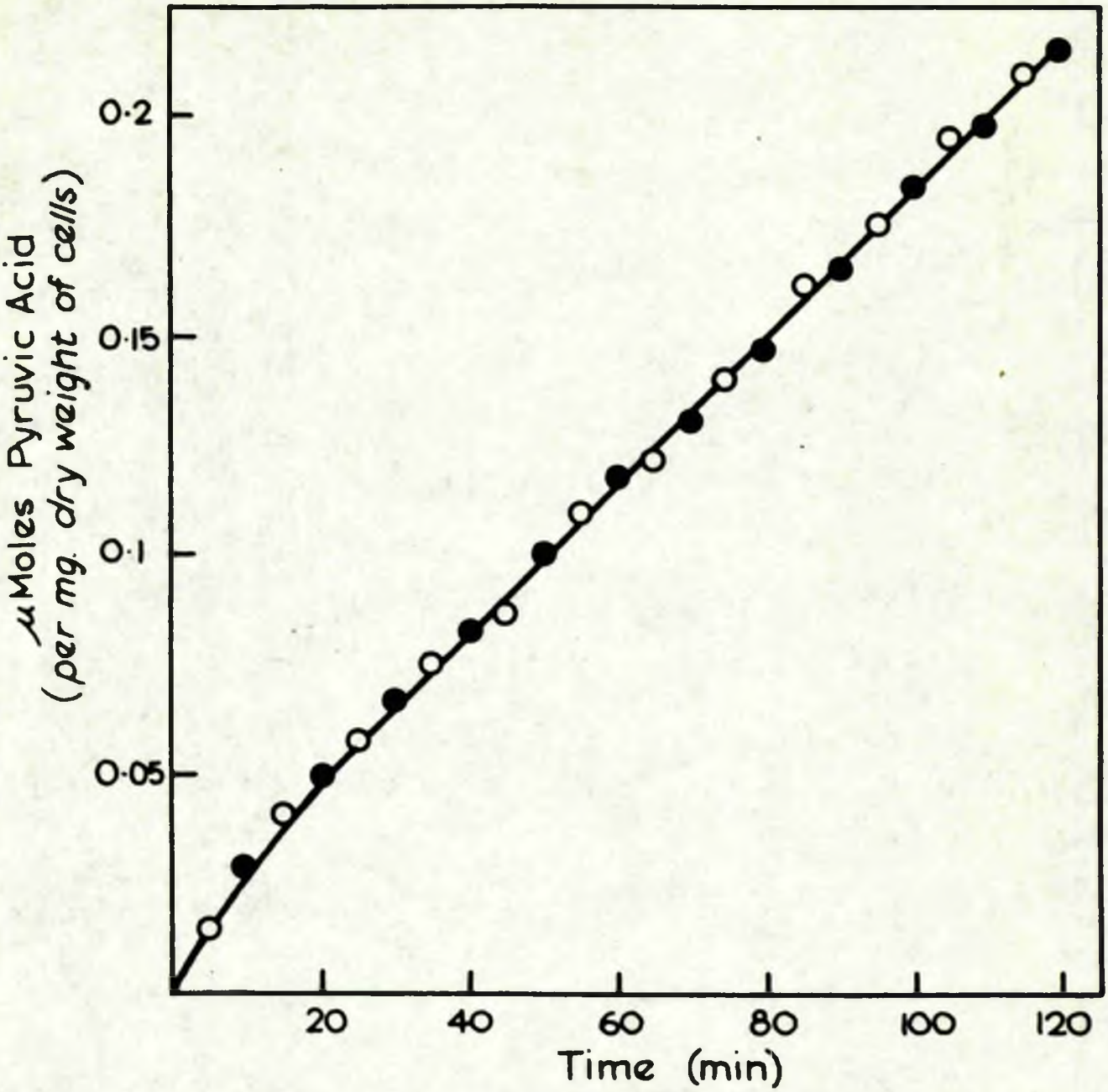
(v) Accuracy of the method. The general method is of a reproducible nature as is shown by fig.23 which represents pyruvic acid production in two experiments from which samples were taken alternately at intervals of 5 min.. The fact that the data from both tubes fall on the same line shows that the same amount of pyruvate is formed in each experiment.

(i) Reduction of 2,3,5-triphenyltetrazolium chloride.

The methods in the literature (Kopper, 1952, 1954) have been criticized previously and this section describes

Figure 23.

Graph showing reproducibility of washed cell suspension method. Production of Pyruvate from Glucose by *Sarcina lutea*.



o—o, *Reaction 'A'*; ●—●, *Reaction 'B'*; Each reaction mixture contained Glucose, 0·033M; KH_2PO_4, 0·032 M per litre, NaOH to pH 7·1; As_2O_3, 5·0 mM; Cells, 20·9 mg. dry weight per ml.; water to 25 ml.

how these criticisms have been met and the amended methods applied to the study of endogenous metabolism (EM) and assimilation studies.

(i) Estimation of reduction. In the methods already described the reaction is stopped and the formazan extracted from the cells by addition of acetone. The mixture is then centrifuged and the colour intensity of the supernatant determined. Even when this process is carried out in stoppered tubes there is a loss of acetone due to its volatility. In this work the acetone (b.p. 56°) is replaced by n-propanol (b.p. 97°) which is equally efficient both in stopping the reaction and in extracting the TPF. Because of its lower volatility the reaction mixtures are centrifuged in open centrifuge tubes, which is a practical advantage.

(ii) Calibration. The calibration procedure of Kun & Abood (1949) described above was criticized because it gave erroneous results due to precipitation of colloidal sulphur. This difficulty was overcome by basing the calibration on the pure sample of TPF obtained as described elsewhere. A known weight of this compound was dissolved in n-propanol and portions of the solution added to 2 ml. phosphate buffer (pH 7.1, 9 g. KH_2PO_4 per litre) and 2 ml. water and the whole made up to 10 ml. with n-propanol. The optical densities of these solutions were read at 484 mμ in a Unicam SP 600 spectrophotometer and the readings found to be proportional

to the concentration of TPF up to 0.1 mM. Changing the pH of the buffer used had no effect between pH 5.0 and 8.5. TPF formation was proportional to the time of incubation from 0 to 5 min. for Sarcina lutea grown and harvested as previously described.

(iii) Application to endogenous metabolism. The method is very simply applied to the measurement of EM by adding a TTC solution to a suspension of cells in buffer and allowing reduction to take place for a predetermined time. The reaction is stopped by addition of n-propanol, the TPF extracted by shaking and the solution of TPF separated from the denatured cells by centrifugation. The amount of reduction, which is assumed to be proportional to the EM, is estimated by measuring the intensity of colour in the TPF solution. Various protocols have been used in experiments of this type but the following is typical of the principles involved. 1 ml. of cell suspension (15 mg. per ml.) is pipetted into a 15 ml. centrifuge tube standing in a bath at 37°; 2 ml. of phosphate buffer (pH 7.1, 9 g. KH_2PO_4 per litre) are added and, when temperatures have equilibrated, 1 ml. of freshly prepared TTC solution (0.1% (w/v), preincubated at 37°), is added and the contents mixed by stirring with a fine glass rod. After any desired time interval, 6 ml. n-propanol are added and the TPF extracted by vigorous shaking. The tube is then centrifuged, the supernatant decanted and E_{484}

of the supernatant read in the spectrophotometer. This type of experiment can be used to determine changes in EM, such as those that occur when a washed cell suspension in water is vigorously aerated, or the system can be modified to examine the effect of inhibitors, temperature changes and pH variation on EM. In short, the cells may be treated in any way whatsoever and the effect of the treatment on EM studied by TTC reduction in a system similar to the one described above.

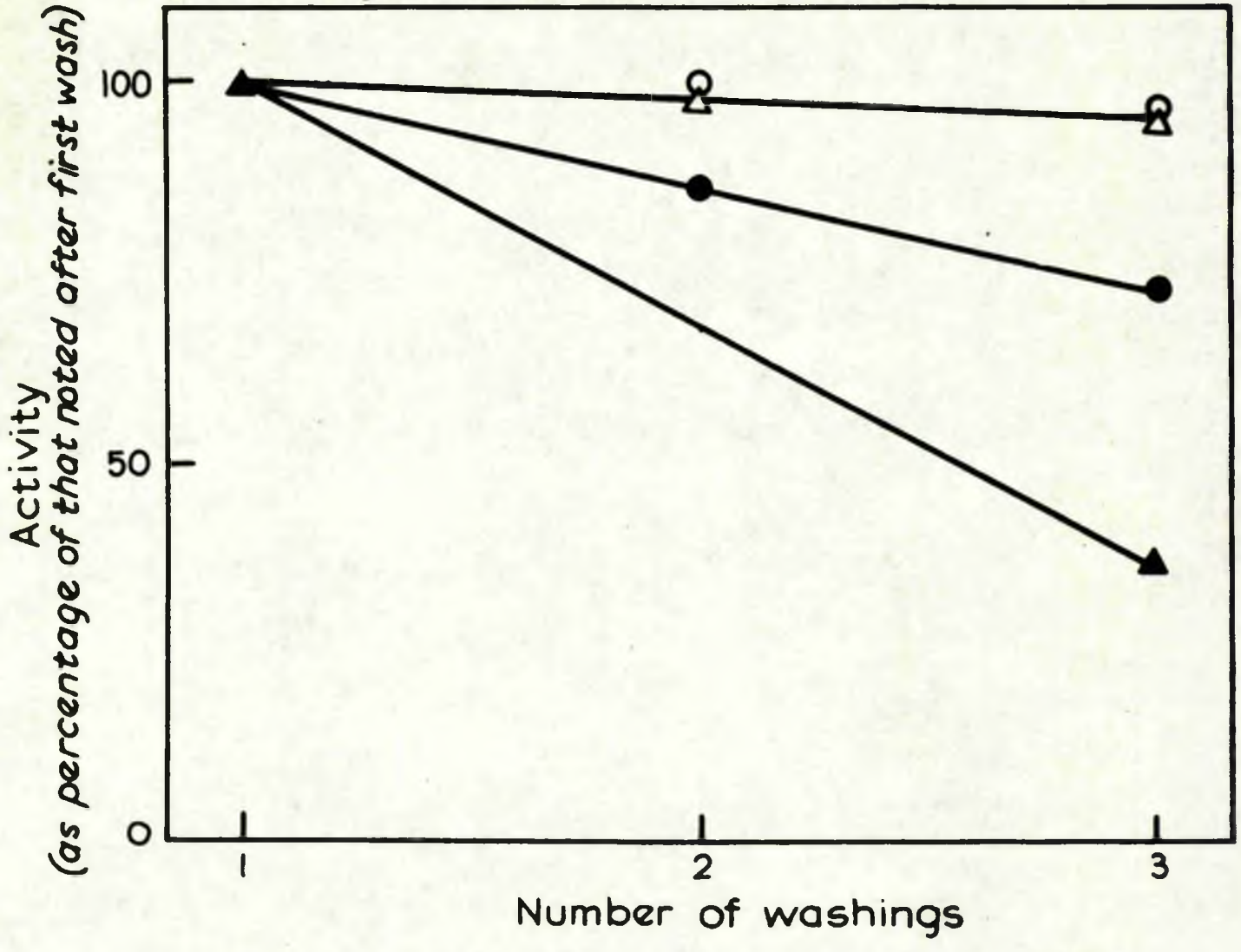
(iv) Application to assimilation. Kopper (1954) studied the assimilation of substrates by Escherichia coli in the following way:- a portion of cell suspension, in phosphate buffer, was incubated with substrate at 37° for 20 min. when the cells were spun down. The cell pellet was taken up in distilled water and centrifuged again. The packed cells were then taken up to a standard volume in water, buffer and TTC added, and the reducing power of the cells determined by a process similar to that described above. The increase in TPF formation due to preincubation with substrate is taken as an index of the assimilation of that substrate into the "reserve food" supply. Several criticisms have been made (X, b) of this approach which will be dealt with here on a practical basis.

Firstly, is the "assimilated substrate" chemically bound within the cell or is it perhaps physically attached or

contributing to some general pool of soluble intermediates? It would seem that washing the cells several times after their incubation with substrate would leach out material which was not strongly held. A preliminary experiment in which cells of Sarcina lutea, which had been incubated with glucose, were washed several times with water at room temperature showed that the TTC reducing activity decreased with each washing. When these data were examined it became obvious that another feasible explanation was that the assimilated material was being metabolised during the washing procedure. To settle this point a suspension of cells was incubated with U-^{14}C-glucose and thereafter divided into two portions which were washed three times with water at 0° and 35° respectively. After each wash the Q_{O_2}, TTC reducing capacity and ^{14}C-content of the cells were determined and the results expressed as a percentage of the value obtained after the first wash, as shown for TTC reduction and ^{14}C-content in fig.24. The Q_{O_2} values follow the same trend as ^{14}C-activity at each temperature. Several interesting features of this graph will be discussed later but, for the moment, it should be noted that the TTC reducing activity is only slightly diminished at 0° but is decreased by approximately two thirds in three washings at 35°. This indicated that the diminution in TTC reducing activity is a metabolic as opposed to a physico-chemical phenomenon. Because of this it is

Figure 24.

Effect of repeated washings at different temperatures on cells of *Sarcina lutea* which have previously metabolized U-^{14}C- Glucose



Open symbols, 0°; Closed symbols, 35°.
Δ, TTC reduction; O, ^{14}C- activity.

desirable to carry out washing procedures with ice-cold water, and this was done in subsequent work. In addition, this result indicates that the phenomenon being measured bears some relation to an assimilatory process in which material is more or less firmly bound to the cell.

The second objection to the work of Kopper (1954) is that he assumes the activity at 20 min. to be related to the speed of uptake when comparing the action of various substrates. But as different substrates may raise the TTC activity to different levels (and different rates) some of these values may be measured while the activity is still rising and others after it has reached a maximum. Comparison under these conditions is valueless and in this work a time-course of the reaction of each substrate was prepared to permit the calculation of relative activities.

In the light of the above findings a general procedure was adopted: cell suspension in buffer was incubated with substrate at 37° for definite time intervals and the cells spun down; the cell pellet was taken up in ice-cold distilled water and spun down again; the cell pellet was taken up to a definite volume in water and the TTC assay procedure carried out as described above (ii). Volumes and weights were varied to suit the experiment and, in the case of time-course experiments, it was found convenient to carry out the incubation

with the substrate in bulk in a conical flask (which was shaken in a bath at 37°) and to withdraw samples for centrifugation at the time intervals required.

(k) Preparation of cell-free extracts.

Attempts were made to obtain cell-free extracts by several methods including:-

(i) crushing cells in the Hughes' press at -25°,

(ii) grinding with alumina and with emery flour,

(iii) extraction of an acetone powder,

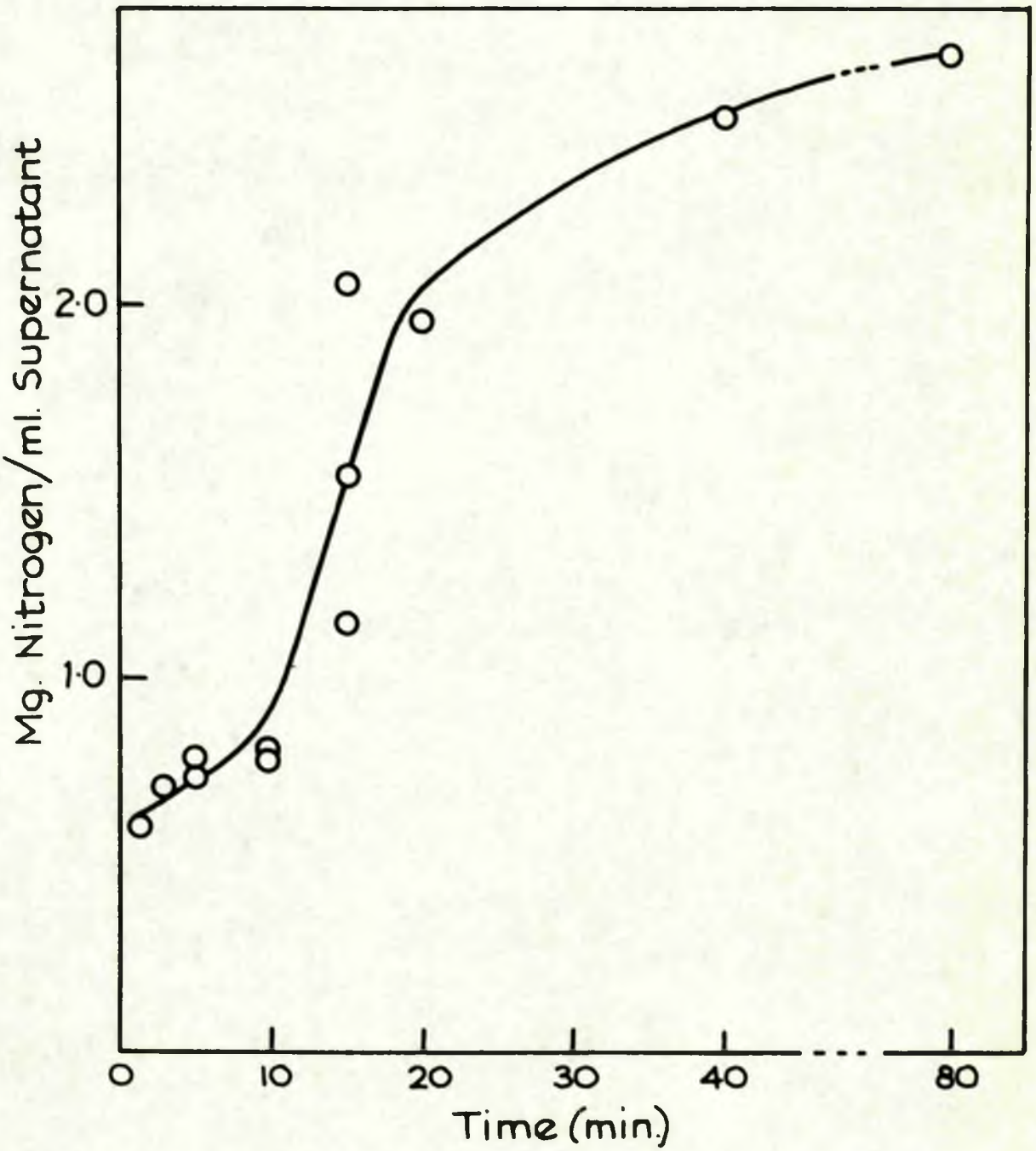
(iv) action of lysozyme, and

(v) shaking with glass beads in the Mickle disintegrator at 4°.

Cell-free extracts were obtained in every case except the Hughes' press, but it was impossible to demonstrate oxidative activity manometrically (Holms & Dawes, 1955). Extraction of enzymes as reflected by glucose-6-phosphate dehydrogenase (G-6-P dh.) activity was greatest in the extracts obtained from the Mickle shaker, but this method was considered unsatisfactory because of the long period of shaking required. A better method is that of Lamanna & Mallette (1954) in which cells and glass beads are rapidly agitated together in a homogenizer and the undisrupted cells and glass beads centrifuged down. Fig.25 shows the nitrogen liberated per ml. from such a system containing 400 mg. lyophilised

Figure 25.

Liberation of Nitrogen on disruption of *Sarcina lutea* by glass beads in homogeniser.



cells made up to 8 ml. with water and 4 ml. (dry volume) of Ballotini No. 12 glass beads. The apparatus used was an M.S.E. homogenizer (Melco), fitted with masticator and Universal 25 ml. container. It will be seen from the graph that there is an immediate release of nitrogen-containing material which remains fairly constant for the first 10 min., after which the value more slowly approaches a maximum. The metabolic activities of various fractions prepared in this way are described later.

(1) Enzyme assay systems.

Standard methods as described (Colowick & Kaplan, 1955) in the literature were used for all enzyme assays. In general these methods depended on the increase in E_{340}, which occurs when either DPN or TPN are reduced, or on the liberation of inorganic phosphate from organo-phosphorus compounds, or on the increase in acidity consequent on the phosphorylation of a hydroxyl group by ATP.

(i) E_{340} methods. All systems directly involving the reduction of the two coenzymes, DPN and TPN, or the oxidation of their reduced forms can be followed spectrophotometrically. Experiments of this nature were carried out in a Unicam SP 500 or Uvispek spectrophotometer in quartz cuvettes of 1 cm. light path. The general procedure is to set up two cuvettes each containing cell-free extract and buffer and then to add

water to one and substrate to the other. Observation of E_{340} at this stage indicates if any "endogenous" activity is occurring, such as would be noted if any amount of coenzyme were present in the extract. Coenzyme is now added to each cuvette and measurement of the change in E_{340} indicates the extent of dehydrogenation. The cuvette without substrate serves as control to the reaction.

Very many reactions other than simple dehydrogenations can be measured by this technique. For example, if an extract contains G-6-P dh and it will reduce TPN when presented with glucose and ATP, it must also contain hexokinase. By coupling together sequences of this type assays may be devised for a variety of enzymes.

(ii) Liberation of inorganic phosphorus. Many enzymes split off phosphorus from organo-phosphorus compounds. Measurement of this release by the methods for phosphorus estimation described above (b) can be used to follow the reaction.

(iii) Phosphorylation by ATP. In kinase action, where ATP is the phosphate donor, the reaction may be described at neutral pH as:

$$\text{Substrate} + \text{ATP} \longrightarrow \text{Substrate-P} + \text{ADP} + H^+.$$

The reaction can be followed by investigating the redistribution of phosphorus or by measuring the formation of H^+.

This latter can be done in several ways but one of the most convenient is to carry out the reaction in a buffer containing $NaHCO_3$ from which the H^+ formed displaces CO_2. The evolution of gas is then measured manometrically (Colowick & Kalckar, 1943).

(m) Chromatography.

Standard techniques were employed throughout using Whatman No.1 chromatography paper with both ascending and descending solvent irrigation.

(1) Carbohydrates. Solvents used were those of Morris & Campbell (1949):

Methanol:ethanol:water (45:45:10), and ethanol:water (90:10).

In addition a special system was used for phosphorylated carbohydrates (Burrows et al., 1952):

Formic acid:acetone:water (14:60:26).

Simple carbohydrates were detected by alkaline silver nitrate (Partridge, 1946) and aniline hydrogen phthalate (Partridge, 1949). The former method was modified as a dipping procedure: the paper is dipped in a saturated solution of silver nitrate in acetone, dried and then dipped in a saturated solution of potassium hydroxide in absolute ethanol. The latter spray consists of aniline, 0.93 g.; phthalic acid, 1.66 g.; water-saturated n-butanol to 100 ml. After spraying the paper is heated at 105° for 5 min.

Chromatograms were examined for seven-carbon sugars by the specific spray of Klevstrand & Nordal (1950) containing orcinol, 0.5 g.; trichloracetic acid, 15 g.; water-saturated n-butanol to 100 ml. After spraying the paper is heated at 105° for 15 - 20 min. Phosphorylated derivatives were detected by the spray of Burrows et al. (1952) containing ammonium molybdate, 1 g. in 8 ml. water; conc. hydrochloric acid, 3 ml.; perchloric acid (72% w/v), 3 ml.; acetone to 100 ml. The paper is dried in the cold when inorganic phosphate appears as a yellow spot. On irradiation with U.V. light phosphate esters appear as blue spots.

(ii) Keto-acids were separated as their 2,4-dinitrophenylhydrazones (XI, e) by the method of Cavallini et al. (1949). The solvent system consists of n-butanol, 80 ml.; ethanol, 22 ml.; ammonium carbonate buffer, 38 ml. The buffer is 1.5M with respect to both ammonium hydroxide and ammonium carbonate.

(n) Chemicals used.

Wherever possible chemicals of analytical reagent or laboratory reagent quality were used. The procedure for freeing substrates of calcium or barium has been described previously. The following less common or specially treated chemicals were used (supplier's name in brackets):

Gluconate, as sodium salt or lactone (B.D.H.).

2-Ketogluconate, as calcium salt (Light).

D-Ribose, purity checked by optical rotation (Light).

Glucosamine, as hydrochloride (B.D.H.).

Pyruvate, distilled in vacuo before use (B.D.H.).

∝-Ketoglutarate, recrystallised from acetone/benzene (Light).

iso-Citrate, as lactone (Light).

cis-Aconitate, as anhydride (Light).

6-Phosphogluconate was a gift from Dr. G.E. Glock.

Ribose-5-phosphate, as barium salt (Light).

ATP, as barium salt: purity checked by labile/total phosphorus ratio (Sigma Chem.Co.).

Hexosediphosphate, as calcium salt (Light).

Glucose-6-phosphate, as barium salt heptahydrate (Sigma Chem. Co.).

TPN 80, 80% pure (Sigma Chem. Co.).

DPN 96, 95% pure (Boehringer & Soehne).

Orcinol was recrystallised from benzene.

Peptone was Difco Bactopeptone.

Zwischenferment, 0.15 Kornberg units/mg. (Sigma Chem. Co.).

(c) Synthesis.

Both phenazine methosulphate and 2,3,5-triphenylformasan (TPF), being unavailable commercially, were synthesized.

(1) Phenazine methosulphate was synthesized according to the

method of Hillemann (1938). 8 g. of phenazine in 150 ml. dry, warmed nitrobenzene were heated on a boiling water bath for 7 min. with 37.9 ml. redistilled dimethyl sulphate and then cooled in ice. The precipitated crystals were harvested at the pump, washed with ether and recrystallized twice from absolute ethanol. The product had a m.p. of 160° (uncorrected) as compared with 155 - 7° quoted. It was stored in a vacuum desiccator in the cold.

(ii) TPF was synthesized by reduction of 2,3,5-triphenyltetrazolium chloride (TTC). Reduction by ascorbic acid (Jerchel & Fischer, 1949) and by sodium dithionite (Kuhn & Weitz, 1953) are quoted in the literature. The latter method was not used because it had already been noted that its use often caused the formation of a precipitate of colloidal sulphur. In addition, the m.p. noted after two recrystallizations from n-propanol and two from benzol was still 16° below that quoted for the product of ascorbate reduction. Accordingly, the following modification of the procedure of Jerchel & Fischer (1949) was adopted.

Two grams TTC are dissolved in 80 ml. distilled water and 1.2 g. ascorbic acid in 20 ml. distilled water added. Gradual addition of 2N NaOH gives first a red coloration and then a red precipitate of TPF. The precipitate is harvested at the pump and dried overnight in the desiccator (m.p. 142 - 8° uncorr.). (A portion of the TPF, in a pre-

liminary trial, gave a m.p. of 163 - 5° (uncorr.) when recrystallized from acetone. This process being unsatisfactory, a further modification was introduced.). The bulk of the precipitate is dissolved in acetone at room temperature and distilled water added until a copious precipitate is obtained. The suspension is then heated on a boiling water bath until most of the precipitate is dissolved, when the liquor is filtered hot. On cooling in ice a copious precipitate of small crystals of TPP is obtained which is harvested at the pump and dried in a vacuum desiccator. The m.p. of this product is 168 - 171°.

XII. EXPERIMENTAL METHODS WITH ^{14}C.

(a) Introduction.

The difficulties of interpretation of isotopic data are discussed later and this section deals with practical problems encountered during the course of this work. These are of two main types, namely, those associated with the counting apparatus used and the random nature of the phenomenon itself, and with the preparation and characteristics of the samples. Two main texts have been consulted - Calvin *et al.* (1949) and Sakami (1955).

(i) Apparatus. Counting is done by a combination of an end-window Geiger-Müller tube held in a lead castle and a Panax scaling unit. Planchettes are of normal design having an effective area on the raised portion of 1.5 cm.^{2}. They are cleaned by rubbing the surface with water and detergent powder using the finger. They are then washed with tap and distilled water and dried by pressing between sheets of filter paper. Planchettes are used only once. The method of plating CO_2 is described below (c).

(ii) The counting error of the instrument when presented with a known sample decreases with the time of counting. This is because both the actual count and the normal background measured by the instrument are random processes. In the present work times of counting were chosen in relation

to the activity of each particular sample so that this type of error was insignificant compared with the other experimental errors. In samples of high activity the number of impulses emitted may be so high that the recording mechanism cannot cope with them and the counter is said to have run into "dead time". In practice this error is avoided by the use of the lower stages in the lead castle which lowers the count registered. The conversion factors to correct counts obtained in this way to the theoretical value they would have attained on the top stage are calculated from the data obtained by counting standard samples on the different stages.

(iii) Self-absorption errors. With a weak β-emitter such as ^{14}C some of the particles are absorbed by the material itself. This phenomenon is of particular importance in thick layers of material and, indeed, a critical thickness exists for each system to which addition of further material does not increase the observed count. This state is known as "infinite thickness". It is possible to draw a self-absorption curve for any given system by plating and counting increasing amounts of labelled material and expressing the results graphically. If the quantities have been well chosen the counts are initially proportional to the amount of material plated, but with increasing quantities diminished returns are observed. From these observations two methods

of correcting or eliminating self-absorption are apparent. Firstly, if a self-absorption curve has been drawn and if the thickness of the sample is known, a correction may be applied. Secondly, if no curve is available, diminishing amounts of material may be plated until successive counts are proportional to the weight plated. At this point self-absorption has been reduced to negligible proportions. Both these methods are used in this work. An additional method is to add a known amount of ^{14}C-activity to a planchette which has been already counted, and if the increase in observed count is less than that added the diminution is directly attributable to self-absorption and a correction can be made. By these procedures all counts quoted in this thesis have been corrected for self-absorption.

(iv) Plating of samples. In plating samples uniform distribution of material on the planchette is obviously necessary to maintain reproducibility. If there is any localisation of deposition, unknown self-absorption errors may also be introduced. Placing a drop of ethanol on the planchette prior to adding the radioactive solution disperses the material uniformly and was adopted routinely. The general method employed with aqueous solutions is as follows. A drop of ethanol is placed on the planchette followed by 0.2 ml. of the radioactive solution added from a 0.2 ml.

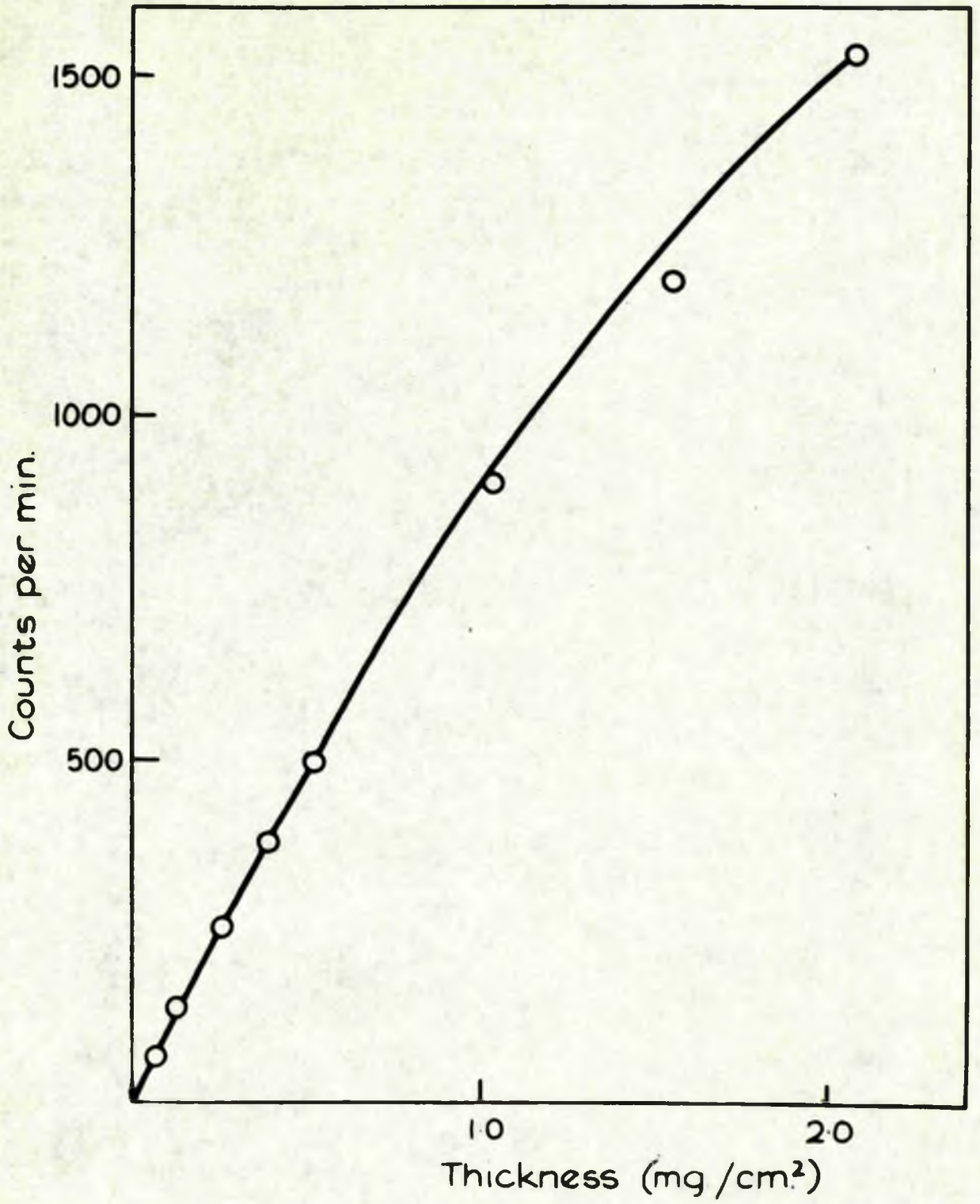
graduated pipette (E-Mil Gold Line). The tip of the pipette touches the surface of the ethanol layer at the beginning and end of delivery. The planchette is then dried under an infra-red lamp. For non-aqueous solutions the ethanol is unnecessary as these generally spread easily and, indeed, with solvents such as ethyl acetate, less than 0.2 ml. must be used otherwise the liquid spreads over the edge of the planchette.

(b) Cells.

(i) Self-absorption. The self-absorption curve of ^{14}C-labelled Sarcina luten was obtained by plating increasing amounts of cells which had been separated from a suspension in which they had metabolized U-^{14}C-glucose. The result is shown in fig.26 where it will be seen that the self-absorption is quite surprisingly small. The weights added to each planchette are derived from the weight of the original cell material which was an actual weight of lyophilized cells. Now the dry weight obtained by processes other than freeze-drying is always higher and if fig.26 were expressed as a function of the actual weight of material (which contains water) on the planchette, the self-absorption would be proportionally lower. As lyophilized cells were invariably used in the isotopic experiments, the curve presented is the one relevant to the experimental data.

Figure 26.

Self-absorption Curve for S. lutea Labelled with ^{14}C.



(ii) Preparation of samples. In general, a portion of the reaction mixture is centrifuged and the pellet of cells taken up in ice-cold distilled water and centrifuged again. The washed cells are then transferred quantitatively to a volumetric flask which is made up to volume and a portion of the suspension is plated for counting. Almost invariably the following sequence is involved:- 3 ml. reaction mixture, containing 100 mg. dry weight of cells, are pipetted into a well-chilled centrifuge tube and spun down rapidly; the supernatant is decanted off and the cell pellet taken up in 10 ml. ice-cold water and transferred to a larger well-chilled centrifuge tube; after centrifugation the supernatant is decanted off and the washed cells transferred in ice-cold water to a 25 ml. volumetric flask and made up to 25 ml. with water; 0.2 ml. of this suspension is immediately plated as described above, dried and counted. The actual amount plated is equivalent to 0.53 mg. per cm.^{2} at which level self-absorption is negligible (fig.26).

(g) Solutions and supernatants.

(i) Preparation of samples. Solutions and supernatants are generally prepared by the procedure outlined above using ethanol to ensure uniform distribution. The amount plated is usually 0.2 ml. When unbuffered supernatants containing volatile fatty acids are plated a drop of weak alkali is

added to the planchette to prevent possible loss of material.

(ii) Self-absorption. In many cases the amount plated is so small that self-absorption is negligible and can be proved by plating half the quantity and showing that the count is halved. In other cases, such as when buffer or large amounts of inhibitor are present, self-absorption is significant and is corrected in the following way. 0.2 ml. of supernatant is plated and counted (x counts/min.) and 0.1 ml. of a suitable dilution of carrier-free glucose-U-^{14}C is also plated and counted (y counts/min.). The amount of glucose-U-^{14}C is so minute that there is no chance of self-absorption. 0.1 ml. of the same glucose-U-^{14}C solution is now added to the first planchette and this combination counted again (z counts/min.). Now, as self-absorption is significant on this planchette, z is less than x + y, and the percentage of self-absorption is given by $100(z - x)/y$. The factor thus obtained is then used to correct the value of x.

(d) Steam-volatile acids.

(i) Separation. Steam-volatile acids are separated from reaction supernatants by addition of sulphuric acid to approx. N and carrying out steam-distillation in a Markham still. The distillate is collected in portions of approximately six times the volume of supernatant used and neutralized by titration with a micro-burette containing 0.02 N NaOH. As

the volumes of distillate obtained are very large in comparison with the content of acids, it is necessary to concentrate them. However, if an internal indicator is used in the neutralization procedure it would also be concentrated and the amount of dye be large in proportion to the active material. Accordingly a pH meter (Mairhead & Co.Ltd., type E417 A) is used for the titration. Unfortunately the contact to the calomel half-cell is by means of a 3.5M KCl bridge and, as KCl contains a significant proportion of the radioactive isotope ^{40}K, it is undesirable to add even a small amount of this material to the system. The difficulty is obviated by leading the KCl bridge into a large reservoir (500 ml.) of 3.5M NaCl and thence a NaCl bridge is led to the titration assembly. The distillates are titrated to pH 8.5 and then all the fractions containing a significant amount of titratable acidity are evaporated to dryness in a continuous vacuum distillation apparatus at 45°. Two drops of 0.04% (w/v) phenol red are added at this stage to serve as an indication that complete neutralisation has been achieved. The residue obtained is taken up in a small volume of water.

(ii) Fractionation. The concentrated steam-distillate is then fractionated by the chromatographic procedure of Phares et al. (1952). This is a column method in which the solid support is celite 545 (10 g. + 8 ml. 0.5N H_2SO_4) and the

liquid phase is ether saturated with 0.5N H_2SO_4. The effluent is titrated with dilute NaOH and the peaks identified by reference to histograms of known materials, by reference to the position of the peak in relation to the emergence of the small amount of phenol red which is eluted from the column, and by specific colour reactions for certain compounds (e.g., pyruvic acid). The method was applied with the following modifications. The concentrated steam-distillate obtained above is taken up in 10 ml. distilled water, acidified with 1 drop of 2N H_2SO_4 and applied to the top of a column 45 x 1 cm. Elution is by ether saturated with 0.5N H_2SO_4 and 5 ml. portions of the effluent are collected in 1 x 2 in. specimen tubes containing 2 ml. distilled water. These tubes are transferred to the microburette, a stream of CO_2-free air is passed for 3 min., 1 drop of 0.04% (w/v) phenol red added and the whole titrated with approx. 0.02N NaOH to the end-point. The NaOH is standardized daily by titration against standard HCl. The remaining top layer of ether is evaporated off by immersing the tube in warm water and a portion of the aqueous phase is plated and counted. By these methods acetic and pyruvic acids have been separated from reaction mixtures and their specific activity measured. In all cases a peak corresponding to fumaric acid has been isolated but has not been further examined.

(iii) Pyruvic acid. A simpler method has been worked out to obtain the specific activity of pyruvic acid. The 2,4-dinitrophenylhydrazone is isolated and purified by differential extraction into toluene or ethyl acetate, then into 10% sodium carbonate and finally into ethyl acetate. The amount of material in the final ethyl acetate solution is determined spectrophotometrically as described previously and a portion is also plated for counting. These data yield a measure of specific activity. As most of the isotopic argument is based on the specific activity of pyruvic acid, and as much of the experimental time was devoted to its determination, it may well be asked why this simple method was not used to the exclusion of the more cumbersome method described above. It did seem possible that some unknown, non-keto acid material could be carried with the hydrazone in the solvent extraction procedure. This material could feasibly be labelled and thus yield falsely high specific activities. Comparison of the specific activities of pyruvic acid isolated from an actual experimental supernatant by the two methods gave identical values for specific activity, thus proving the validity of each method beyond reasonable doubt.

(e) Carbon dioxide.

The collection and counting of $^{14}CO_2$ is, in our

experience, one of the most difficult isotopic techniques to master. Much time was spent in devising and checking the methods described in this section.

(i) Collection of CO_2. Evolved CO_2 is trapped in CO_2-free NaOH which is made by filtering saturated NaOH (in which Na_2CO_3 is insoluble) through a sintered glass filter and rapidly diluting a portion with CO_2-free water (which is freed of CO_2 by boiling). All solutions in contact with the atmosphere are protected by soda lime tubes. When $^{14}CO_2$ is evolved in a Warburg flask it may be trapped in the centre well and at the end of the experiment the filter paper concertina removed to a graduated vessel, the centre well washed several times with CO_2-free water and the whole made up to a given volume. In our work $^{14}CO_2$ was more frequently evolved in a system which was aerated with CO_2-free air. In these cases the $^{14}CO_2$ is collected in alkali held in specially made traps placed at the end of the aeration train. The alkali is then transferred quantitatively with CO_2-free water to a volumetric flask and made to the mark.

(ii) Precipitation of $BaCO_3$. The $^{14}CO_2$ is counted as the insoluble carbonate of barium which is precipitated on to a filter paper disk. The apparatus used is similar to that described by Sakami (1955) except that it is made of brass and holds a highly polished stainless steel sleeve. This

cylinder is tightly held on a sintered glass filter disk by coiled springs and between the two are clamped two disks (2 cm. in diameter) of Whatman No.50 filter paper. The filtrate is collected in a Buchner flask to which suction may be applied. The principle of the apparatus is that any precipitate formed in the cylinder may be deposited on the top of the two filter papers held at its base. The physical characteristics are such that little or no filtration occurs unless suction is applied. The internal diameter of the cylinder is such that the area of the deposit formed on the filter paper disk is identical with that of the deposit obtained on the planchette in the normal process of plating samples.

The apparatus is used as follows: 3 ml. of CO_2-free 5% (w/v) $BaCl_2$ are pipetted into the cylinder without suction being applied. 1 ml. of the $^{14}CO_2$ solution, obtained as described above, is now added dropwise and the mixture stirred for 1 min. with a fine glass rod. Suction is applied and the precipitate of $BaCO_3$ washed to the bottom of the cylinder with CO_2-free water and then the cylinder is removed. The filter paper with its cake of $BaCO_3$ is now held to the sintered glass disk by the suction of the pump and is washed successively with water, acetone/water, and acetone. It is sucked dry, the suction is interrupted and the filter paper transferred to the counting tray. It is

held on a polythene disk in the centre of the tray by a brass ring in such a position that the surface of the filter paper occupies the same position relative to the end window of the counter as does a metal planchette. The diameter of the $BaCO_3$ cake is the same as that of a planchette and as it is placed in the same relative position it may be assumed that the systems are geometrically identical and therefore counts obtained in the two ways are strictly comparable.

(iii) Corrections. Two corrections can be applied when using this method. The first of these corrects for loss of $BaCO_3$ in the filtration apparatus which inevitably occurs by adhesion to the sides of the cylinder. The procedure adopted is to transfer separately the filter paper disk and 1 ml. of $^{14}CO_2$ solution to each of two Warburg flasks and make up to 2.5 ml. with CO_2-free water. After equilibration, 0.5 ml. 5N HCl contained in the side arm is tipped and the evolved CO_2 measured in the usual way. The difference between the two values represents the loss of $BaCO_3$ in the filtration apparatus which may therefore be corrected. The other possible error is that due to self-absorption, but the amount of CO_2 evolved from the filter paper disk is a measure of the amount of $BaCO_3$ present and its thickness may be calculated. Reference to a self-absorption curve for $BaCO_3$ enables a correction to be applied. The $BaCO_3$ self-absorption curve

is made by precipitating increasing amounts of $Na_2{}^{14}CO_3$ using the method described above.

When the $^{14}CO_2$ is very dilute the proportional loss in the plating technique can be extremely high and it is better under these conditions to add a known amount of carrier Na_2CO_3 to the solution when it is made up to volume.

(f) Distribution of ^{14}C within pyruvate.

A simple method may be used to determine the distribution of ^{14}C between the carboxyl and the other two atoms within the pyruvate molecule. This is achieved by the action of ceric sulphate in sulphuric acid (Krebs & Johnson, 1937) which splits off the COOH group as CO_2 in a special apparatus. This may be swept out by a stream of CO_2-free air, the $^{14}CO_2$ collected in CO_2-free alkali and subsequently plated as $BaCO_3$. The amount of $^{14}CO_2$ evolved is usually so small that carrier Na_2CO_3 has to be added as described above.

(g) General experimental procedures.

Isotopic experiments are usually carried out by the washed cell suspension technique described above. Aeration is with air stripped of CO_2 by passing through strong alkali in a Dreschel bottle. The issuing air stream is passed through CO_2-free alkali contained in a CO_2 trap. The general

protocol is as follows:- 12 ml. of cell suspension (50 mg./ml.) and 2 ml. of water (or inhibitor or other addition) are pipetted into the reaction tube (8 x 1½ in.) and which is sealed with a rubber stopper carrying the capillary aeration tube and a gas exit tube. The reaction vessel is then connected to the aeration train, placed in the water bath to equilibrate to temperature, and swept free of CO_2. Connexion is now made to the CO_2 trap and the time of addition of 4 ml. ^{14}C-labelled substrate (e.g., 18 mM glucose) is taken as zero time. The system is then treated in one of two general ways. Firstly, the $^{14}CO_2$ is collected in portions by switching from one CO_2-trap to another by means of a three way glass stopcock, and at the conclusion of the experiment the cell suspension is rapidly chilled and separated into cells and supernatant by centrifugation. The alternative procedure is to withdraw samples (usually 3 ml.) at intervals into chilled centrifuge tubes and to separate the cells and supernatant by centrifugation. In the latter case the $^{14}CO_2$ is collected to avoid contamination of the atmosphere but it is not counted. The various fractions obtained in these ways are then examined as described earlier.

(h) Fractionation of cell material.

Three different methods of fractionation were used in the treatment of ^{14}C-labelled cell material. These are:-

(i) Trichloroacetic acid/ethanol/ether. This method was a modification of that described by Roberts et al. (1955). The general principle is to treat the cells in a centrifuge tube with a solvent, separate the debris from the supernatant, and then treat it with a further solvent. 8 ml. 5% (w/v) trichloroacetic acid (Tca) are added to about 90 mg. of cells (dry weight) in a centrifuge tube which is held at 5° for 30 min. The contents are then centrifuged - the supernatant is the cold-Tca-soluble fraction and the debris is further extracted with 8 ml. 75% (v/v) ethanol for 30 min. at 42°. Centrifugation yields the alcohol-soluble fraction and the debris is taken up in 8 ml. of a mixture of equal parts of 75% ethanol and ether. After 15 min. at 42°, centrifugation yields the alcohol-ether-soluble fraction and the residue is further extracted with 8 ml. 5% Tca for 30 min. in a boiling water bath. Centrifugation yields the hot-Tca-soluble fraction and the residue is the residual protein fraction. The alcohol-soluble fraction may be separated into the alcohol-soluble-ether-soluble and alcohol-soluble-ether-insoluble fractions by addition of equal volumes of ether and water and separation of the two phases thus formed. The various samples and residues are then plated and their radioactivity determined.

(ii) Phenolic extraction, by the method of Westphal et al., (1952) was attempted on a reduced scale. 1.5 g. dry weight of cells are taken up in 50 ml. water and stirred into 50 ml. 90% phenol at 68°. After continuous stirring at 68° for 30 min. the mixture is centrifuged and the aqueous layer removed. A further 50 ml. of water are added to the phenolic liquor and the whole stirred at 68° for a few minutes. After centrifugation, the aqueous layer is removed and added to the first fraction.

The greatest difficulty encountered in this method was the poor resolution of the two phases, and also of importance is the great difficulty inherent in handling 45% phenolic suspensions at 68°.

(iii) Sodium hydroxide. A method of alkaline fractionation was evolved in this laboratory. About 1 g. of cells (dry weight) is extracted at 4° for several days with 1.5N NaOH and then centrifuged down. The separation is only completed at 100,000 g. for 60 min., but less extreme treatment gives a good yield of supernatant. 7.5 ml. of this supernatant are acidified (to pH 1.0) with 1.9 ml. 6N HCl and the precipitate (A) centrifuged down. The supernatant is then brought to pH 7 with 1.5N NaOH, when the addition of an equal volume of ethanol brings out a copious white precipitate (B). B is centrifuged down, separated from the pale yellow super-

natant and washed with ethanol/ether and with water. Qualitative tests show that A is protein in nature but that B definitely contains carbohydrate.

(i) Chromatography.

Hydrolysates of the various fractions of ^{14}C-labelled cell material were examined by two-dimensional chromatography (Roberts et al., 1955). The solvent in the first dimension was sec-butanol (70 ml.), formic acid (10 ml.) and water (20 ml.) and in the second was phenol (80 g.), conc. ammonia (0.3 ml.) and water (20 ml.); the first solvent was used by descending and the second by ascending technique. The carbohydrate sprays described above (XI, m, i) were used and, in addition, amino-acids were detected by dipping in ninhydrin (0.1%, w/v in acetone).

Autoradiograms were made of the developed chromatograms by placing them in contact with X-ray film for periods of up to two weeks.

RESULTS

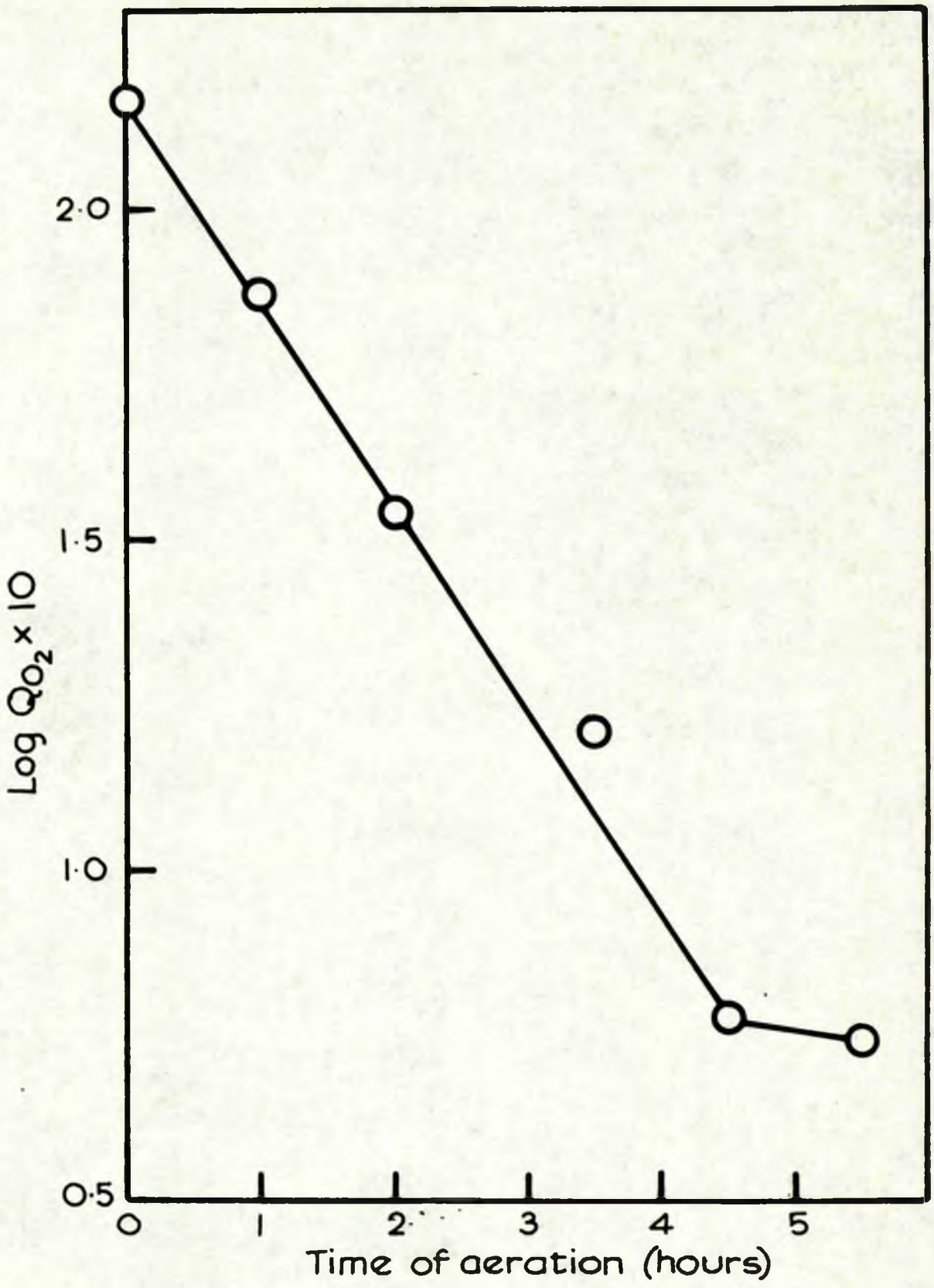
XIII. EXPERIMENTS WITH WASHED CELL SUSPENSIONS.

(a) Manometric Experiments.

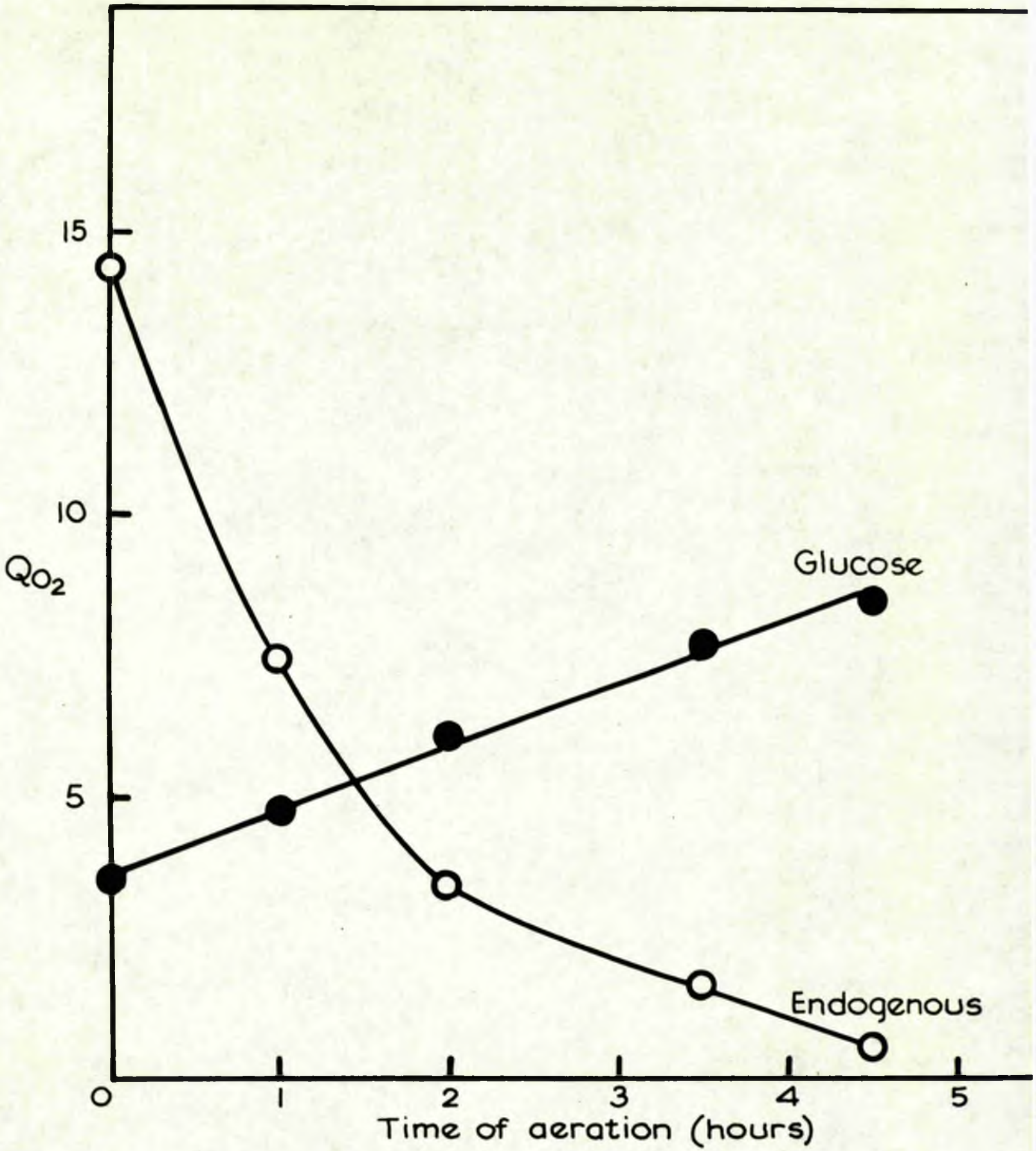
(1) Endogenous metabolism. Cells were harvested from the aerated growth medium described above, washed three times with water and used directly without further treatment. About 50 ml. of the suspension (approx. 30 mg. dry wt. per ml.) were placed in a large tube held in a bath at 37° and briskly aerated with water-saturated air at the rate of 800 ml. per min. 5 ml. samples were collected at intervals and the cells separated from the supernatant by centrifugation. The cell pellets were each taken up in 5 ml. water and the resulting suspensions used to determine the oxygen uptake both endogenously and with addition of glucose or glucosamine. Other portions of suspensions were used to determine the cell density and polysaccharide content by the anthrone method, as described above (XI, d, iii). The protocol for the Warburg experiments was:
cells, 1.0 ml.; 0.066 M phosphate buffer pH 7.1, 1.3 ml.; substrate (10 mM), or water, 0.5 ml. in the side-arm; 0.2 ml. 20% (w/v) KOH in centre well; temperature 37°; shaking, 140 cycles per min.
The results, expressed as Q_{O_2} values, are shown in figs.27 and 28. It will be seen that while ER decreases logarithmically, the Q_{O_2} (glucose) increases arithmetically. The

Figure 27.

Effect of aeration on endogenous oxygen consumption of *S. lutea* 'G'



Effect of previous aeration on Q_{O_2} values of endogenous and glucose metabolism ~ *S. lutea* 'G'



duplicates of anthrone determinations were not in very good agreement but there was no indication of any diminution in polysaccharide as aeration proceeded. Pentose estimations on the supernatant showed a linear production of a chromogen-producing material corresponding to a final concentration of almost 0.2 mM.

(ii) Addition of Substrates. Sarcina lutea exhibits oxygen consumption in the Warburg manometer with a variety of substrates. Cells as harvested show oxygen uptake with hexoses, glucosamine, pyruvate and a few Krebs' cycle intermediates but not with G-6-P, pentoses, gluconate or 2-keto-gluconate. Reduction of ER facilitates the measurement of the low response of these substrates and freeze-drying gives a further variation as shown in table 2. The reaction of Krebs' cycle intermediates in this system is seen in table 3. Reference to Q_{O_2} (glucose) reveals that the cells in the second group of experiments were very much more active than those used in the earlier work. Variation in metabolic activity is frequently found and when comparison between cell batches is desired reference is made to the activity with glucose as, for example, in table 2.

The initial rapid rate of oxygen uptake with glucose as substrate falls off when about 1.5 moles of oxygen per mole of substrate have been consumed. An experiment with

Table 2.

Q_{O_2} values for Sarcina lutea (E.R. reduced).

Substrate	Intact Cells	Dried Cells
None	3.5	2.3
Glucose	12.7	3.9
Glucosamine	9.3	2.0
Glucose-6-phosphate	0.0	0.0
Hexose-1,6-diphosphate	0.0	0.0
Gluconate	1.0	1.3
2-Ketogluconate	0.8	1.6
Ribose	0.7	1.4
Pyruvate	10.7	2.4
Lactate	19.4	-
∝-Ketoglutarate	8.0	-
Citrate	0.6	1.3
Succinate	8.4	2.4
Malate	7.3	2.7 *
Mannose	2.1	-
Galactose	1.1	-
Fructose	2.5	-
2,3,4,6-Tetramethylglucose	0.0	-
Arabinose	0.7	-
Xylose	0.4	-
Glucose-1-phosphate	0.0	-
∝-Glycerophosphate	0.0	-
Sucrose	-	2.0
DL-Alanine	-	2.2

* Values below the line were obtained with different cell samples and corrected by reference to Q_{O_2} (glucose).

Table 3.

Q_{O_2} values for intermediates of the tricarboxylic acid cycle using lyophilized cells.

Substrate	Q_{O_2}
Glucose	10.6
Pyruvate	8.5
Acetate	5.5
Citrate	6.3
cis-Aconitate	0.0
iso-Citrate	0.2
α-Ketoglutarate	0.4
Succinate	16.4
Fumarate	19.8
Malate	8.4
Oxaloacetate	12.8

dried cells showed that the initial rate of uptake could be restored at this point by a further addition of glucose.

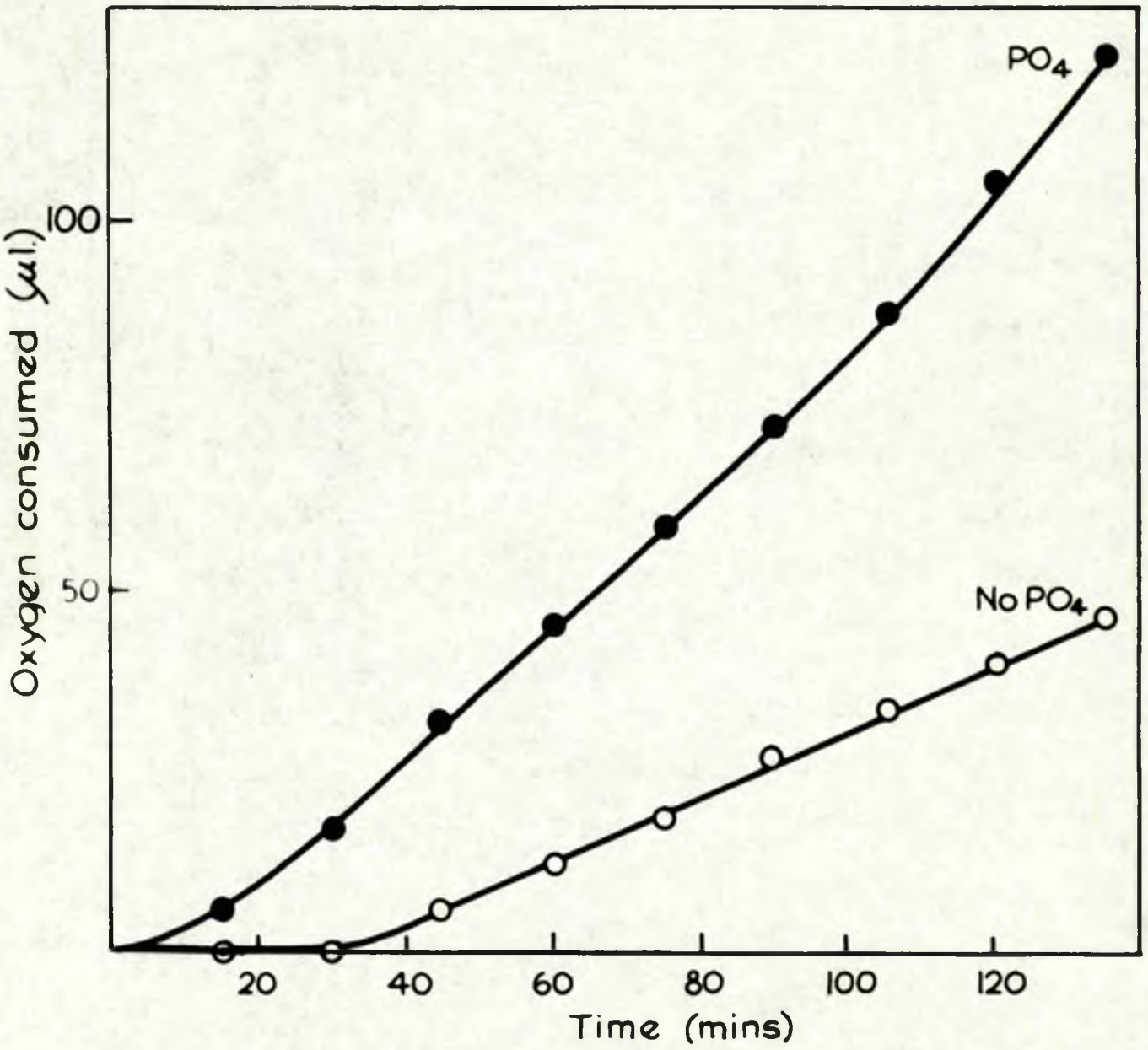
An experiment in which glucose and glucose-6-phosphate were used as substrates singly and in combination revealed that there was no change in the treatment of these substances when added together. Furthermore Murray (1956) demonstrated that a preparation of protoplasts of Sarcina lutea, which oxidises glucose in the same way as whole cells, does not oxidize glucose-6-phosphate.

(iii) Effect of phosphorus. No difference was noted in oxygen uptake with glucose or glucosamine when 0.09 M tris(hydroxymethyl)amino-methane buffer (tris) was substituted for the phosphate buffer when using intact cells. However, freeze-dried cells which had been washed six times at various stages of their preparation showed a very low metabolic activity which could be increased by the addition of inorganic phosphate. A suspension of these cells was dialysed overnight against running tap water and tested manometrically in tris buffer as shown in fig.29. It will be seen that addition of phosphate greatly stimulates oxygen uptake. The 'phosphate-deficient' cells were tested in other systems (q.v.).

Addition or omission of $MgSO_4$ had no effect on oxygen uptake with glucose or glucosamine.

Figure 29.

Oxygen uptake by dialysed S. lutea in 0·033 M tris buffer with glucose as substrate.



Endogenous respiration has been subtracted

●—● 5 µmoles glucose: Final PO_4 concentration of 0·007 M

○—○ 5 µmoles glucose: No PO_4.

(iv) Effect of pH. Changes of pH were studied using the normal protocol but varying the pH of the buffer used. Glucose, tested in a range of phosphate buffers from pH 5.5 to 8.0, gave the fastest oxygen uptake from pH 7.1 to 7.5. It was thought that gluconate and 2-ketogluconate might more easily penetrate the cell at lower pH values and these were tested with glucose as reference compound. The oxygen uptake with gluconate is proportionally increased with reference to both glucose and 2-ketogluconate, as shown in table 4.

(v) Effect of inhibitors. The fall-off in oxygen uptake with glucose as substrate has been mentioned above; in the presence of 2×10^{-3}M arsenite the point of inflection is clearly defined at 1 mole of oxygen per mole of substrate as seen in fig.30. A concentration of iodoacetate of 10^{-4}M or more has the same effect (fig.31). By addition of inhibitors and following oxygen uptake the data given in table 5 were obtained for glucose as substrate.

As will be seen in this table, 0.4×10^{-2}M azide causes 89.4% inhibition of the initial rate of glucose oxidation. The total oxygen uptake is also lowered and at this concentration gives a final oxygen uptake of only 40.1% of that obtained in its absence. Lower concentrations of azide (2×10^{-3}M) do not affect the respiration, while higher concentrations depress both the rate and total oxygen uptake.

Effect of Arsenite on Oxygen Uptake by *Sarcina lutea* at two different glucose levels.

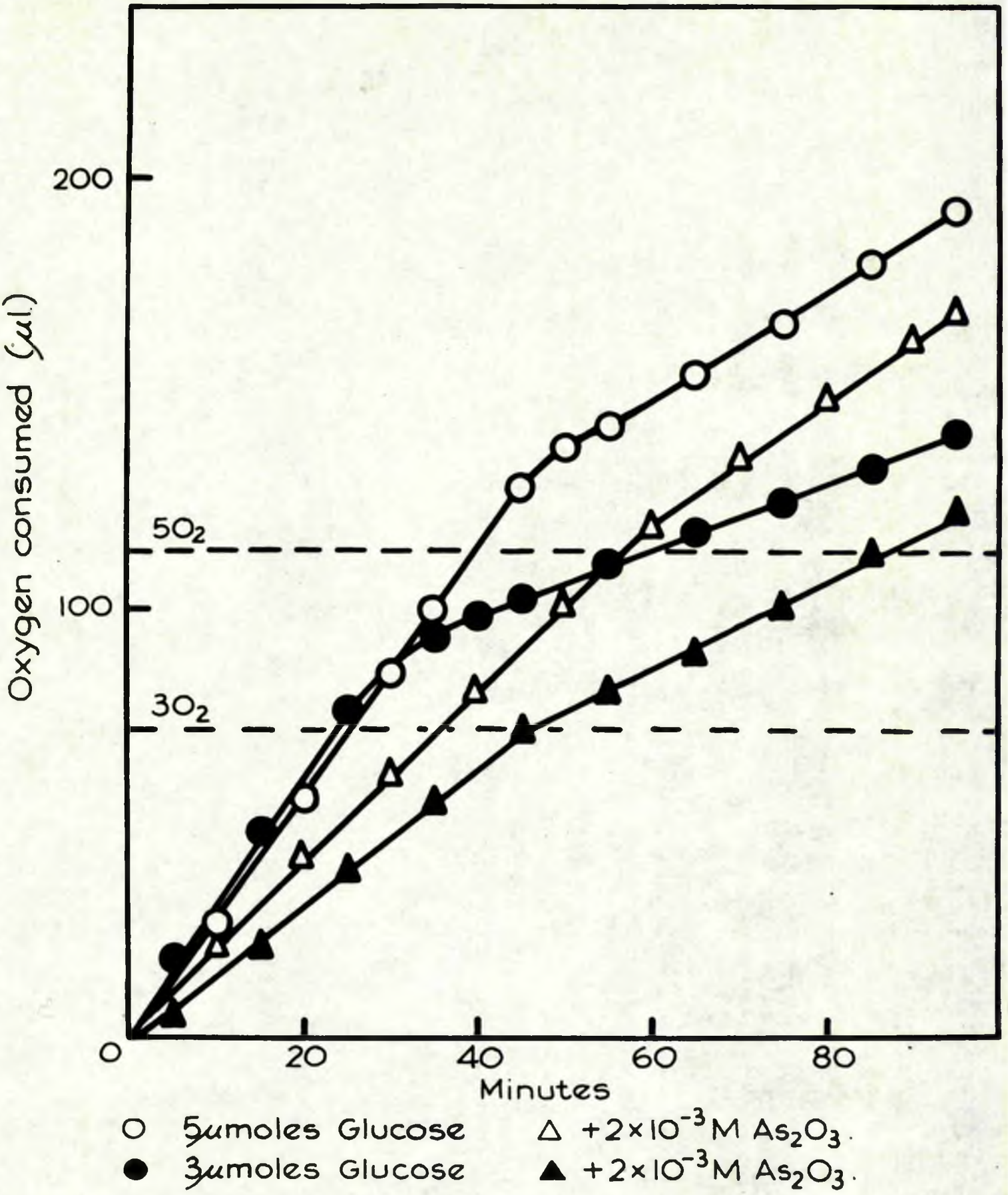


Figure 31.

Effect of Iodoacetate on Oxygen Uptake by *Sarcina lutea* with Glucose as Substrate.

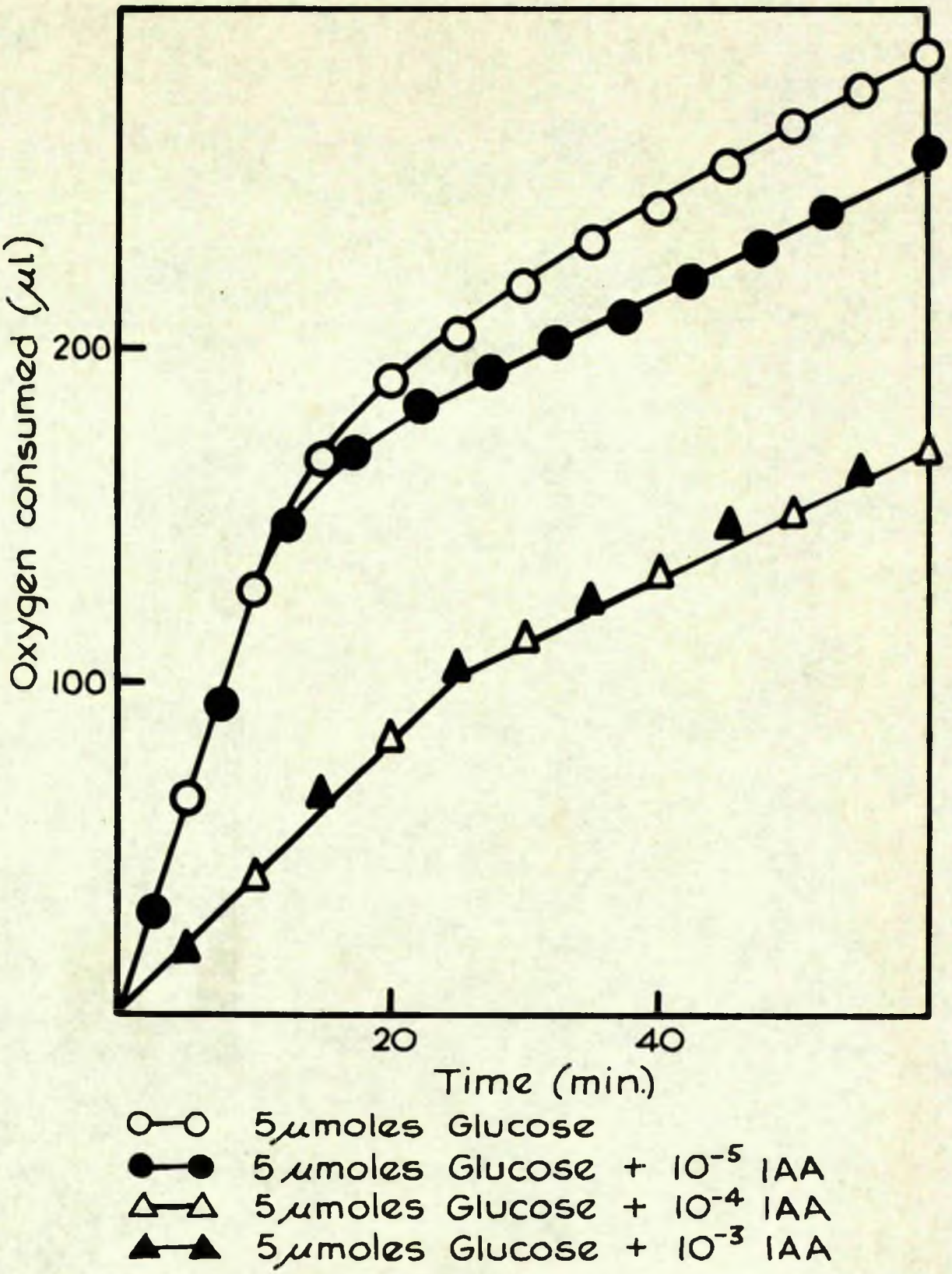


Table 4.

Ratios of oxygen uptakes relative to gluconate, with glucose and 2-ketogluconate at different pH values.

(The values in parentheses are the uptakes on gluconate in µl. per 60 min., based on initial rates.)

pH	Glucose/Gluconate	2-Ketogluconate/gluconate
8.0	5.9 (57.0)	4.6 (57.0)
7.1	-	1.9 (75.5)
5.5	0.98 (61.0)	1.3 (61.0)

Table 5.

Action of inhibitors on oxygen uptake with glucose as substrate.

Inhibitor	Concentration (M) ($\times 10^4$)	Percentage Inhibition
Arsenite	20	30.0
Iodoacetate	1.0	70.0
Sodium fluoride	100	0.0
Cyanide	1.0	43.0
Malonate (pH 6.0)	100	50.0
Azide	40	59.4

In all cases the percentage inhibition is calculated on the basis of initial rates of oxygen uptake with 5 µ moles glucose.

Further experiments of the same nature using 2:4-dinitrophenol (DNP) at 10^{-4} to 10^{-2}M yielded variable results but, in general, the initial rate of uptake was depressed while the final uptake could be increased to as much as 130%.

(vi) Conditions of aerobiosis. Oxygen must be present for metabolic activity in the manometric apparatus as evidenced by lack of carbon dioxide evolution from bicarbonate buffer under anaerobic (95% N, 5% CO_2) conditions.

In this experiment the cells were taken up in 0.01M sodium bicarbonate and the following protocol used: cell suspension, 1 ml.; Krebs-Ringer bicarbonate buffer, 1.5 ml.; 10 mM glucose, 0.5 ml.; gas phase, 95% nitrogen, 5% carbon dioxide.

(b) Chromatographic detection of substrate utilisation and extracellular products.

(i) Endogenous. No compounds can be detected chromatographically in the supernatants of aerated cell suspensions during periods of up to two hours.

(ii) Glucose utilization. Added glucose (2 mM) rapidly disappeared from the supernatant of an aerated aqueous cell suspension. A fairly faint reducing spot appeared with time, and reference to marker compounds indicated that it might be glucose-6-phosphate (G-6-P) or gluconate. Several repetitions of the chromatograms using methanol:ethanol:water

showed that the compound moved slightly more slowly than gluconate (R_f 0.425) and about the same speed as G-6-P (R_f 0.36). With the phosphate ester system of chromatography the compound reacted to the spray as a phosphate ester and had an R_f identical to G-6-P.

(iii). Glucose utilization with arsenite. When an aqueous cell suspension containing arsenite (5 mM) is aerated with glucose (2 mM), large amounts of keto-acids accumulate. These acids may be extracted as their 2,4-dinitrophenylhydrazones and chromatographed, but they are more easily amenable to the analytical procedures described in the following section (XIII c, iv, v). Analysis of the supernatants of this system showed that G-6-P also accumulated in much the same amount found in the absence of arsenite.

(iv). Other products. A search was made for other carbohydrate products in these systems and, in addition, cells aged in aqueous suspension at 4°, and as freeze-dried cells at 37°, for several days were also tested. The compounds sought were gluconate, 2-ketogluconate, sedoheptulose, ribose and their phosphorylated derivatives. None of these was ever detected nor were any other compounds which react either with the carbohydrate or phosphate ester sprays used.

(g) Analytical detection of substrate utilisation and extracellular products.

(i). Endogenous. Very brisk aeration of an aqueous suspension of the organism gave a small increasing concentration of a material which acts as a chromogen in the orcinol pentose determination (already described, XI, d, ii). No other products have been detected and under the more gentle aeration conditions and shorter periods of washed cell experiments even this production was not apparent. Addition of arsenite however caused accumulation of keto-acids (fig. 32). In this case the protocol was: cell suspension, 2.4 mg. dry weight per ml.; glucose, 4 mM; phosphate buffer (pH 7.1), 0.026M; arsenite, 5 mM; $MgSO_4$, 1.6 mM; total volume, 25 ml. As will be seen, the pyruvate was produced at a steady rate but the amount of α-ketoglutarate remined constant. The amount of pyruvate depended on the previous treatment of the cells; "endogenous-reduced" material had a very low pyruvate-producing ability.

(ii). Glucose utilization-phosphorus dependence. With both intact and freeze-dried cells glucose is usually quickly dissimilated in any system. Dialysis of cell-suspensions induced a phosphate-deficiency (cf. XIII, a, iii) which could be demonstrated as shown in fig.33. The system consisted of dialysed cells, 10 mg. dry weight per ml.; tris buffer,

Figure 32.

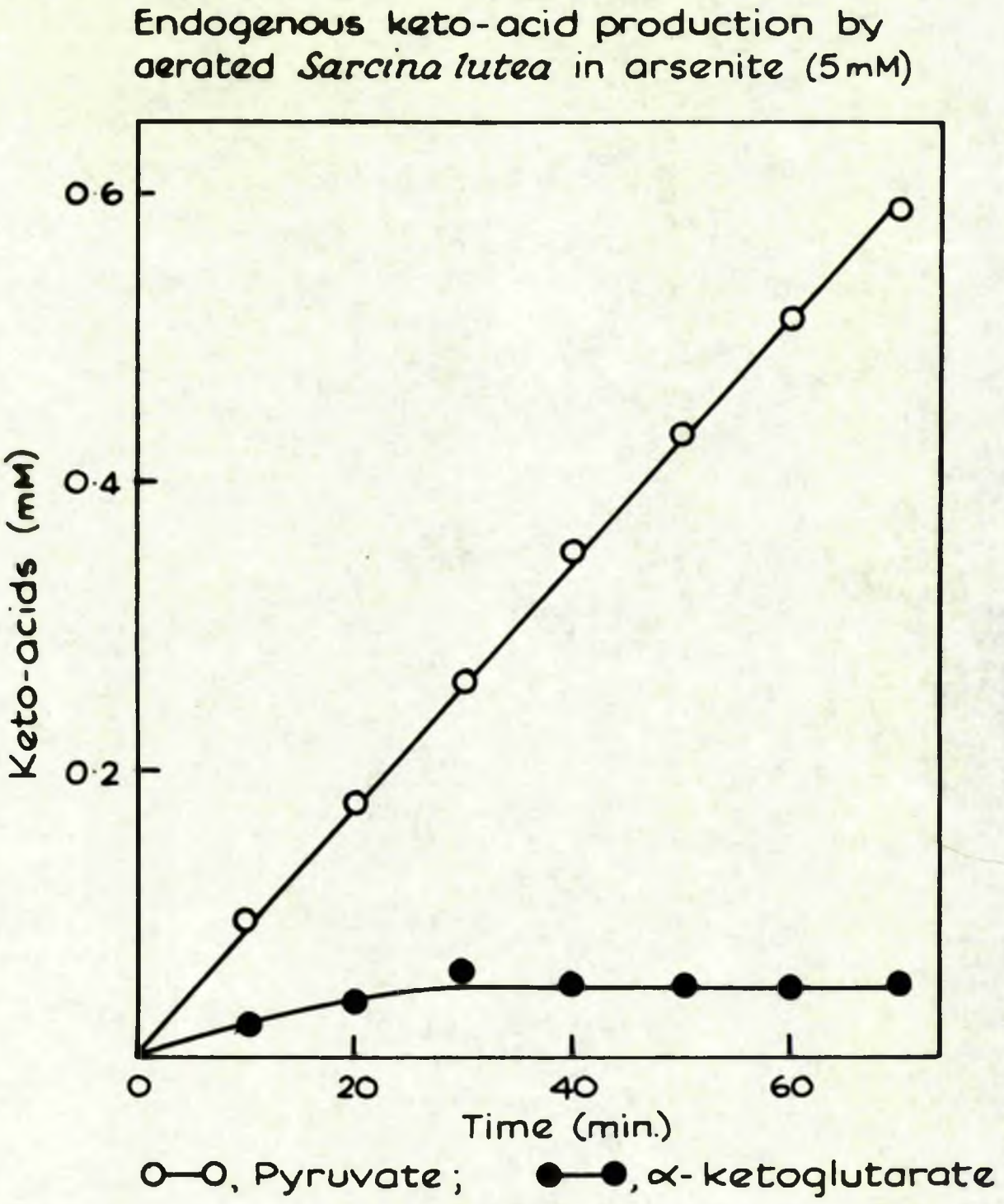
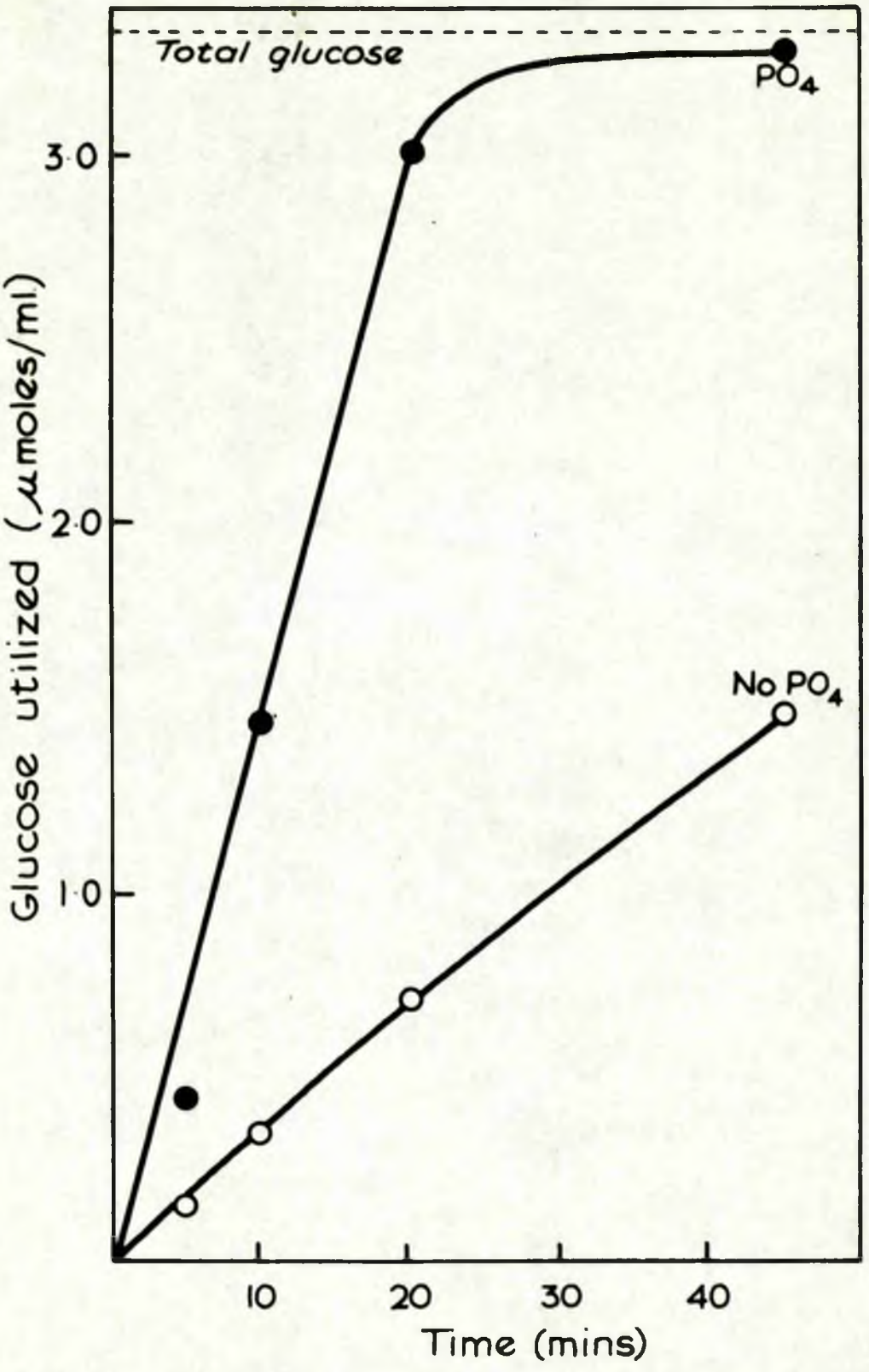

Endogenous keto-acid production by aerated *Sarcina lutea* in arsenite (5mM)
Keto-acids (mM)
0·6
0·4
0·2
0
20
40
60
Time (min.)
O—O, Pyruvate ;
●—●, α-ketoglutarate

Figure 33.

Effect of phosphate on glucose utilization by an aerated suspension of S lutea.



●—● Final phosphate concentration of 0·022M

○—○ No phosphate added.

0.033M, pH 7.1; glucose, 3.34 mM; with addition of 0.022M phosphate (at pH 7.1) as indicated. The result obtained confirms that reported by manometric techniques, in that this preparation requires phosphate for efficient glucose utilization.

(iii). Glucose utilization - effect of aerobiosis. Requirement for oxygen in glucose utilisation is shown in figs.34 and 35. In these experiments nitrogen from a cylinder was bubbled through two Dreschel bottles containing alkaline pyrogallol and then bubbled through the cell suspension to obtain "anaerobic" conditions. Fig.34 shows that substitution of air or oxygen for nitrogen greatly increases the rate of glucose utilisation (a curve for phosphate-deficient cells is included for comparison). Fig.35 demonstrates that the effects can be alternated by switching from one gas supply to the other. The system consisted of lyophilized cells, 10 mg. per ml.; glucose 3.34 mM. The slow but definite uptake in nitrogen was thought to be a reflexion of failure to remove all the oxygen from the gas stream and the experiment was repeated in Thunberg tubes as follows. Portions of cell suspension were placed in the tubes with glucose in the side-arm and the tubes were then evacuated at the oil-pump. During this process the tubes were shaken to permit the evolution of dissolved air. After evacuation,

Figure 34.

Effect of Conditions of Aerobiosis on Glucose Consumption by Sarcina Lutea.

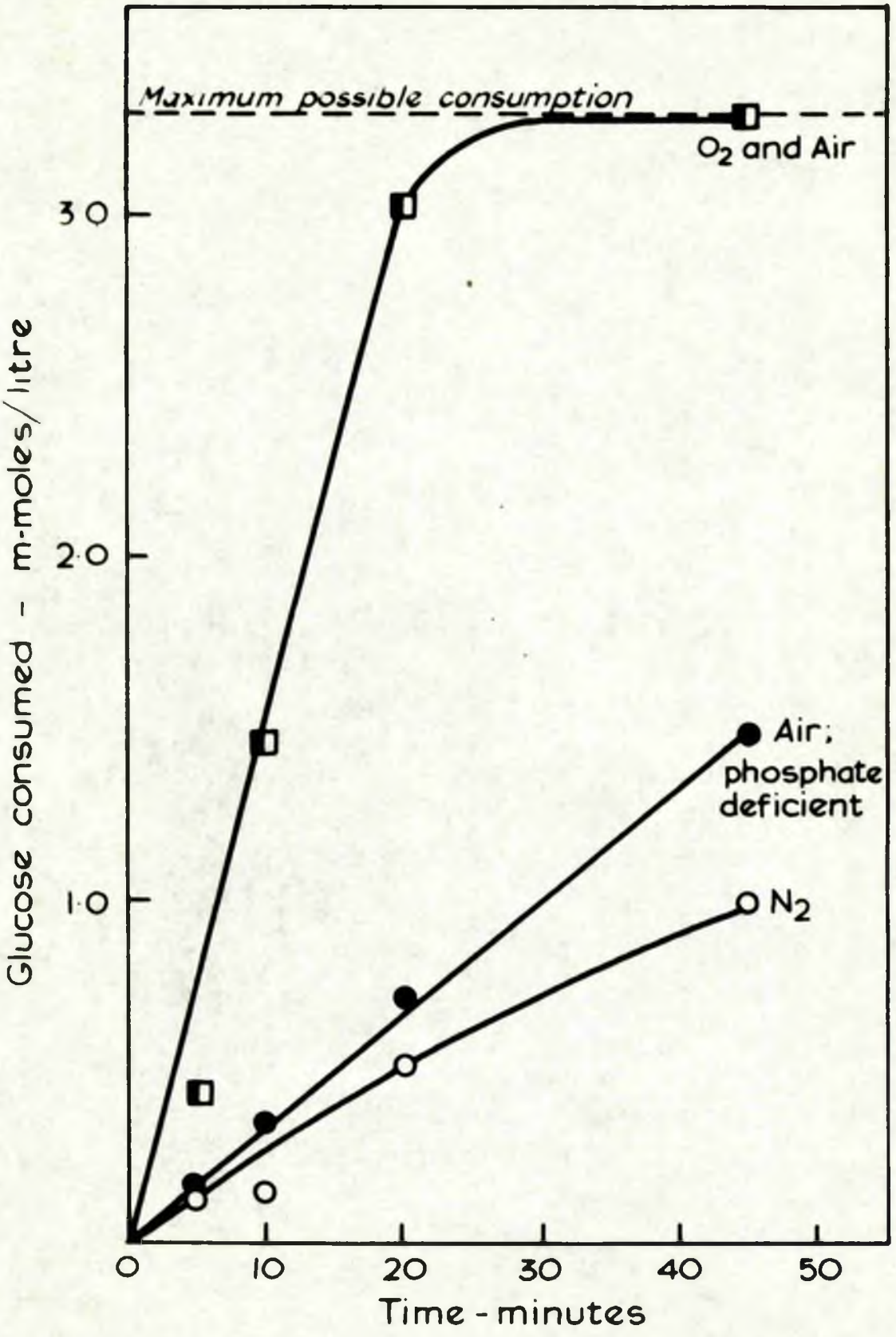
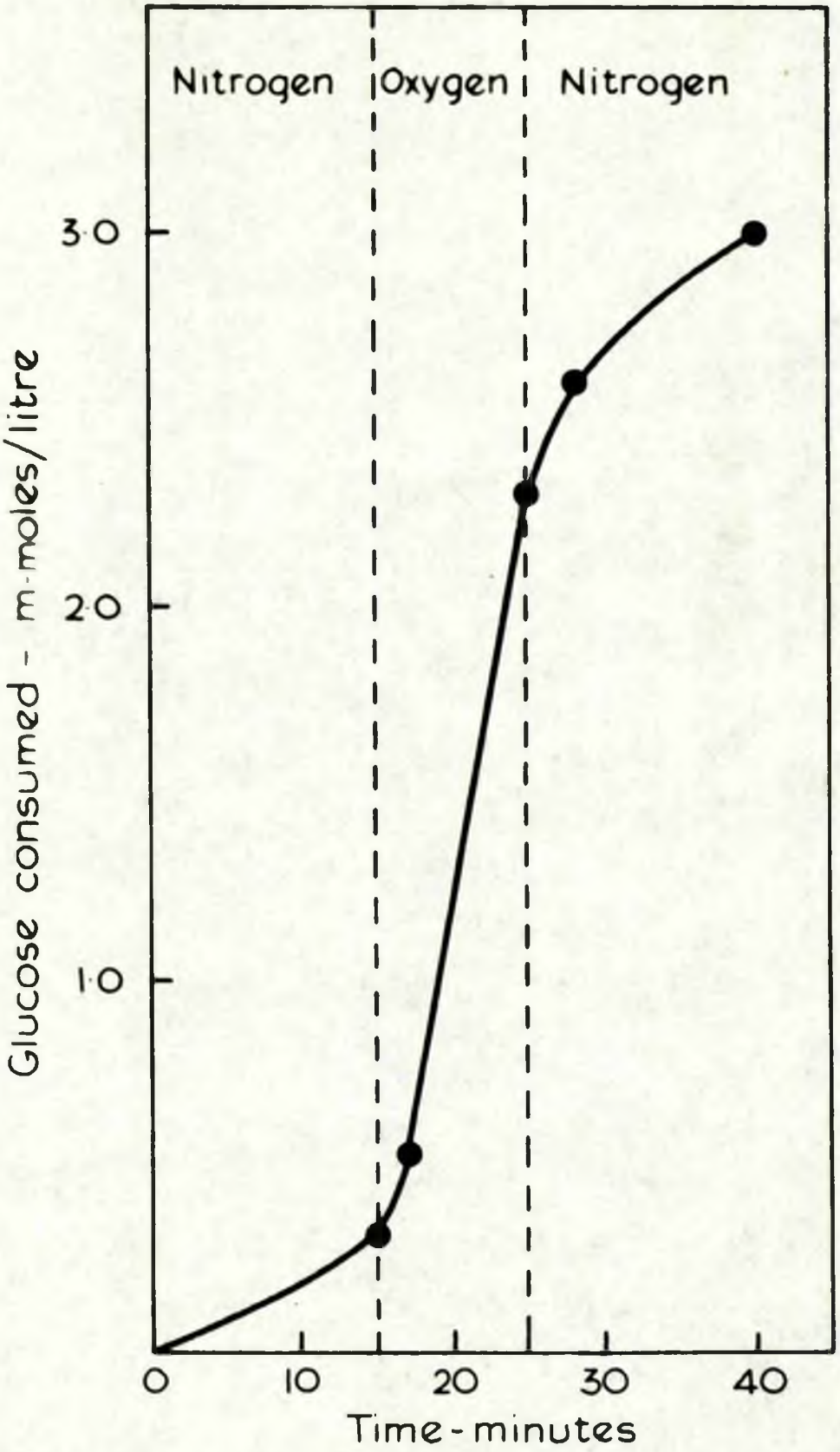


Figure 35.

Effect of Conditions of Aerobiosis on Glucose Consumption by Sarcina Lutea.

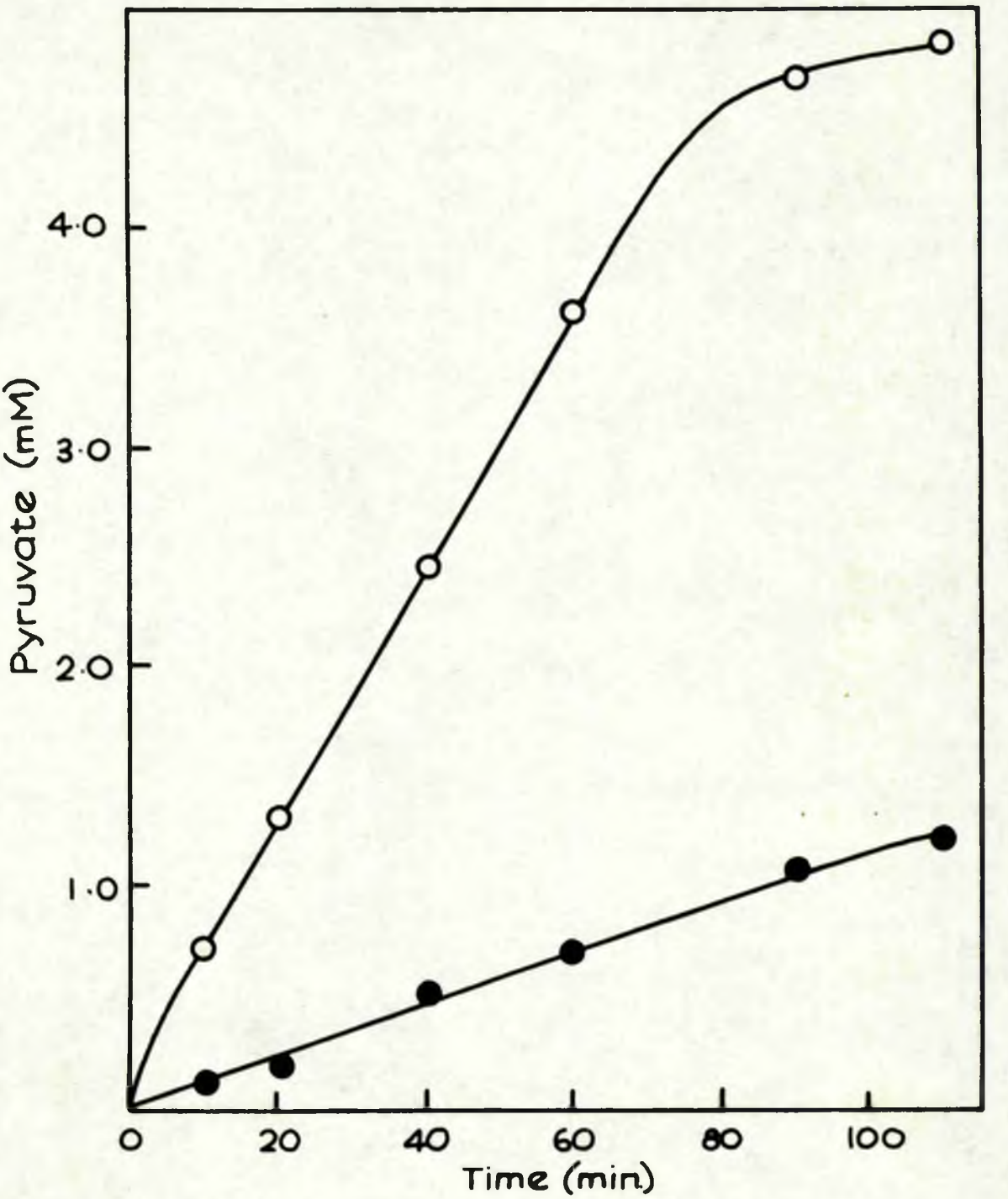


the tubes were incubated with shaking at 37° and after predetermined intervals the reaction was stopped by plunging the tubes into a boiling water bath for 5 min. Analysis of the supernatants showed that no glucose was used in periods of up to one hour. These results confirm those obtained manometrically (XIII, a, vi).

(iv). Glucose utilization - production of keto-acids. Some preparations of intact cells, which had been stored as aqueous suspensions in the refrigerator, produced pyruvic acid when aerated with glucose but the amount was greatly increased by addition of arsenite. This is shown in fig.36, protocol: cells, 7.4 mg. dry weight per ml.; phosphate pH 7.1, 0.026M; glucose, 4 mM; arsenite as indicated, 5 mM. The ability to form pyruvic acid from glucose was variable and uncertain and could not always be reproduced even when using the same cell suspension under apparently identical conditions. Endogenous reduced, lyophilized cells have never been shown to produce pyruvate from glucose in the absence of arsenite. The behaviour of this material was quite characteristic in the presence of arsenite and is displayed in fig.37. The system used differed from the one described above in that it was unbuffered and contained: cells, 33.3 mg. dry weight per ml.; glucose 2 mM; arsenite, 5.6 mM. This system has certain advantages for the study

Figure 36.

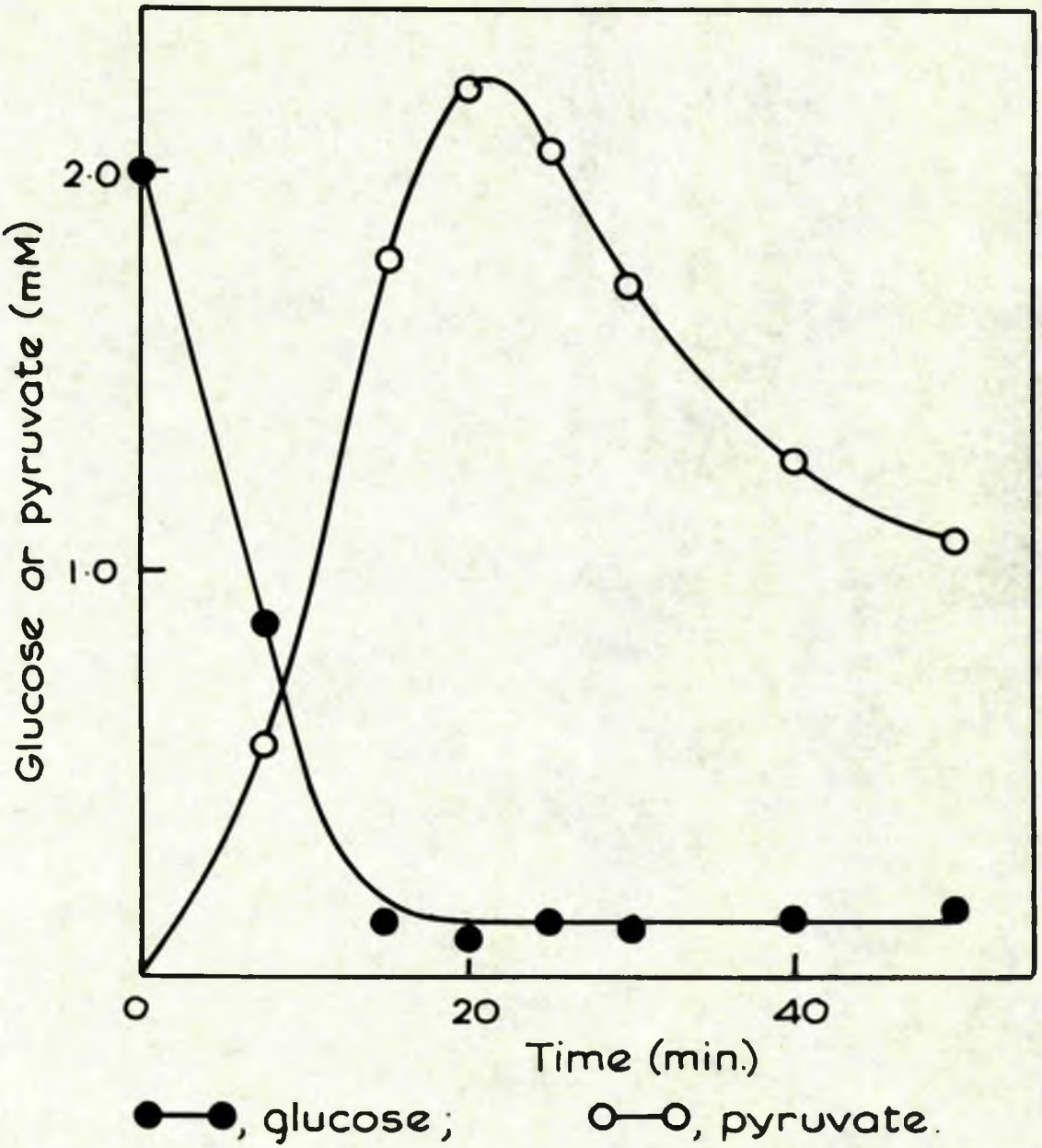
Pyruvate production by aerated *Sarcina lutea* from glucose (4mM)



●—●, no As_2O_3 ; O—O, As_2O_3 (5mM.)
Endogenous values subtracted

Figure 37.

Production of pyruvate from glucose by lyophilized cells in arsenite (5·6 mM).

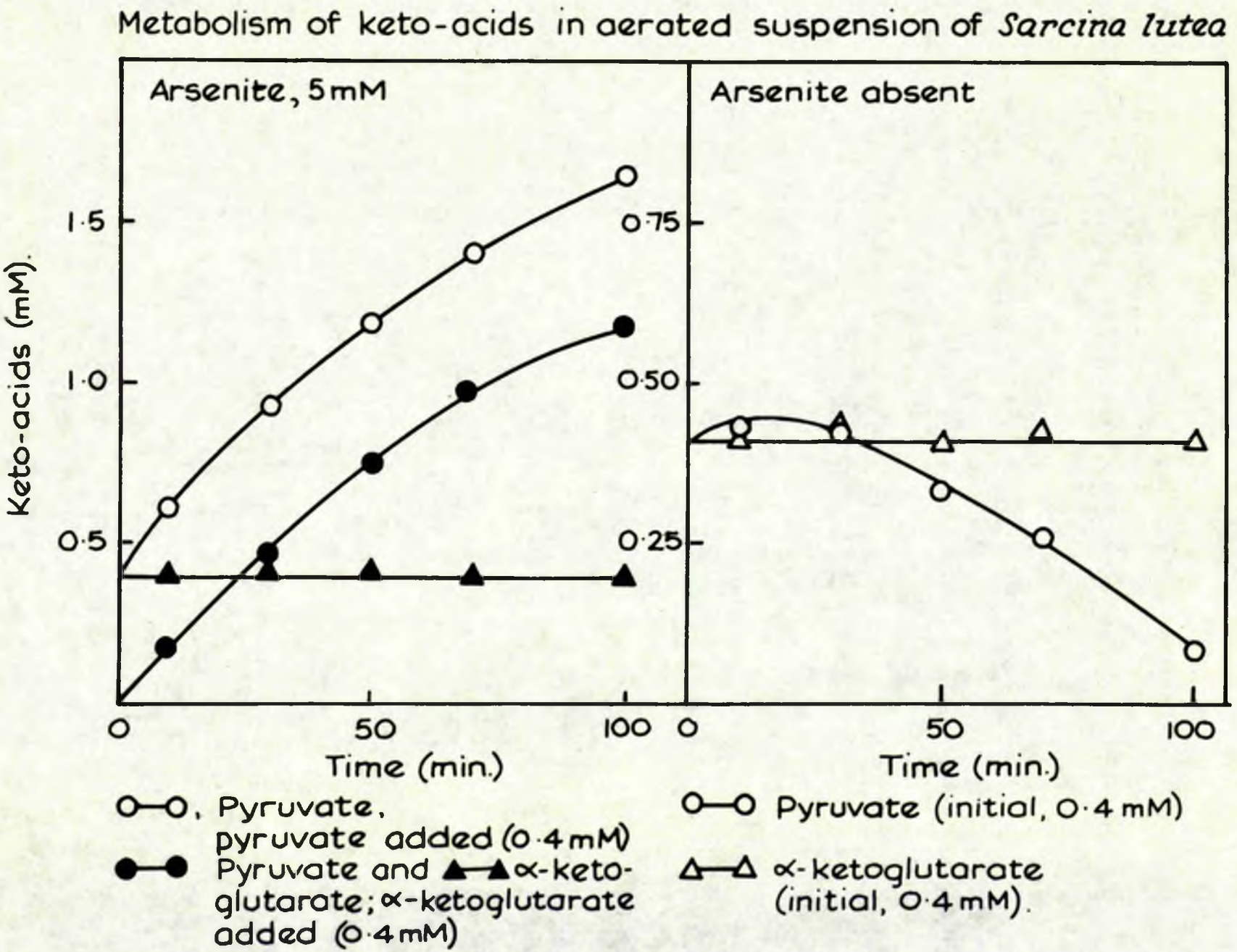


of glucose metabolism: the high cells/glucose ratio involves a rapid utilisation of the substrate; pyruvic acid is rapidly formed but is also utilized and the system therefore approximates more closely to an uninhibited one; because of the nature of the cells used, endogenous metabolism is at a minimum.

(v). Keto-acid utilization. Intact cell suspensions, as described in the preceding section, utilized pyruvate but not ∝-ketoglutarate, but they did not utilize either acid in the presence of arsenite. As will be seen in fig.38, the concentration of added ∝-ketoglutarate remained constant irrespective of arsenite. Pyruvate, on the other hand, was utilized in the absence of arsenite but increased in its presence. The increase in pyruvate was virtually identical when pyruvate or ∝-ketoglutarate was added or when no addition (not shown on graph) was made. Protocols were identical with the preceding section except for the substrates. With lyophilized material, in the presence of arsenite, pyruvate is still metabolized (fig.37). The rate of utilisation is a function of the pyruvate concentration; some 30% of the pyruvate is metabolized every 10 min.

(vi). Glucose utilization - appearance of other products. A search was made for ribose and 2-ketogluconate in the supernatants from experiments in which glucose had been metabolized.

Figure 38.


Metabolism of keto-acids in aerated suspension of *Sarcina lutea*
Arsenite, 5mM
Arsenite absent
Keto-acids (mM).
1·5
1·0
0·5
0·75
0·50
0·25
0
50
100
Time (min.)
O—O, Pyruvate, pyruvate added (0·4 mM)
●—● Pyruvate and ▲—▲ α-keto-glutarate; α-ketoglutarate added (0·4 mM)
O—O Pyruvate (initial, 0·4 mM)
Δ—Δ α-ketoglutarate (initial, 0·4 mM).

Neither was found even when arsenite (5 mM), azide (1 mM) or fluoride (0.22M) were added. Chromatographic analysis (XIII, b, ii and iii) had previously shown the production of small amounts of glucose-6-phosphate (G-6-P) from glucose. A test was made for the presence of G-6-P in the supernatants from the following system: lyophilized cells, 8.9 mg. per ml.; tris buffer pH 7.1, 0.032M; glucose 4 mM. The assay system was that described in the experimental section and contained 1% (w/v) sodium bicarbonate, 0.5 ml.; 0.1% (w/v) Zwischenferment, 0.1 ml.; 2 mM TPN, 0.1 ml.; water, 1.3 ml.; supernatant from experiment 1.0 ml. An increasing reduction of TPN was noted with samples taken at later times (table 6). The amount of G-6-P present is too small to evaluate, but the data show that G-6-P has been formed and the chromatographic result is confirmed.

(d) Experiments with 2,3,5-triphenyltetrazolium chloride.

The calibration and assay procedure for 2,3,5-triphenyltetrazolium chloride (TTC) reduction are given in the experimental section. The work described here is at present being extended in this laboratory to substrates other than glucose.

(i). Endogenous. The time and cell density relationships for 2,3,5-triphenylformazan (TPF) formation are shown in

Table 6.

Appearance of glucose-6-phosphate in supernatant during metabolism of glucose by Sarcina lutea.

Time (min.)	$\triangle E_{340}$ at 10 min. in assay system
0	0.020
5	0.037
10	0.037
30	0.055
45	0.053
60	0.065

fig.39. The protocols were: (a) cells (14 mg. per ml. in 6 g. per l. KH_2PO_4 buffer, pH 7.1), 3 ml; TTC (0.1%, w/v), 1.0 ml.; period of incubation as indicated: (b) cells (in 6 g. per l. KH_2PO_4 buffer, pH 7.1), as indicated, in total volume of 3 ml.; TTC (0.1%, w/v), 1.0 ml.; the period of incubation was in inverse proportion to the cell density used, except that lower levels of cells exhibit a lag. This latter observation has already been recorded for Escherichia coli (Kepper, 1952).

Estimation of the pH-optimum for TTC-reducing activity showed that while freshly harvested cells had maximum activity about pH 7.2, there was a shift to pH 8.05 during storage as a suspension at 4°.

During reduction of endogenous activity by aeration Q_{O_2} and TTC-reducing values behaved in a similar manner (fig.40). In this experiment a suspension (50 ml.) of cells, as described above, was aerated at 37° with 860 ml. of water-saturated air per min. and samples removed at the time intervals stated. The samples were immediately centrifuged and resuspended in buffer for assay of Q_{O_2} and TTC-reduction. Despite the falling values for both Q_{O_2} and TTC-reduction, it proved impossible to detect any fall in cellular polysaccharide by either of the two methods described in the experimental section.

Figure 39.

Relationship of incubation time (1) and cell density (2) to TTC-reduction.
For *Sarcina lutea*

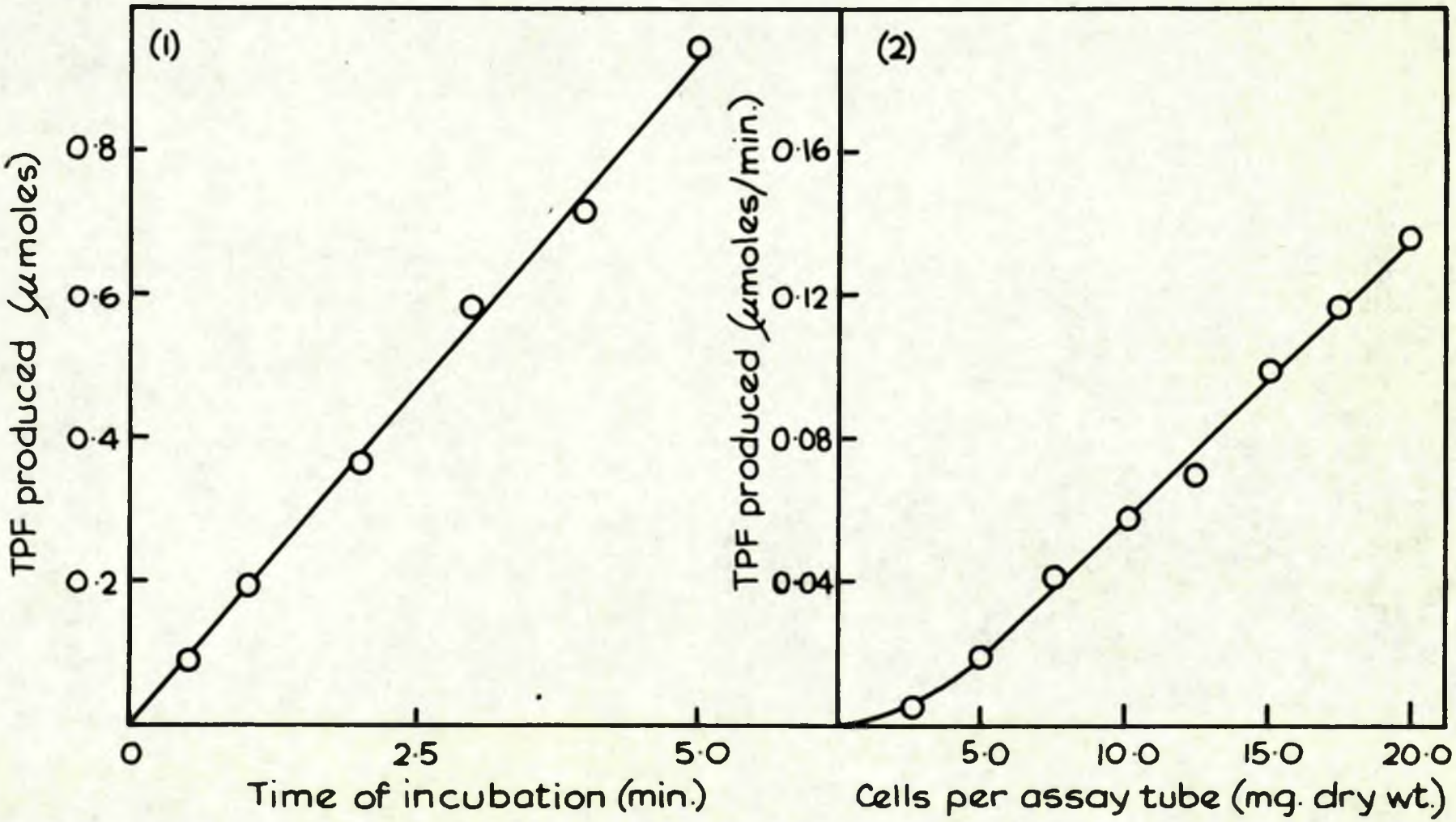
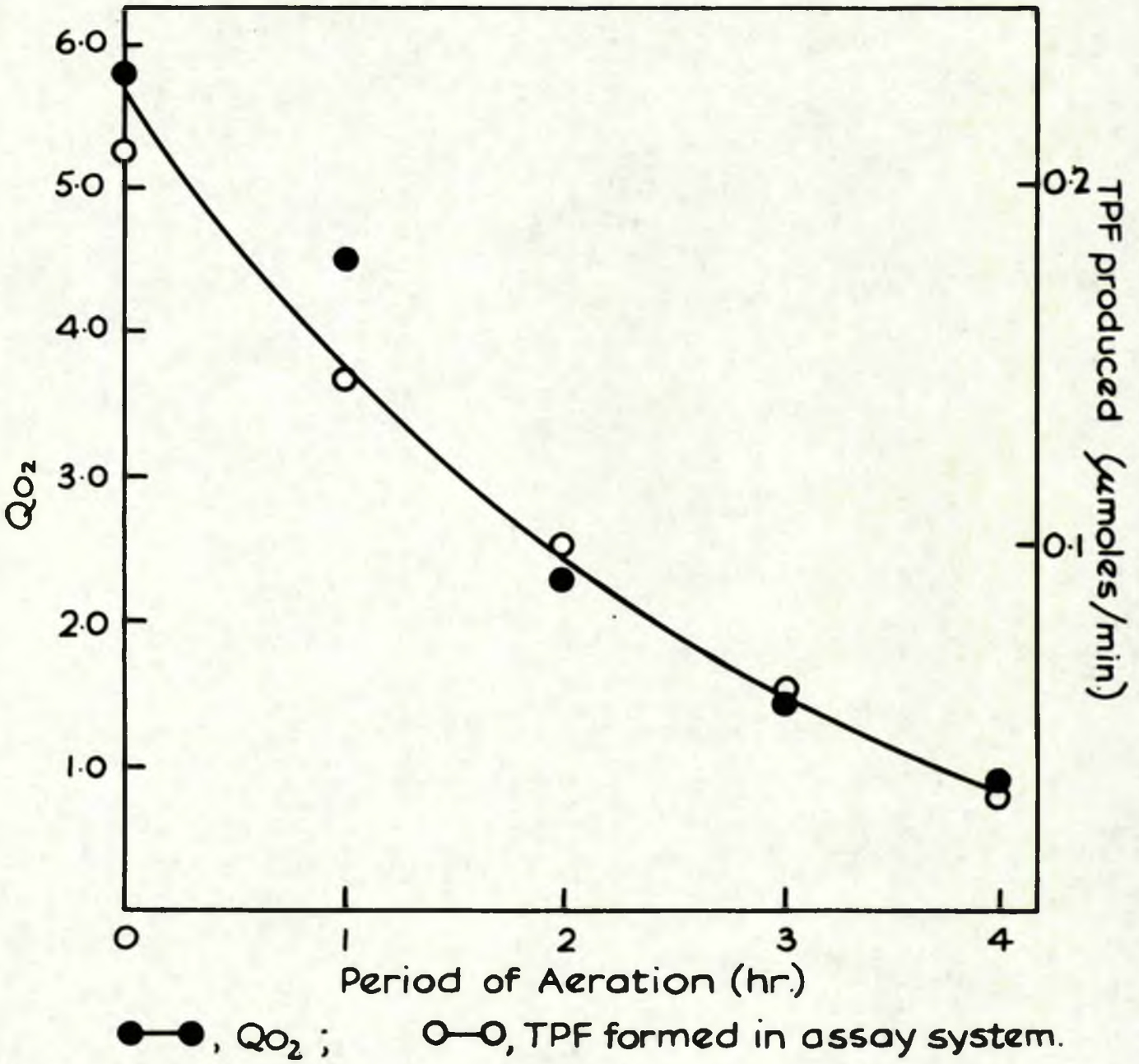


Figure 40.

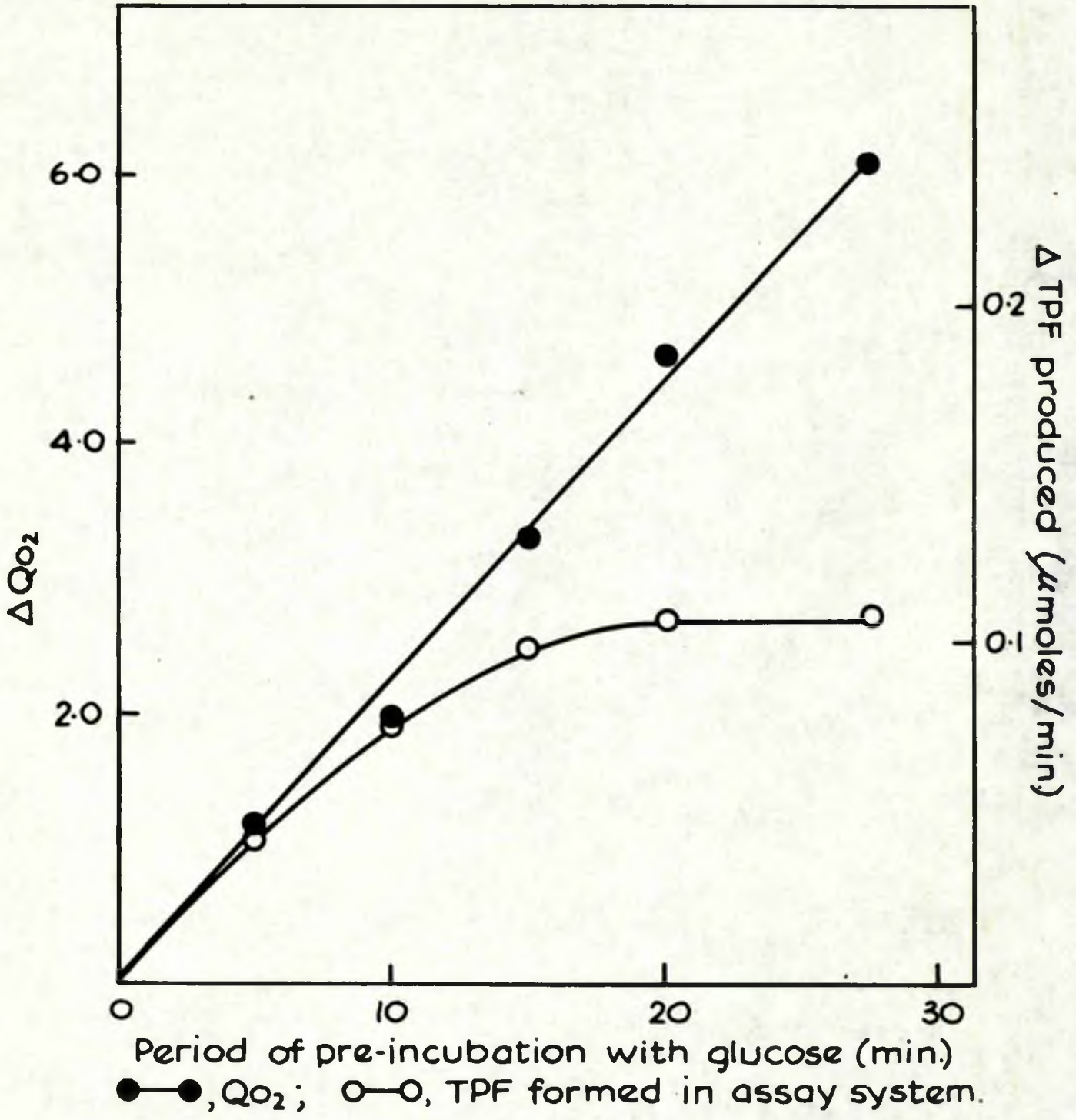
Effect of aeration of a suspension of *Sarcina lutea* on Qo_2 and TTC-reducing values.



(ii). Effect of pre-incubation with glucose. When endogenous-reduced cells were incubated with glucose prior to assay both the Q_{O_2} and TTC-reducing values were increased. However, the TTC-reducing value reached a maximum in about 15 min., while the Q_{O_2}-value was still increasing at 30 min., as seen in fig.41. The system used was that described in the experimental section except that a higher concentration (21.0 mM) of glucose was used. This ensured that lack of glucose was not a limiting factor, and analysis of the supernatant showed that glucose (16 mM) was still present at 20 min.

Figure 41.

Effect of glucose on Qo_2 and TTC-reducing values of endogenous reduced *Sarcina lutea*.



XIV. EXPERIMENTS WITH CELL-FREE SYSTEMS.

(a) Manometric methods.

(i). Oxidation of substrates. Many attempts were made to detect oxidative systems in cell-free supernatants. Glucose, G-6-P, gluconate, glucosamine, and iso-citrate were used as substrates. Methylene blue or phenazine methosulphate were added in an effort to bridge any gap in the chain of enzymes to molecular oxygen. Hog liver extract, yeast extract, DPN and TPN were added to supply possible deficiencies in coenzyme requirements. Tris, glycylglycine and phosphate buffers were used with supernatants from the Mickle shaker and the Nelco homogeniser. Under none of these circumstances was a convincing oxygen uptake noted. If the supernatant was supplemented with some of the debris from which it had been separated, an uptake was always observed with glucose as substrate. This observation could, of course, depend on whole cells remaining in the debris.

(ii). Kinases. In the manometric method for following phosphorylation by ATP, use is made of the fact that the process involves an increase in acidic groups (XI, 1, iii). When this method was applied to cell-free extracts of Sarcina lutea no difference in acid production, as measured by CO_2 evolution, was noted when glucose, gluconate, 2-ketogluconate, glucosamine or ribose were added as substrates or when no

addition was made. The amounts of CO_2 evolved being substantial and identical in each manometer indicates that a powerful ATP-ase is hydrolyzing the ATP and thus may prevent the action of any kinases that are present.

(b) Spectrophotometric methods.

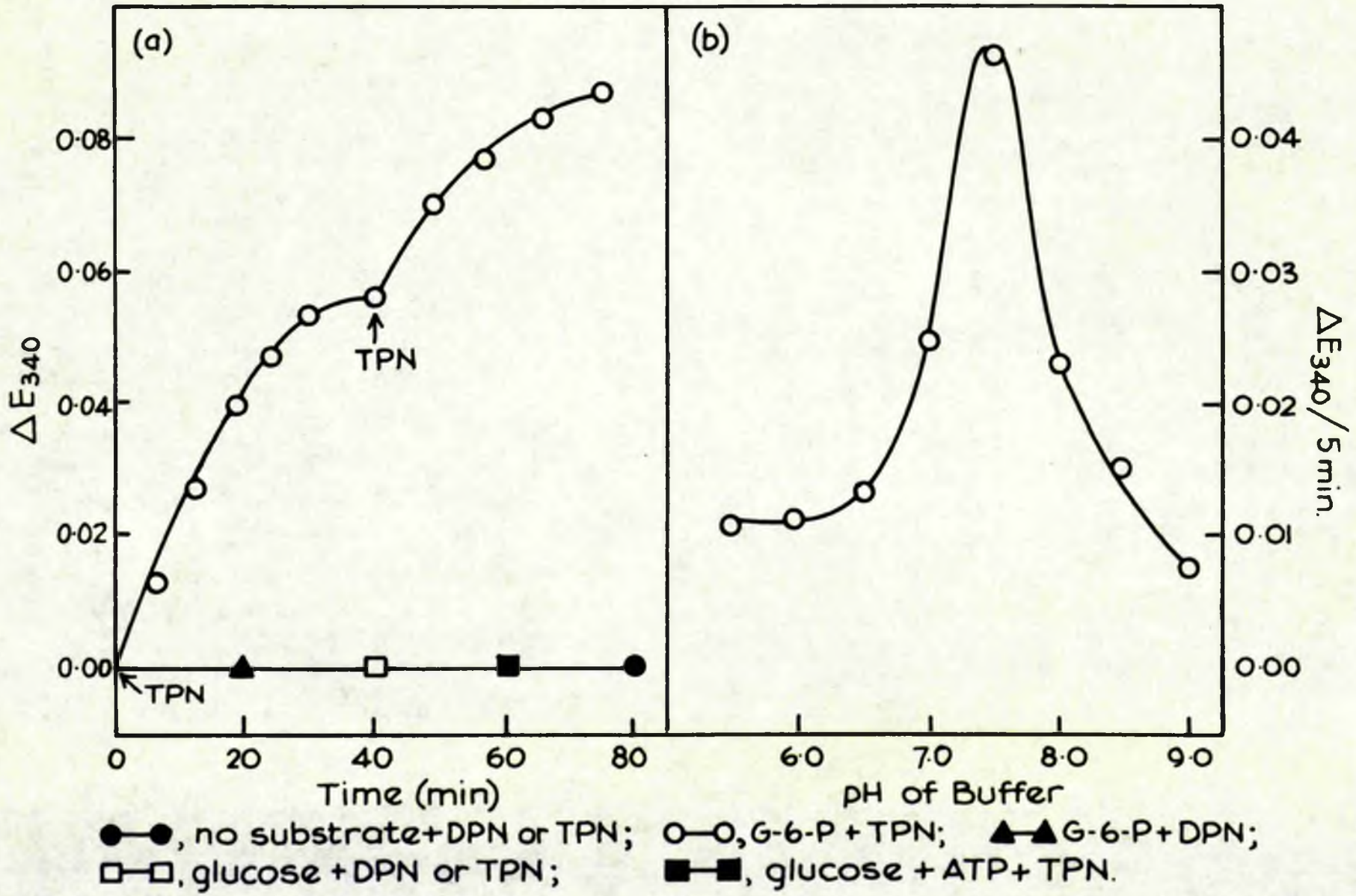
The methods used in this section are described above (XI, 1, 1) and, in general, substrate, composition of buffer and coenzyme are the only variables mentioned here.

(1). Glucose-6-phosphate dehydrogenase (G-6-Pdh) has been detected as a TPN-linked enzyme in all extracts in tris, phosphate, glycylglycine or bicarbonate buffer. A system where limiting TPN was added in two portions is shown in fig.42 (a) where phosphate buffer was used. Activity was greatest in glycylglycine buffer and fig.42 (b) shows the pH optimum curve for this system. Variation of the final concentration of glycylglycine between 0.027 and 0.170M had no effect on the G-6-Pdh activity. The activity was increased by addition of $MgSO_4$ (0.015M) and decreased by versene (0.01M) when glycylglycine was used.

As seen in fig.42 (a), glucose could not be linked to any DPN or TPN-dependent system, nor did it enter the G-6-Pdh system by the action of ATP. The absence of an effective kinase in these extracts confirmed the result obtained manometrically (XV, a, ii).

Figure 42.

Glucose-6-phosphate dehydrogenase of *Sarcina lutea* and its pH optimum.



(ii). 6-Phosphogluconate dehydrogenase (6-PGAdh) was detected as a TPN-linked enzyme (fig.43). Glycylglycine buffer (pH 7.5) was used and G-6-Pdh activity is included for comparison. Ribose-5-phosphate (R-5-P) had no activity in this system.

(iii). Glycolytic enzymes. Hexokinase was not active in extracts (fig.42, a) but may have been denied ATP by the hydrolysing system already noted (XIV, a, ii). With hexosediphosphate (HDP) as the substrate DPN was reduced (fig.44). This may be interpreted as indicating the presence of aldolase and triosephosphate dehydrogenase. The fall in E_{340} in this system showed that a DPN.H oxidase was active.

(iv). Krebs' cycle enzymes. A TPN-specific iso-citric dehydrogenase was demonstrated (fig.45), as was aconitase, by the substitution of cis-aconitate and citrate as substrates in this system. Phosphate buffer (pH 7.1) was used but even when this was supplemented with coenzyme A (0.67 mM) and glutathione (0.033M) a DPN-linked α-ketoglutarate dehydrogenase could not be demonstrated. The absence of this enzyme, in these extracts, was confirmed by the detection of α-ketoglutarate as its 2,4-dinitrophenylhydrazone in extracts which had oxidised iso-citrate. Aconitase was not always so active, as is shown in fig.45; many samples which gave the almost instantaneous reaction characteristic of iso-citric dh

Figure 43.

6-phosphogluconate dehydrogenase of *Sarcina lutea*

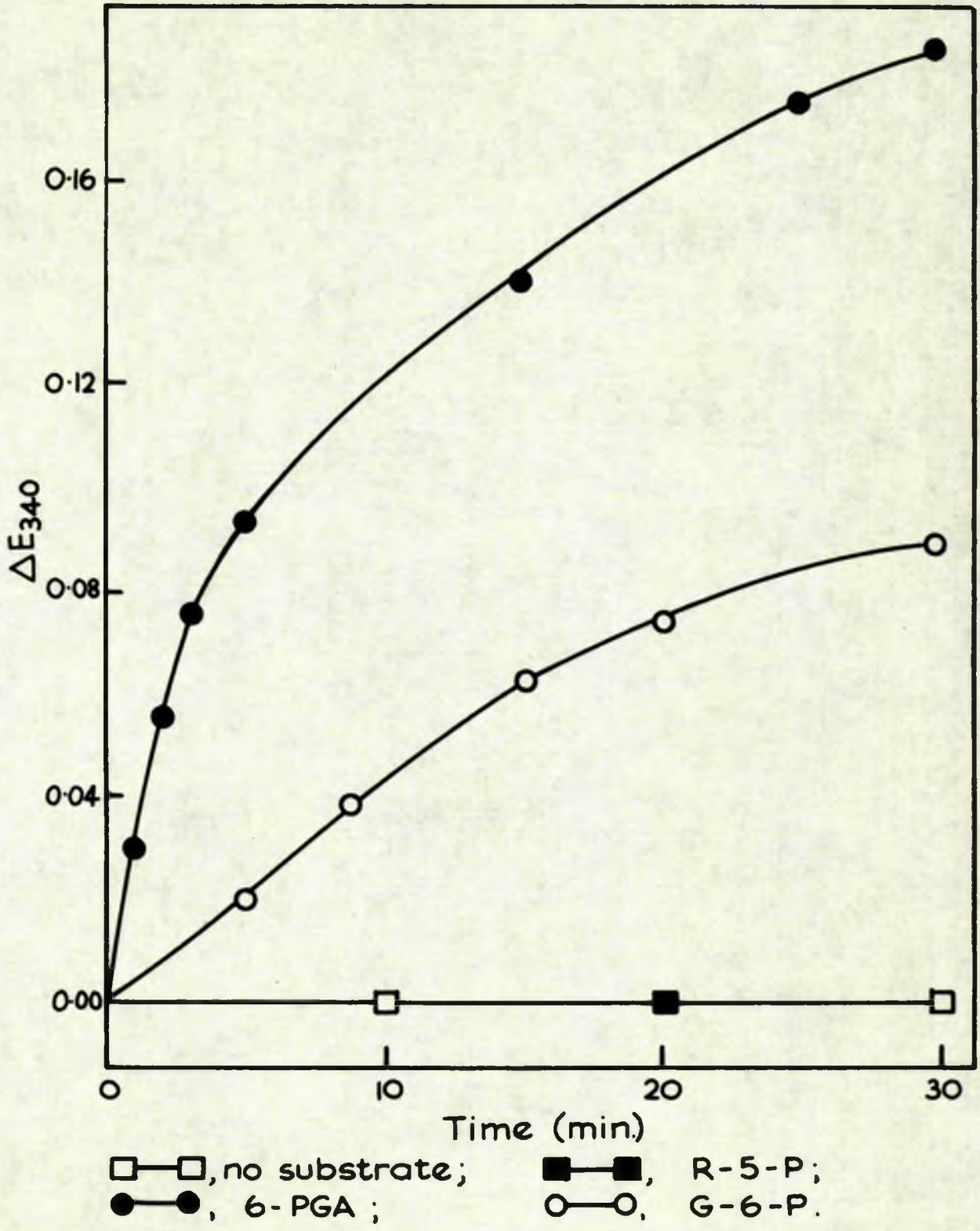


Figure 44.

Aldolase and triosephosphate dehydrogenase of *Sarcina lutea*

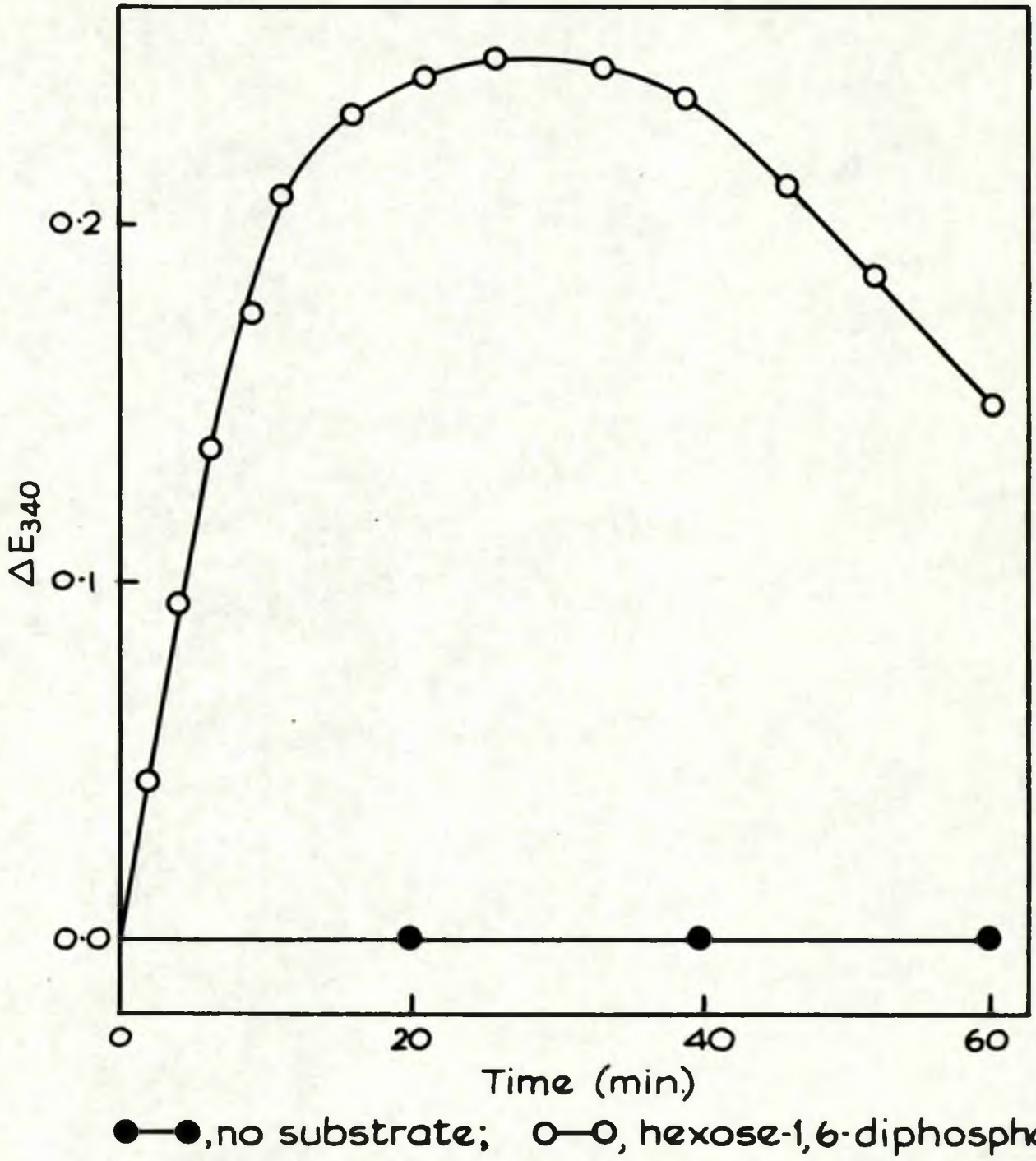
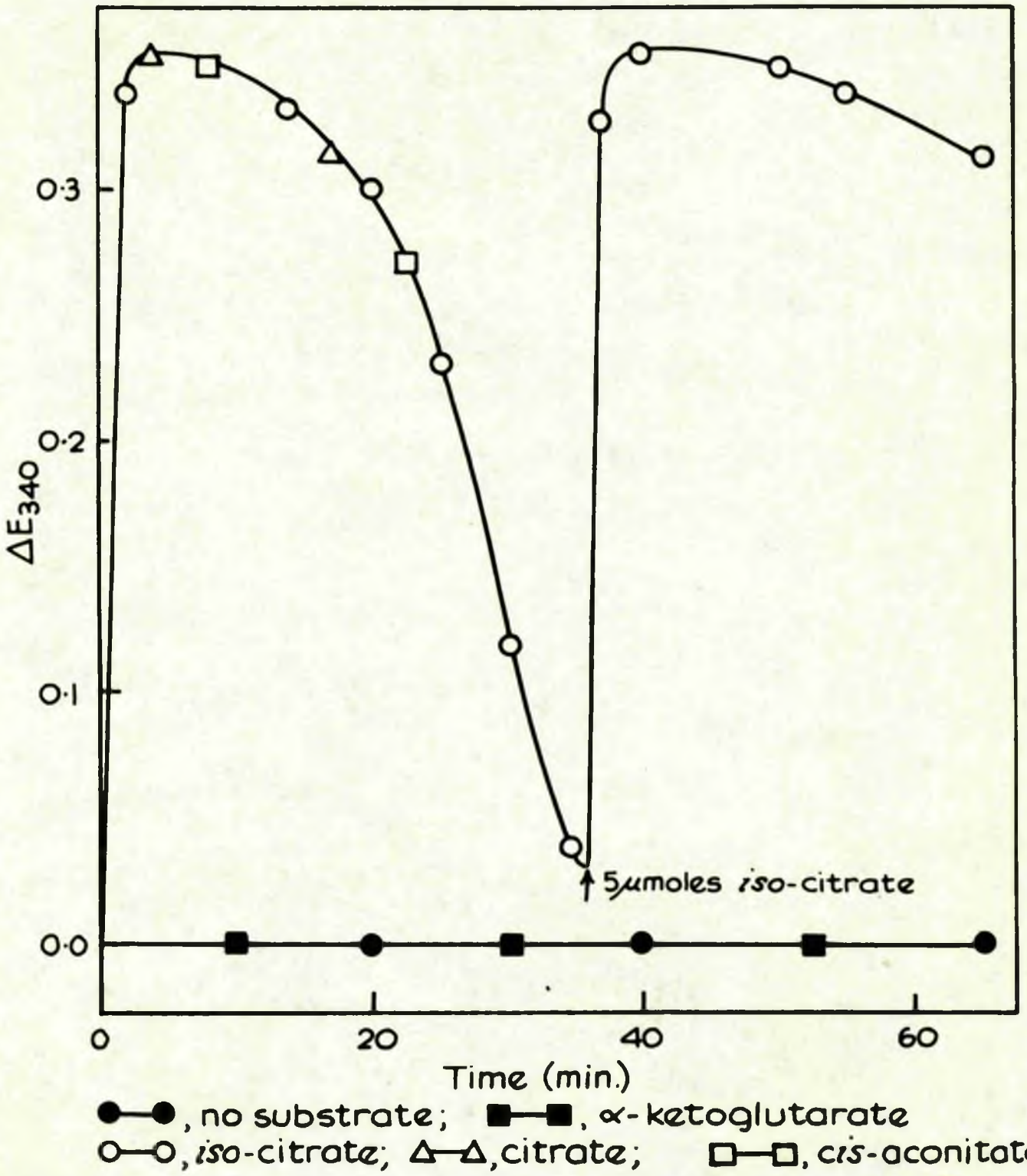


Figure 45.

Aconitase and *iso*-citric dehydrogenase of *Sarcina lutea*.



exhibited a slower reduction with both citrate and cis-aconitate.

The eventual decrease of TPN.H recorded in fig.45 demonstrates the presence of TPN.H oxidase which makes it all the more remarkable that extracts will not give an oxygen uptake in manometric experiments (XIV, a, i).

(c) Analytical Methods.

In view of the results obtained when looking for kinases, the extracts were examined for phosphatase activity. (i), Protocol. The general method involved the incubation at 37° of extract and phosphorylated substrate. The order of addition of components to the reaction mixture was standardized: water, to make the final volume to 3.6 ml.; sodium bicarbonate (0.04M), 1.5 ml.; magnesium sulphate (0.012M), 0.1 ml.; water or substrate (10 mM), 0.5 ml.; sodium fluoride (0.36M), 0, 0.25, 0.5 or 1.0 ml.; cell-free extract, 0.5 ml.; perchloric acid (35% w/v), 0.4 ml. For zero time samples the perchloric acid was added before the enzyme solution. After the addition of perchloric acid the samples were centrifuged and the inorganic phosphate content of the supernatant determined as previously described (XI, b, i). Taking into consideration the blanks run without substrate and without incubation, the results were ex-

pressed as μ moles phosphate liberated by the action of extract on substrate for 30 min. at 37°.

(ii). Results. With glucose-6-phosphate as substrate, very little phosphate is liberated in the system described above. With ATP, however, phosphate is liberated as shown in table 7. The results show that more than two of the phosphate residues are split off from each molecule of ATP in the presence of molar sodium fluoride. The activity without fluoride is less but still considerable.

Table 7.

ATP-ase action of extracts of Sarcina lutea.

Concentration of fluoride (M)	Inorganic phosphate liberated (μ moles)
0.0	2.12
0.25	2.22
0.5	3.50
1.0	12.20

ATP added was 5 μ moles in each experiment.

XV. EXPERIMENTS WITH ^{14}C-LABELLED GLUCOSE.

(a) The general reaction.

(i). Glucose utilization and ^{14}C-assimilation. In the system described in the experimental section (XII, g), glucose-U-^{14}C behaves as shown in fig.46. The observable counts decline in the supernatant as they increase in the cells. At the same time the total counts diminish owing to the evolution of $^{14}CO_2$. Estimation of the glucose present in the supernatant yielded a curve which always fell slightly below that of the residual radioactivity, showing that there were radioactive products other than glucose in the supernatant. When arsenite was added this discrepancy was accentuated because of the accumulation of keto-acids. The results of experiments with glucose-U-^{14}C are shown in tables 8(a) and (b). Table 8(b) gives the various products as a percentage of the counts or concentration of glucose at zero time. Pyruvate is expressed by reference to the maximum possible amount that can be obtained from glucose (i.e., 2 moles per mole). Table 8(a) contains the data from which table 8(b) is derived, and is included to give some idea of the actual counting levels. Dilution factors to relate these values to the whole reaction mixture were derived as follows:

Supernatants: The reaction mixture contained 35.3 mg. dry weight of cells per ml. and hard centrifugation of a portion

Figure 46.

Dissimilation of Glucose-U-^{14}C by S lutea.

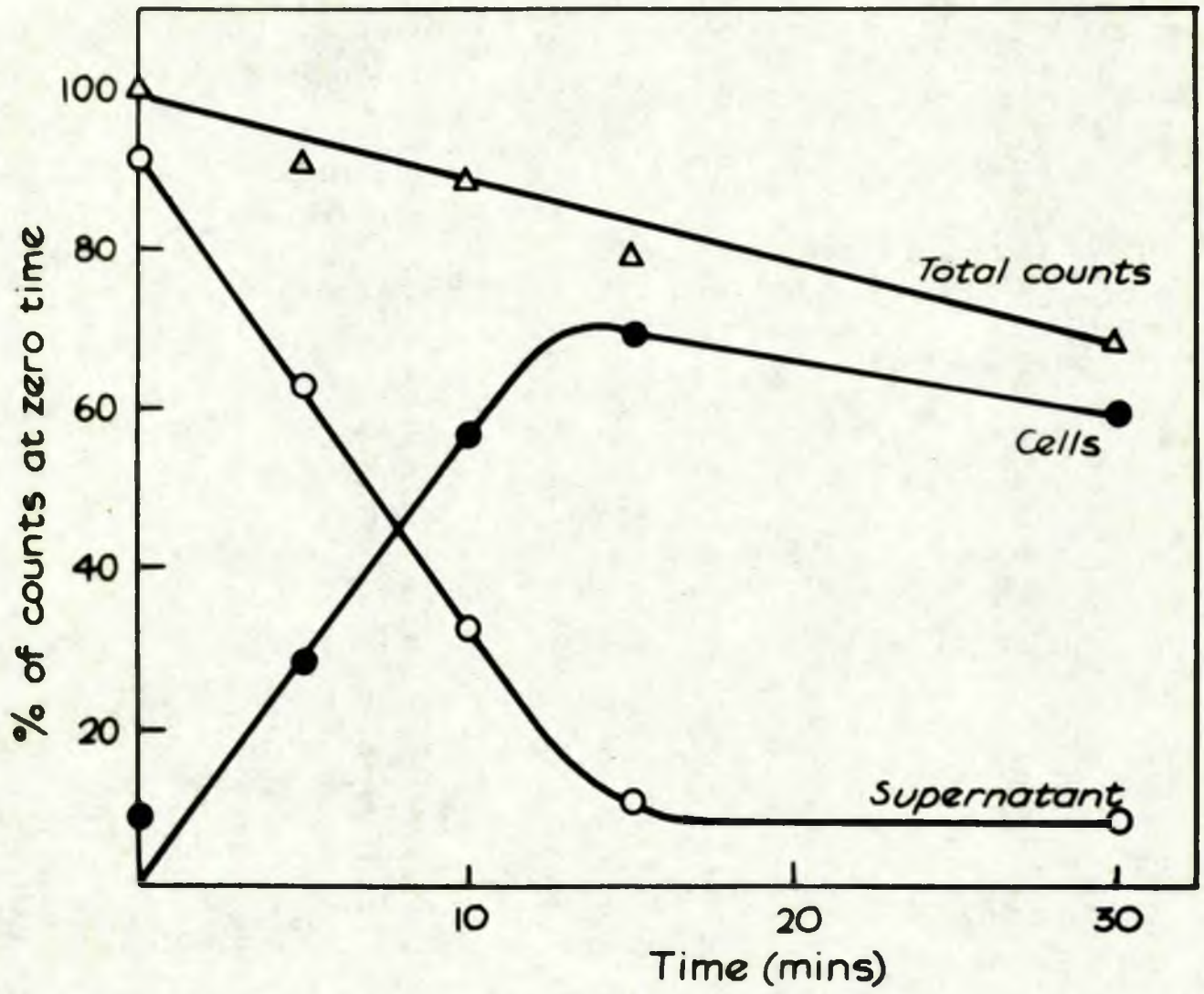


Table 8(a).

Dissimilation of glucose-U-^{14}C by Sarcina lutea.

Actual determined activities of plated portions of supernatants and cells are recorded (see text for factors to relate to total activity).

Time (min.)	Glucose-U-^{14}C *			Glucose-U-^{14}C * plus As_2O_3 (5.6 mM)			
	Glucose (mM)	Supt. (counts/min.)	Cells (counts/min.)	Glucose (mM)	Supt. (counts/min.)	Cells (counts/min.)	Pyruvate (mM)
5	2.72	2,280	104	2.15	1,900	78	0.82
10	1.17	1,180	190	0.93	1,370	139	1.40
15	0.16	3,900	229	0.17	8,700	166	1.80
30	0.15	3,160	206	0.00	8,260	164	2.70

*Total counts per min. added to the system were respectively 256,200 and 229,900.

Table 8(b).

The data of table 8(a) expressed as percentages of the added glucose.

Time (min.)	Glucose-U-^{14}C			Glucose-U-^{14}C plus As_2O_3 (5.6 mM)			
	Glucose	Supt.	Cells	Glucose	Supt.	Cells	Pyruvate
5	60.7	62.5	30.4	52.5	58.0	25.5	11.0
10	26.1	32.3	55.5	22.7	41.8	45.3	17.1
15	3.6	10.7	67.0	4.1	26.6	54.1	21.0
30	2.7	8.6	60.2	0.0	25.2	53.4	25.1

in a graduated centrifuge tube indicated that 33.3 mg. dry weight of cells in suspension occupy 0.22 ml. Therefore in the reaction mixture of 18 ml. the cells occupy 3.96 ml. and the supernatant fluid 14.04 ml. As 0.2 ml. of supernatant is taken for plating, the value thus obtained must be multiplied by 70.2 (5 x 14.04) to give the count for the whole supernatant.

Cells: The 3 ml. portions of reaction mixture taken for centrifugation contain 100 mg. dry weight of cells and, after washing, are taken up in 25 ml., of which 0.2 ml. is plated. Thus, 0.8 mg. of cells are counted and the total reaction mixture contains 600 mg. The dilution factor is, therefore, 750 (600/0.8).

Glucose and pyruvate are expressed as mM in the results obtained using the analytical methods already described (XI, d,i and e,i).

In the same way tables 9(a) and (b) describe the reactions with glucose-1-^{14}C. Several interesting features may be noted: ^{14}C from each substrate is assimilated to a considerable extent both in the presence and absence of arsenite; the loss in supernatant activity is less in the presence of arsenite due to formation of keto-acids. The amount of pyruvate present cannot account for the activity remaining in the supernatant, so there must be other labelled

Table 9(a).

Dissimilation of glucose-1-^{14}C by Sarcina lutea.

Actual determined activities of supernatants and cells are recorded (see text for factors to relate to total activity).

Time (min.)	Glucose-1-^{14}C *			Glucose-1-^{14}C * plus As_2O_3 (5.6 mM)			
	Glucose (mM)	Supt. (counts/min.)	Cells (counts/min.)	Glucose (mM)	Supt. (counts/min.)	Cells (counts/min.)	Pyruvate (mM)
5	2.12	1,640	118	3.10	2,370	27	0.82
10	0.61	820	168	2.40	1,970	44	1.40
15	0.07	520	199	1.87	1,590	57	1.80
30	0.11	490	189	0.23	780	88	2.70

*Total counts per min. added were respectively 226,100 and 193,400.

Table 9(b).

The data of table 9(a) expressed as percentages of the added glucose.

Time (min.)	Glucose-1-^{14}C			Glucose-1-^{14}C plus As_2O_3 (5.6 mM)			
	Glucose	Supt.	Cells	Glucose	Supt.	Cells	Pyruvate
5	44.3	51.0	39.2	74.5	86.0	10.6	9.9
10	12.8	25.4	53.9	57.5	71.5	17.0	16.8
15	1.5	16.2	66.3	44.9	57.7	22.3	21.6
30	2.3	15.2	62.8	5.5	28.3	34.0	32.5

substances present and, in the absence of arsenite, the decrease in supernatant activity is less than the decrease in glucose, therefore other labelled compounds must be formed.

(ii). Total recoveries. Not many experiments were conducted to achieve a complete recovery of added ^{14}C as this was not necessary for the type of work attempted. Nevertheless, a few experiments were run as a guide to the overall accuracy of the method and it was found that the most serious source of error occurred in plating the supernatant, due to loss of ^{14}C in volatile compounds and to self-absorption. Table 10 shows a typical result obtained with glucose-1-^{14}C in the presence of arsenite. Column (a) represents a recovery of 88.5% when no corrections are applied; when the supernatant is plated with a small amount of alkali (b) the recovery is raised to 83.4%; correction of the self-absorption of the supernatant by adding a known ^{14}C-solution to (b) gives a total recovery of 92.6%. In this experiment the deficit is almost certainly due to a low carbon dioxide value as this was not corrected for loss in the plating technique (XII, e, iii).

Table 10.

Recovery of ^{14}C from glucose-1-^{14}C.

(Expressed as percentage of total added).

	(a)	(b)	(c)
Carbon dioxide	13.3	13.3	13.3
Cells	20.5	20.5	20.5
Supernatant	34.7	49.6	58.8
Recovered	68.5	83.4	92.6

(a) Supernatant plated without NaOH.

(b) Supernatant plated with addition of NaOH.

(c) Correction for self-absorption applied.

(b). $^{14}CO_2$ production.

No experiments were conducted on this problem as such, but the $^{14}CO_2$ was collected routinely in the course of other experiments and it was found that $^{14}CO_2$ was always preferentially released from 1-labelled (as opposed to U-labelled) glucose. This observation was unaffected by the presence of arsenite and was particularly evident in experiments of short duration (table 11).

(c). Specific activity of pyruvate.

Pyruvate was isolated from reaction mixtures as the 2,4-dinitrophenylhydrazone and its specific activity determined as previously described (XII, d, iii). The results of an experiment of this type are shown in table 12. In addition, pyruvate was separated from the reaction mixture by steam distillation, isolated by column chromatography and estimated titrimetrically (XII, d, ii). The results of an experiment in which both methods were used are presented in table 13, and it will be seen that there is good agreement between the specific activities of pyruvate isolated by the two techniques. The two figures quoted for pyruvate estimated titrimetrically are those for the major peaks in the effluent. The other two acids isolated in this way, which were suspected to be fumarate and acetate,

Table 11.

Evaluation of $^{14}CO_2$ from labelled glucose.

(The figures in brackets are percentages of added activity).

	Glucose-U-^{14}C (counts/min.)	Glucose-1-^{14}C (counts/min.)
Activity added	873,400 (100)	816,400 (100)
$^{14}CO_2$ produced in 15 min.	36,000 (4.1)	68,000 (8.3)
Cells after 15 min.	406,400 (46.5)	246,100 (30.1)
Supernatant after 15 min.	409,600 (46.9)	450,000 (55.1)

	Glucose-U-^{14}C (plus 5.6 mM As_2O_3) (counts/min.)	Glucose-1-^{14}C (plus 5.6 mM As_2O_3) (counts/min.)
Activity added	38,310 (100)	37,680 (100)
$^{14}CO_2$ produced in 10 min.	309 (0.8)	649 (1.7)
$^{14}CO_2$ produced in 30 min.	2,370 (6.2)	3,110 (8.2)
Cells after 30 min.	14,740 (38.5)	12,830 (34.0)
Supernatant after 30 min.	15,690 (41.0)	17,510 (46.5)

Table 12.

Specific activity of pyruvate derived from glucose-1-^{14}C.

Material	Counts/min.	μ moles/ planchette	Specific activity (counts/min./μ mole)
Glucose-1-^{14}C	12,100	3.6	3,360
	11,960	3.6	3,320
	12,170	3.6	3,380
			Mean 3,350
Pyruvate *	1,230	0.108	1,140
	620	0.054	1,150
			Mean 1,145

* Isolated and estimated (E_{350}) as the 2,4-dinitrophenylhydrazone.

Table 13.

Determination of specific activity of pyruvate derived from glucose-1-^{14}C.

Material	Counts/min.	μ moles/ planchette	Specific activity (counts/min./ μ mole)
Glucose-1-^{14}C	1,160	0.144	8,060
	1,160	0.144	8,060
			Mean 8,060
Pyruvate *	439	0.151	2,910
	435	0.151	2,880
			Mean 2,895
Pyruvate +	266	0.0091	2,920
	348	0.0122	2,850
			Mean 2,885

* Isolated and estimated (E_{350}) as the 2,4-dinitrophenylhydrazone.

\+ Isolated by steam distillation and column chromatography; determined titrimetrically.

gave variable specific activities depending on the fraction. Because of this the data obtained from these peaks are considered unreliable and, unlike pyruvate, there was no other method available for their characterisation.

(d). Degradation of pyruvate.

From the point of view of the argument developed later, it was of interest to know if any of the pyruvate formed from glucose-1-^{14}C was labelled in the carboxyl group. Accordingly, pyruvate was isolated as the 2,4-dinitrophenylhydrazone from a system metabolizing glucose-1-^{14}C and was decarboxylated as previously described (XII, f). A control was carried out in a similar manner using glucose-U-^{14}C as the starting material. The data are given in table 14 and it will be seen that a complete recovery is not obtained from the uniformly labelled pyruvate - this might be connected with the fact that endogenous metabolism was evident in this system. Only a very small amount of the activity is found in the carboxyl group of pyruvate when it is derived from glucose-1-^{14}C. The significance of these results is discussed later.

Table 14.

Decarboxylation of metabolically produced pyruvic acid.

	Substrate	
	Glucose-U-^{14}C	Glucose-1-^{14}C
Specific activity of pyruvate*	2,070	2,600
Quantity of pyruvate* taken for decarboxylation (u mole)	0.705	2.87
$^{14}CO_2$ counted (counts/min.)	345	178
Correction factor for loss of $BaCO_3$ in filtration apparatus	1.48	1.68
Correction factor for self-absorption	1.05	1.06
Corrected counts/min. of $^{14}CO_2$	538	299
Specific activity of $^{14}CO_2$	763	104
Recovery of $^{14}CO_2$ (as percentage of activity per C-atom of pyruvate)	85.7	
Recovery of $^{14}CO_2$ (as percentage of activity of pyruvate)	-	4.0

* As the 2,4-dinitrophenylhydrazone.

(e). Fractionation of ^{14}C-labelled cell material.

(i). Tca/ethanol/ether fractionation of ^{14}C-labelled cell material (XII, h, i) gave results as expressed in table 15. Data for cell material separated from systems metabolizing either glucose-U-^{14}C (a) or glucose-1-^{14}C (b) are included on the basis of total counts/min./fraction and as a percentage of the initial count. The total counts were derived from the experimental data by applying dilution factors (XV, a, i) which had values of 125 for residues and 40 or 53.3 for supernatants (depending on the amount plated). In each experiment 100 mg. dry weight of cells were used and four portions were carried through to different stages which permitted the recovery to be calculated for several stages of the process. This also enabled an internal check of the manipulations to be calculated and the recoveries ranged from 92 to 104.5%. Inspection of table 15 shows that there is no significant difference between the two materials tested and that the largest single fraction is ethanol-soluble (about 65%) with the hot Tca fraction in second place (about 17%). A portion of the ethanol-soluble fraction was diluted with an equal volume of water and extracted twice with the same volume of ether. This yielded the alcohol-soluble-ether-insoluble and the alcohol-soluble-ether-soluble fractions of which the former contained some 95% of the activity of the

Table 15.

Fractionation of ^{14}C-labelled cell material.

(a) Cells labelled by metabolism of glucose-U-^{14}C.

Fraction	Residues		Supernatants		Recovery Percentage
	Counts/min.	Percentage	Counts/min.	Percentage	
Starting material	88,250	100	-	-	100
Cold Tca	77,250	81.9	7,570	8.5	90.4
75% Ethanol	-	-	60,800	68.7	-
Ethanol/ether	21,750	24.4	2,450	2.8	104.5
Hot Tca	2,500	2.8	17,280	19.6	102.5

(b) Cells labelled by metabolism of glucose-1-^{14}C.

Fraction	Residues		Supernatants		Recovery Percentage
	Counts/min.	Percentage	Counts/min.	Percentage	
Starting material	51,250	100	-	-	100
Cold Tca	43,250	84.4	3,960	7.7	92.1
75% Ethanol	-	-	33,206	64.8	-
Ethanol/ether	11,750	22.9	1,760	3.4	99.2
Hot Tca	1,375	2.7	6,920	13.5	92.1

alcohol-soluble fraction.

(ii). Sodium hydroxide was used to fractionate cell material (XII, h, iii) and a "polysaccharide" fraction isolated. By hydrolysis and determination of the reducing power this material analysed as 20% carbohydrate, and a quantitative biuret reaction indicated 3.1% protein. Estimation of the total nitrogen by micro-Kjeldahl technique gave a value (0.51%) consistent with the protein content. The composition of the larger part of this fraction remains to be determined.

(iii). Chromatography (XII, j). By techniques already described, hydrolysates (by HCl or H_2SO_4) of the ethanol-soluble fraction, the "polysaccharide" obtained by phenolic extraction (XII, h, ii) and the "polysaccharide" described above were examined for amino-acids, reducing material and radioactivity (all materials labelled by metabolism with glucose-U-^{14}C). Of these, the first two had radioactive glutamate and alanine with perhaps traces of activity in the other amino-acids. None of the hydrolysates contained reducing sugars. The bulk of the radioactivity (as shown on autoradiograms) moved with the solvent fronts in the systems used and it was concluded that most of the radioactive components had been degraded in the course of hydrolysis.

DISCUSSION

"False facts are highly injurious to the progress of science, for they often endure long; but false views, if supported by some evidence, do little harm, for everyone takes a salutary pleasure in proving their falseness" .....

Charles Darwin.

XVI. DISCUSSION OF RESULTS.

(a) Endogenous respiration of Sarcina lutea.

(i). Relationship of endogenous respiration (ER) to glucose metabolism. The original investigation of ER was concerned with diminishing an annoying phenomenon which, by virtue of its magnitude, interfered with the manometric investigation of substrate utilisation. The simple process of aerating a bacterial suspension was effective in lowering ER (fig.27) and was of great practical value. However, the observation that while the Q_{O_2}(endogenous) was decreased logarithmically, the Q_{O_2}(glucose) was increased arithmetically (fig.28), has considerable theoretical significance. The values for Q_{O_2}(glucose) in fig.28 were obtained by subtracting the endogenous uptake from that obtained in the presence of glucose. If this were not done, the values of Q_{O_2}(glucose) would, of course, have been higher but would have decreased rapidly in the first two hours of aeration and then remained constant. There seems to be no feasible explanation for such behaviour and one therefore concludes that the assumptions inherent in the construction of fig.28 are correct. If, then, it is legitimate to subtract endogenous oxygen uptakes from those obtained in the presence of external substrate, it follows that the metabolism of this material does not suppress the ER. Several attempts were made to confirm the

result by the methods of Norris et al. (1949) previously mentioned (X, a, ii). Unfortunately these techniques could not be applied as they depend on the rate of oxygen uptake, subsequent to the addition of substrate, reverting to the endogenous level in the course of time. With Sarcina lutea using glucose as substrate (at low concentrations) it was found that, even when the duration of the experiment was extended to ten hours, this condition could not be achieved. Despite this difficulty, the conclusion that added substrate does not suppress ER appears to be soundly based and it is interesting to reconsider fig.28 in this light. The steady increase in Q_{O_2}(glucose) can only be explained on the assumption that ER suppresses oxygen uptake on this substrate. Here again the picture is complicated because it might be considered reasonable to expect a logarithmic increase in Q_{O_2}(glucose) if this conclusion were correct. Despite this inconsistency, it is felt that the interpretation of the data is valid in a general way, and a possible fundamental explanation of the observations might be that endogenous and external substrates are competing for enzymes common to the oxidation of both. This conclusion is of significance in that all previous work (X, a, ii) has indicated that ER rather than substrate oxidation is suppressed under these conditions.

(ii). Relationship of ER to assimilation. Measurement of endogenous metabolism (EM) as ER was supplemented by experiments with TTC-reduction. As fig.40 shows, both these indices of EM decrease in the same manner when a cell suspension is aerated. One might therefore conclude that they are measuring the same property. That this is untrue is indicated by fig.41 where it will be seen that on EM-reconstitution by incubation with glucose TTC-reduction rapidly approaches a maximum value while ER continues to increase in the same time interval. Differences were also noted between these two values in the experiment devised to check the validity of Kopper's (1964) assumption that TTC-reduction measured assimilation. The results are expressed in fig.34 and indicate that repeated washings at 35° lower TTC-reduction at a rate greater than ER or ^{14}C-retention (assimilated from radioglucose). It would appear, then, that ER and TTC-reduction are similar for EM in cells as harvested. However, when the ER is reduced by aeration and the cells are then incubated with glucose, an increase in EM is noted. During this assimilatory process the cells behave differently towards TTC-reduction and Q_{O_2} measurement and, in the lowering of this artificially increased EM, TTC-reduction is diminished more quickly than ER and ^{14}C-retention, which are lowered at the same rate.

From the observations on assimilation it seems

reasonable to assume that TTC-reduction reflects the size of a pool of oxidizable substances. Fig.41, in which TTC-reduction reaches a maximum while Q_{O_2} (endogenous) is still increasing, can then be explained on the assumption that assimilation of the substrate glucose rapidly fills the hypothetical pool to its maximum capacity while the reserve substrate of ER is still being laid down. This theory does not, of course, imply that the TTC-reduction pool is necessarily an intermediate on the pathway of endogenous reserve (ERes.) synthesis although this is also an attractive possibility. On this hypothesis it is more difficult to understand why lowering the level of ER by aeration should also lower the size of the TTC-reduction-pool in the same proportion. The problem of the exact significance of TTC-reduction in relation to ER and assimilation is not yet settled, but the hypothesis that it reflects an intracellular pool of oxidizable substances is the basis of further work being carried out in this laboratory.

(iii). <u>The substrate of endogenous respiration</u>. Many workers have found that the manometric and other data of oxidative assimilation indicate that carbohydrate is laid down within the cell during this process (X, b). Accordingly the ERes. is, in many instances, considered to be of a carbohydrate nature. This is not always the case as shown by Stickland's

(1956) observations that carbohydrate levels are not related to ER in baker's yeast. The observation that no measurable diminution in cell polysaccharide of Sarcina lutea is noted during reduction of ER by aeration (XII, a, i) is of considerable interest. It can be calculated from fig.28 that the oxygen taken up by 1 mg. dry weight of cells in the course of 4½ hours aeration is about .1 μmole. If this represented the oxidation of reserve carbohydrate it would correspond to 30 μg. of polysaccharide (calculated as glucose equivalents). The amount of polysaccharide measured is of the order of 90 μg/mg. dry weight of cells and the calculated amount of polysaccharide would be 33% of this value. To put it another way, the change in E_{620} in the assay procedure would be 0.13. These changes would easily be evaluated within the accuracy of the methods used and the fact that they are not detected is conclusive proof that there is no diminution in polysaccharide content during reduction of ER. However, experiments carried out in this laboratory (Dickson, 1957) gave R.Q. values of nearly unity for both glucose oxidation and ER, and ERes. must be of the same oxidation level as glucose.

When endogenous-reduced cells of Sarcina lutea are incubated with radioglucose, ^{14}C is assimilated (XV, a, i) and ER and TTC-reduction are raised. Fractionation, hydrolysis and chromatographic analysis of the cell material indicate that some of the ^{14}C is fixed in glutamate and alanine, but

the bulk of the activity is contained in fast-moving unidentified spots (XV, e, iii). No radioactive reducing sugars could be detected.

The information available regarding the ERes. of Sarcina lutea is not complete but it seems to be quite clearly established that it is not of carbohydrate nature and is thus analogous to baker's yeast, as reported by Stickland (1956).

(b) Glucose utilisation by Sarcina lutea.

(i). Utilization of substrates. Washed cell suspensions of Sarcina lutea consume oxygen in the presence of glucose, fructose, galactose, mannose, glucosamine, gluconate, 2-ketogluconate, ribose, arabinose, xylose, pyruvate and lactate. No uptake was noted with phosphorylated intermediates such as G-6-P, G-1-P, hexosediphosphate and α-glycerophosphate. Little can be deduced from these data, but the metabolism of gluconate, 2-ketogluconate and the pentoses indicates that pathways other than glycolysis must exist. The lack of oxygen uptake on certain substrates may be caused by permeability or transport effects which are certainly present, as shown by the increase of Q_{O_2} for certain substrates (e.g., gluconate) consequent on lyophilization of cell material. That phosphorylated intermediates do not give any oxygen uptake may be due to these permeability barriers which cannot reside in the cell wall as proved by the inability of prote-

plasts to oxidize G-6-P. These observations naturally raise the question of whether a direct non-phosphorylated route is the main form of carbohydrate metabolism (see below, ii).

That all the glucose is not oxidized is shown by the rapid fall in rate of oxygen consumption before the theoretical maximum uptake has been reached (XIII, a, ii). The fact that the initial rate of oxygen consumption can be restored by addition of more glucose, and also the total oxygen consumption be increased by addition of DNP (XIII, a, v), suggest that substrate is being assimilated. Conclusive proof of this is found in the results with radioglucose where it is seen that up to about 70% of the substrate activity may be bound within the cell (XV, a, i). The nature of the bound material is unknown (XV, e, iii).

It was thought, in the earlier stages of this work, that the point of inflexion in the rate of oxygen consumption was of some significance. In particular, the reproducible finding that this change in rate of oxygen consumption always occurred at exactly one mole of oxygen per mole of substrate in the presence of arsenite or iodoacetate was of great interest. This level of oxygen consumption corresponds to the formation of pentose or 2-ketogluconate but, as it is now known that a large portion of glucose has been assimilated at this stage, the derived relationship no longer stands.

(ii). The position of phosphorus in relation to glucose utilisation by Sarcina lutea has been clarified to some extent by the discovery of phosphorus-deficient cell material (XIII, a, iii; c, ii). This material was obtained accidentally when a batch of cells was washed with water rather more frequently than was usual and it was later found that the phosphorus-deficiency could be increased by dialysis against water. Material prepared in this way oxidized glucose very slowly, as shown by oxygen uptake and substrate utilization (figs.29 and 33), and this defect may be abolished by addition of phosphate. The same material did not assimilate ^{14}C from radioglucose and it was because of this that it was first realized to be different from the cells normally used. (This last observation is not included in the Results as sufficient material was not available to carry out the necessary control experiment with added phosphate.) Thus it may be concluded that phosphate is required for normal glucose metabolism, but this does not mean that glucose is metabolized through a series of phosphorylated derivatives, as the phosphorus might be required for other processes such as oxidative phosphorylation. This point is of considerable importance because of the failure to detect kinases in cell-free extracts (XIV, a, ii; b, i). Nevertheless, a phosphate requirement has been proved and the observation that cell suspensions metabolising glucose excrete small amounts of glucose-6-phos-

phate into the medium (XIII, v, iv; c, vi) support the occurrence of phosphorylated derivatives in carbohydrate metabolism by Sarcina lutea. The lack of detectable kinase activity could be due to the powerful ATP-hydrolysing activity found in extracts (XIV, c, ii).

It may be assumed, then, that glucose metabolism in Sarcina lutea involves phosphorylated intermediates, and that the lack of oxygen uptake by whole cells, when these intermediates are substrates, is due to permeability effects.

(iii). The effect of aerobiosis. Glucose is not dissimilated under anaerobic conditions by Sarcina lutea. This was shown by the absence of acid production (VII, a, vi) and also by direct analysis for glucose utilization (XIII, c, iii). Aerobically glucose is metabolized readily and the rate was measured both by oxygen uptake (XIII, a, ii) and by direct analysis for glucose in reaction mixtures (XIII, c, iii). There is no difference in the rate of glucose dissimilation in air or in oxygen, thus showing that oxygen tension is not limiting within these broad limits (fig.34).

(iv). The effect of inhibitors was investigated principally by manometric techniques (table 5). The partial inhibition by iodoacetate can be attributed to action on EMP while the apparently contradictory result with fluoride could be explained on the basis of a manganese-activated enolase unaffected by fluoride. Terminal respiration is probably by TCA as malonate

causes a 50% inhibition of oxygen uptake on glucose, and a 43% inhibition with cyanide implicates the cytochromes as hydrogen carriers. The observation that Sarcina lutea contains cytochromes (Smith, 1954) was confirmed for our strain (Murray, 1956).

(v). Enzymes of carbohydrate metabolism were sought in cell-free extracts. The failure to detect kinases is a fundamental deficiency in this aspect of the research but there are good reasons for believing that phosphorylated derivatives occur in Sarcina lutea (XVI, b, ii). Aldolase and triose-phosphate dehydrogenase were detected as representatives of EMP (XIV, b, iii) while glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PGA) dehydrogenases (XIV, b, i & ii) indicate one of the alternative pathways. The inability to detect any of the 'non-phosphorylated' dehydrogenases supports the occurrence of phosphorylated pathways. If one accepts the normal functioning of the enzymes detected, the occurrence of subsequent enzymes in the respective pathways must be assumed as no products of these reactions accumulate in normal cellular metabolism (XII, b, iv; c, vi). In this context it is interesting to note that the activity of 6-PGAdh is greater than G-6-Pdh so that, in the HMP at least, there would be little possibility of an accumulation of the primary dehydrogenation product.

(vi). Products of glucose utilisation. The only products of glucose metabolism that could be detected were glucose-6-phosphate (XIII, b, iv; c, v) and pyruvic acid (XIII, c, iv). As the first of these comes at the beinning of both EMP and HMP, and as pyruvate can be the product of either of these pathways, no deductions as to route can be drawn. The amount of pyruvate produced (figs.36 & 37), however, is too great to be explained on the basis of the sole operation of HMP, and the existence of EMP or EDS may be presumed.

(vii). Conclusions as to the route of glucose utilisation are difficult to draw on the data obtained. Phosphate is of importance and phosphorylated derivatives are probably involved. If glycolysis exists it does not act anaerobically. Enzymes of EMP and HMP are present and substrates of the direct pathways are oxidised. These meagre conclusions indicate that the approach on the substrate and enzymatic level was rather fruitless and, although further experiments could have been attempted, it was decided that greater opportunity lay in the application of tracer techniques as described below (XIII, d).

(c) Terminal respiration in Sarcina lutea.

(i). Utilisation of substrates. Many substrates associated with terminal respiration are oxidised by Sarcina lutea. These are pyruvate, acetate, citrate, iso-citrate, α-keto-

glutarate, succinate, fumarate and oxaloacetate (XIII, a, ii). Cis-aconitate was the only Krebs' cycle intermediate which was not oxidized, although oxalosuccinate was not available for investigation. The position of ∝-ketoglutarate is of interest in that it is oxidized only by whole cells as opposed to lyophilised material and many preparations which will utilize pyruvate will not utilize ∝-ketoglutarate (fig. 38). It would appear that the system for dissimilation of this intermediate is somewhat labile in Sarcina lutea.

(ii). Enzymes of terminal respiration were sought in cell-free extracts (XIV, b, iv). The absence of ∝-ketoglutaric dehydrogenase may be considered to be due to the lability of this system noted above. However, the unequivocal demonstration of iso-citric dehydrogenase and associated aconitase (fig.45) place in perspective the low or zero Q_{O_2} values obtained with two of the tricarboxylic acids. The strong activity of these enzymes in cell-free systems almost certainly indicates that the low oxygen uptakes observed may be due to permeability factors rather than low levels of oxidative enzymes.

(iii). Conclusions. Sarcina lutea now joins that small group of micro-organisms in which the Krebs' cycle is definitely known to operate. This assertion is based on the observation that most of the individual members of the cycle are

oxidised by preparations of organisms or, in the cases where oxygen uptakes were low or absent, the relevant enzymes have been shown to be extremely active within the cell. Not only is the existence of the cycle proven but its importance in the utilisation of glucose is shown by the inhibitory action of malonate on glucose oxidation (table 5). Thus, although investigation at the substrate and enzymic level has not established the primary route of glucose metabolism, the approach has been vindicated for the study of terminal respiration, and the role of the Krebs' cycle is firmly established.

(d) Quantitative evaluation of pathways of glucose utilization in Sarcina lutea.

(i). The occurrence of alternative pathways is clearly demonstrated by the preferential release of CO_2 from C-1 of glucose (table 11). No evaluation is based on these data but they are of importance in showing that essentially the same result is obtained in the presence and absence of arsenite. Justification of the use of an inhibitor in experiments of this nature is, of course, essential as it can be argued that addition of toxic substances might derange different routes of catabolism to varying degrees. This possibility is unlikely in the present instance as arsenite is known to affect EMP, EMP and KDS only at a stage which is

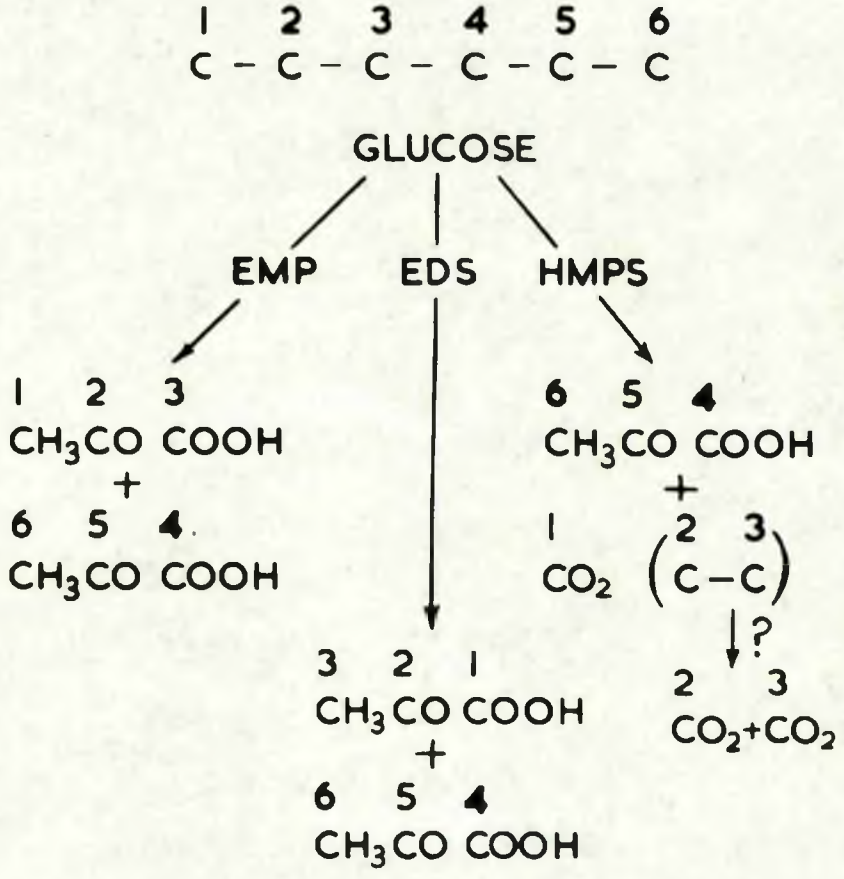
common to all three pathways and, at any rate, the data on CO_2 evolution show that there is no essential change consequent on arsenite addition.

(ii). The evaluation of the hexosephosphate oxidative pathway (HMP). Two experiments are quoted in this context (tables 12 and 13) and these will be dealt with separately. In the first, the specific activities were 3,350 and 1,145 for glucose-1-^{14}C (substrate) and pyruvate (product) respectively. The distribution of the carbon atoms of glucose in relation to several pathways has been described (figs.12, 13 and 14) and is summarized in fig.47 which shows the carbon redistribution in metabolically produced pyruvate. We may now consider the case where the parent glucose was labelled with ^{14}C in the first or aldehydic carbon atom. If the assumptions inherent in fig.47 are correct, labelled pyruvate can arise only via EMP or EDS. Furthermore, each of these pyruvate molecules is accompanied by an unlabelled molecule formed by the same route, and the amount of pyruvate arising from one glucose molecule via EMP or EDS is twice that arising via HMP.

All the activity of glucose-1-^{14}C is located in a single carbon atom and the specific activity of this carbon (on a per atom basis) is the same as that of the whole molecule (on a per molecule basis); in this case the value is 3,350.

Figure 47.

Distribution of the carbon atoms of glucose in pyruvic acid by various pathways.



Thus if all the pyruvate molecules contained a labelled carbon atom this would contribute 3,350 counts/min. to each molecule and the specific activity would be 3,350 per molecule. But the observed specific activity of pyruvate is 1,145 and dilution with non-labelled material must have taken place.

Let there be y labelled molecules per 100 molecules of pyruvate then there will be (100 - y) unlabelled molecules. Now the specific activity of the pyruvate is 1,145 and this is obtained by dividing the count recorded by the amount of material plated. 100 molecules of pyruvate, containing y labelled molecules, will give a count of 3,350y and the specific activity is 3,350y/100, which in this case is estimated as 1,145. Thus,

Percentage of labelled pyruvate molecules = $y = \frac{1,145 \times 100}{3,350}$

$= \underline{34\%}$. (1)

But as each labelled pyruvate molecule arises via EMP or EDS and is accompanied by an unlabelled pyruvate molecule, it follows that:

Percentage of pyruvate molecules via EMP & EDS = 2 x 34

$= \underline{68\%}$. (2)

If HMP is the only other pathway producing pyruvate from glucose:

Percentage of pyruvate molecules via HMP = 100 - 68

$= \underline{32\%}$. (3)

Now, one glucose molecule yields two pyruvate molecules via EMP or EDS but only one by HMP. Therefore 68 molecules of pyruvate via EMP and EDS represent 34 molecules of glucose, and 32 molecules of pyruvate via HMP represent 32 molecules of glucose. Thus for every 34 molecules of glucose catabolized via EMP and EDS, 32 are degraded via HMP. The percentage of glucose molecules which goes through HMP can now be calculated:

$$\text{Percentage of glucose catabolized via HMP} = \frac{32}{34 + 32} \times 100$$

$$= 48.5\% \qquad (4)$$

This calculation assumes that carbon atoms 2 and 3 of the substrate glucose do not yield pyruvate via HMP (fig.47) and the value obtained is thus a maximum. If, however, one assumes that these two atoms can be recycled (fig.9) and yield pyruvate, a minimum value can be calculated on the basis that one glucose molecule yields $1\frac{2}{3}$ molecules of pyruvate. This value is:

$$\frac{32 \times \frac{3}{5}}{(32 \times \frac{3}{5} + 34)} \times 100 = \underline{36\%}. \qquad (5)$$

The data contained in table 12 (glucose-1-^{14}C, 8,060; pyruvate, 2890) can be treated in the same way with the following result:

$$\text{Percentage of labelled pyruvate} = \frac{2,890 \times 100}{8,060}$$

$$= \underline{38.8} \qquad (1a)$$

Percentage of pyruvate molecules via EMP & EDS = 71.6 (2a)

Percentage of pyruvate molecules via HMP = 28.4 (3a)

Percentage of glucose catabolized via HMP = 44.0 (4a)

Percentage of glucose via HMP (minimum) = 32.0 (5a)

(iii). The position of the Entner-Doudoroff system (EDS) was ignored in the previous section in that it was not differentiated from EMP. Examination of fig.47 reveals that this route yields carboxyl-labelled pyruvate when glucose-1-^{14}C is the substrate. The data in table 14 show that only 4 per cent. of the activity in pyruvate produced from glucose-1-^{14}C is found in the carboxyl group. This might indicate minor participation of EDS in glucose catabolism but it is more likely that this figure represents either a randomization of ^{14}C within the substrate hexose chain or fixation of $^{14}CO_2$ in the pyruvate molecule. The question of fixation of $^{14}CO_2$ is important and it is for this reason that the data for pyruvate derived from glucose-U-^{14}C are included in table 12. One important difference in the protocols for the two experiments should be noted; when glucose-U-^{14}C was taken as substrate, a sample of cells was used which had a small endogenous activity, while in the case of glucose-1-^{14}C the cells had no endogenous activity. The rationale for this difference is that non-labelled CO_2 can be formed from glucose-1-^{14}C but not from glucose-U-^{14}C and by carrying out the reaction with

the latter substrate, in the system used, a supply of non-labelled CO_2 was made available.

It is possible for the carboxyl carbon atom of pyruvate to equilibrate with carbon dioxide. This could occur by carboxylation of the pyruvate to yield oxaloacetate which, via malate, is in enzymatic equilibrium with the symmetrical molecules of fumarate or succinate. Reversal of the process could yield pyruvate in which the original carboxyl group is lost as carbon dioxide and is replaced by the carbon dioxide fixed in oxaloacetate. That something of this nature occurs is shown by the observation that the carboxyl group of pyruvate derived from glucose-U-^{14}C, in the system described above, only possesses 86 per cent. of the activity calculated on the basis of a uniformly labelled pyruvate molecule (table 14). It may be assumed that the diminution of activity in this carbon atom is due to equilibration with non-labelled carbon dioxide. The appearance of ^{14}C in the carboxyl-group of pyruvate formed from glucose-1-^{14}C can be explained on the same basis if we assume that the carboxyl-group is unlabelled and that it equilibrates with CO_2 containing $^{14}CO_2$ derived from C-1 of the substrate glucose. There is, however, another possible mechanism which could explain the appearance of ^{14}C in this grouping, without involving the enzymes of EDS. This requires the mediation of transaldolase (TA) and trans-

ketolase (TK) which must be present if EMP is operative (VI, a, iv & v). The reactions are:

$$1\text{-}^{14}\text{C-hexose-P} + \text{triose-P} \overset{TK}{\rightleftharpoons} 1\text{-}^{14}\text{C pentose-P} + \text{tetrose-P}$$

$$1\text{-}^{14}\text{C-pentose-P} + \text{hexose-P} \overset{TK}{\rightleftharpoons} 3\text{-}^{14}\text{C heptulose-P} + \text{tetrose-P}$$

$$3\text{-}^{14}\text{C-heptulose-P} + \text{triose-P} \overset{TA}{\rightleftharpoons} 3\text{-}^{14}\text{C hexose-P} + \text{tetrose-P}$$

The tetrosephosphate produced in these reactions could be metabolized by transketolase action and the 3-^{14}C-hexosephosphate could be degraded by the enzymes of EMP to give 1-^{14}C-pyruvate.

To summarize, the appearance of ^{14}C in the carboxyl group of pyruvate derived from glucose-1-^{14}C can be explained on the bases of randomisation of the substrate by TK and TA, equilibration of the product with $^{14}CO_2$ formed from the C-1 of glucose-1-^{14}C or by the action of EDS. Formation by EDS is rather unlikely as, to date, this mechanism is thought to be confined to Gram-negative micro-organisms. Randomization of the substrate is possible and equilibration with $^{14}CO_2$ is feasible as this type of reaction has been shown to take place with pyruvate-U-^{14}C.

(iv). Assumptions and conclusions. Before drawing any final conclusions from these experiments it is desirable to consider the assumptions inherent in their design and interpretation.

Firstly, it is assumed that glucose is metabolized by Sarcina lutea both in the presence and absence of arsenite, in essentially the same manner, to the stage of pyruvate. This is considered feasible because arsenite is thought to exert its effect at a point common to all known routes of glucose utilisation. In addition, cells fix radioactivity from glucose-U-^{14}C in the same manner irrespective of the presence of arsenite, and the ratio of $^{14}CO_2$ obtained from glucose-U-^{14}C and glucose-1-^{14}C is virtually identical whether arsenite is added or not. Furthermore, the amount of arsenite added is not sufficient to block pyruvate utilisation completely and, because of this, approximates more closely to an uninhibited system. This is also upheld by the observation that the formation of the small amounts of glucose-6-phosphate, which is noted in systems metabolising glucose, is unaffected by addition of arsenite.

The second assumption is that only EMP, EDS and HMP can occur in glucose utilisation by Sarcina lutea. This is probably justified as none of the data obtained by the various approaches attempted is incapable of explanation on this basis. The fate of the carbon atoms degraded via HMP is assumed to be as shown in fig.47. On this formulation maximum participation of HMP is calculated and the minimum value presented allows for the possible contribution of C-atoms 2 and 3 of glucose in pyruvate production. It is not impossible that

the maximum value is correct, and the minimum too low because it is based on the assumption of recyclization of these two C-atoms to hexose which is then degraded by EMP. If some of the hypothetical recyclized hexose were degraded via HMP this would increase the calculated value of this pathway above the minimum quoted.

Thirdly, it is assumed that randomisation of substrates or products is not affecting any major trend in isotope distribution. This point has been discussed above and it should be noted that if EDS had occupied a major role in glucose catabolism the randomization of the pyruvate-1-^{14}C formed from glucose-1-^{14}C would have partially vitiated the interpretation of the data. Fortunately, it is unlikely that EDS occurs to a quantitatively important extent and this potential source of error does not arise. There is a small amount of $^{14}CO_2$ fixation in the carboxyl group of pyruvate which has been ignored in the calculation but which, if so small an amount could be measured accurately, would raise the values for HMP participation if the proper correction were applied. Thus, assuming that 4 per cent. of the activity is located in the carboxyl group (table 14), and applying the correction to the data of table 13, the maximum participation of the shunt is raised from 44 to 50 per cent. The calculation is identical with those already presented but the specific

activity of the isolated pyruvate is taken as 96 per cent. of the experimentally derived value. That this small discrepancy in the specific activity of the pyruvate causes a proportionally greater variation in the calculation of pathway contribution is inherent in the mathematics of the calculation. This must be borne in mind in the final interpretation of the data.

The final assumption is that the specific activity of the isolated pyruvate is not depressed by the endogenous production of unlabelled material. Such errors can, of course, be corrected by estimating endogenous pyruvate production experimentally, but for the critical experiments presented in tables 12 and 13 cell material was used which did not display any endogenous pyruvate production.

Bearing in mind the possible sources of error, it is reasonable to conclude that both EMP and HMP occur in Sarcina lutea. They are responsible for approximately equal proportions of the glucose utilized, and, if EDS occurs, it can only be of minor significance.

(e) Questions raised by the results.

The writer is aware that as many questions have been raised as have been answered by the work presented. In the field of endogenous metabolism it would be of fundamental

importance to discover the chemical nature of the endogenous reserve and to ascertain its relation to oxygen uptake and TTC-reduction. It is hoped that the first part of this problem might be solved by fractionation of cell material and that the result obtained will point the way to a further investigation of the two phenomena related to endogenous metabolism.

The observation that the Krebs' cycle operates in Sarcina lutea raises the question of whether its primary function is the supply of energy or intermediates for growth. This problem has been tackled by several workers using other systems and it is not intended to pursue the matter further at the present time.

Perhaps the most interesting observation is that the occurrence of EMP in Sarcina lutea is now established. Why, then, is this organism a strict aerobe and why does it not dissimilate glucose anaerobically? In the first instance, it is intended to tackle this problem by making a complete survey for the enzymes of EMP in cell-free extracts of Sarcina lutea. It is hoped that when this is accomplished some peculiarity in the enzymatic complement will be revealed which might explain the strictly aerobic nature of glycolysis in this organism.

APPENDIX I.

A considerable portion of the literature on *Sarcina lutea* is not relevant to this thesis; the more important papers in this category are included in this appendix.

(a) Classification, growth and nutrition of *Sarcina lutea*: Boersch, 1921; Guerin & Thiry, 1913; Katznelson & Lockhead, 1952; Koser & Bottger, 1919; Nys, 1928; Petit, 1931; Ritter, 1912; Weinberg, 1950.

(b) *Sarcina lutea* and the Gram stain: Finkelstein & Bartholomew, 1956; Mittwer & Bartholomew, 1956.

(c) Action of antibiotics, disinfectants etc. on *Sarcina lutea*: Bouillenne & Bouillenne-Walrand, 1951; Brown & Binkley, 1956; Culler *et al.*, 1948; Fuller & Rvgosa, 1935; Gray & Kazin, 1946; Kuzyurina, 1946; Orlandi *et al.*, 1950; Shema *et al.*, 1949; Todd & Smith, 1932.

(d) *Sarcinae* in the brewing industry: de Smedt, 1934; Engelhard, 1937; Fuchs, 1929, 1936; Gunnison & Marshall, 1937; Janensch, 1936a, 1936b; Kretschmer, 1935; Landisperaky, 1937; Laufer & Siegel, 1934; Lund, 1947; Petit, 1935; Rascumov & Rubenstein, 1936; Satava, 1929; Schnegg & Schachner, 1936; Shimwell & Kirkpatrick, 1939; Silbernagel, 1928; Stockhausen, 1925.

APPENDIX II.

Microbiological systems have been assumed to utilize the system of glycolysis as the result of four main types of experiment:

1. Production and utilization of EMP intermediates,
2. Extraction of the enzymes of EMP,
3. Sensitivity to inhibitors,
4. Fermentation of isotopically labelled substrates.

The principal applications of these methods found in the literature are as follows:

Method 1.

Aerobacter aerogenes, Wiggert & Workman, 1938;

Aerobacter indologenes, Stone & Workman, 1937;

Aspergillus niger, Kiessling, 1950;

Asteromyces mycoides, Rodwell & Rodwell, 1954;

Azotobacter vinelandii, Stone & Workman, 1937;

Bacillus mycoides, Stone & Workman, 1937;

Bacillus subtilis, Garry & Bard, 1952; Stone & Workman, 1937;

Brucella suis, Roessler et al., 1952;

Clostridium acetobutylicum, Bolcato et al., 1950;

Clostridium butyricum, Gavard, 1952;

Escherichia coli, Antoniani, 1933; Endo, 1938; Stone & Workman, 1937; Tikka, 1955;

Euglena gracilis, Albaum et al., 1950;

Lactobacillus casei, Virtanen & Tikka, 1930;

Lactobacillus delbrueckii, Neuberg & Kabel, 1933;

Lactobacillus pentoaceticus, Stone & Werkman, 1937;

Lactobacillus plantarum, Katagiri & Murakami, 1939; Stone & Werkman, 1937;

Lactobacillus sake, Katagiri & Murakami, 1939;

Leuconostoc mesenteroides, Katagiri & Murakami, 1939;

Propionibacterium arabinosum, Stone & Werkman, 1937; Werkman et al., 1937;

Propionibacterium pentosaceum, Stone & Werkman, 1937; Werkman et al., 1937;

Staphylococcus albus, Fosdick & Rapp, 1943; Stone & Werkman, 1937.

Streptococcus paracitrovorus, Stone & Werkman, 1937;

and various yeasts, Katagiri & Murakami, 1939.

Method 2.

Achromobacter fischeri, Friedman, 1954;

Agrobacterium tumefaciens, Hill & Mills, 1954;

Asterococcus mycoides, Rodwell & Rodwell, 1954;

Bacterium tularense, Hill & Mills, 1954;

Chlorella pyrenoidosa, Holzer & Holzer, 1952;

Clostridium butyricum, Gavard, 1952, 1954;

Clostridium perfringens, Bard & Gunsalus, 1950;

Escherichia coli, Still, 1940;

Microbacterium lacticum, Vandemark & Wood, 1956;
Streptococcus haemolyticus, McIlwain, 1948;
Streptomyces coelicolor, Cochrane, 1955;
and various yeasts, Dixon & Atkins, 1913.

Method 3.

Ashbya gossypii, Nickelson, 1950;
Bacillus subtilus, Gary & Bard, 1952;
Clostridium perfringens, Bard & Gunsalus, 1950;
Propionibacterium pentosaceum, Volk, 1954;
and Streptomyces coelicolor, Cochrane, 1955.

Method 4.

Aspergillus niger, Shu et al., 1954;
Bacillus subtilis, Neish, 1953;
Butyribacterium rettgeri, Pine et al., 1954;
Clostridium thermoaceticum, Barker et al., 1945; Wood, 1952;
Escherichia coli, Cohen, 1951;
Fusarium lini, Heath et al., 1956;
Lactobacillus casei, Bernstein et al., 1955; Gibbs et al., 1950; Wood et al., 1945;
Leuconostoc mesenteroides, Bernstein et al., 1955;
Pasteurella pestis, Santer & Ajl, 1955;
Rhizopus oryzae, Gibbs & Gastel, 1953;
and various yeasts, Blumenthal et al., 1954; Gibbs et al., 1955; Gilvarg, 1952; Koshland & Westheimer, 1950.

APPENDIX III.

The Krebs' cycle has been assumed to operate in microbiological systems as the result of four main types of experiment:

1. Utilisation, accumulation and interconversion of TCA intermediates,
2. Extraction of enzymes of TCA,
3. Sensitivity to inhibitors,
4. Distribution of isotopes from labelled substrates.

Method 1.

Acetobacter pasteurianum, King et al., 1956;

Acetobacter peroxydans, Atkinson, 1956;

Achromobacter guttatum, butyri & superficiale, Sguros & Hartsell, 1952;

Ashbya gossypii, Mickelson & Schuler, 1953;

Azotobacter vinelandii, Stone & Wilson, 1952a;

Brucella abortus, Altenbern & Housewright, 1952;

Corynebacterium creatinovorans, Fukui & Vandemark, 1952;

Micrococcus lysodeikticus, Saz & Krampitz, 1955;

Mycobacterium tuberculosis, Millman & Youmans, 1954, 1955;

Neisseria gonorrhoeae, Tonbazy & Pelczar, 1953;

Pasteurella tularensis, Kann & Mills, 1955;

Penicillium chrysogenum, Casida & Knight, 1954;

Propionibacterium pentosaceum, Delwiche et al., 1953;

Pseudomonas aeruginosa, Campbell & Stokes, 1951;

Vibrio 01, Dagley et al., 1952; Dagley & Rodgers, 1953;
Dagley & Walker, 1956;

and various yeasts, Foulkes, 1951; Lynen, 1939; Lynen & Necifullah, 1939; Virtanen & Sundman, 1942; Wieland & Sonderhoff, 1932.

Method 2.

Acetobacter pasteurianum, King et al., 1956;

Aspergillus niger, Neilson, 1955; Ramakrishnan & Martin, 1954;

Azotobacter vinelandii, Alexander & Wilson, 1956;

Blastocladiella emersonii, Cantino & Hyatt, 1953;

Corynebacterium creatinovorans, Fukui & Vandemark, 1952;

Escherichia coli, Wheat et al., 1956;

Micrococcus lysodeikticus, Sas & Krampitz, 1955;

Mycobacterium phlei, Blakely, 1952;

Mycobacterium tuberculosis, Millman & Youmans, 1955;

Neisseria gonorrhoeae, Tonhazy & Pelczar, 1953;

Pasteurella pestis, Engelsberg & Levy, 1955;

Penicillium chrysogenum, Casida & Knight, 1954; Hockenhull et al., 1954;

Propionibacterium pentosaceum, Delwiche et al., 1953;

Pseudomonas fluorescens, Barrett & Kallio, 1953; Kogut & Podoski, 1953;

Rhodospirillum rubrum, Eisenberg, 1953;

Streptomyces coelicolor, Cochrane & Peck, 1953;
and various yeasts, Eaton & Klein, 1954; Foulkes, 1951; Hirsch, 1952; Jacobsohn, 1931; Kornberg & Price, 1951; Krebs et al., 1952; Mossel, 1954; Novelli & Lipmann, 1950.

Method 3.

Ashbya gossypii, Nickelson & Schuler, 1953;
Brucella abortus, Altenbern & Housewright, 1952;
Corynebacterium creatinovorans, Fukui & Vandemark, 1952;
Micrococcus lysodeikticus, Sax & Krampitz, 1955;
Pasteurella pestis, Kann & Mills, 1955;
Penicillium chrysogenum, Goldschmidt et al., 1956;
and various yeasts, Krebs et al., 1952.

Method 4.

Aerobacter aerogenes, Ajl & Wong, 1951;
Azotobacter vinelandii, Stone & Wilson, 1952;
Escherichia coli, Ajl & Wong, 1955; Swim & Krampitz, 1954;
Micrococcus lysodiekticus, Ajl et al., 1951; Sax & Krampitz, 1955;
Penicillium chrysogenum, Goldschmidt et al., 1956;
Pseudomonas aeruginosa, Claridge & Workman, 1954;
Rhodospirillum rubrum, Glover et al., 1952;
and various yeasts, Wang et al., 1953; Weinhouse et al., 1947; Sonderhoff & Thomas, 1937; Lynen, 1947.

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