THE METABOLISM OF AMINO SUGARS.

Thesis submitted by C.J. Bates for the Degree of Doctor of Philosophy. The research was carried out in the Department of Biochemistry, Oxford, under the supervision of Dr. C.A. Pasternak.

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ABSTRACT

1. The incorporation and distribution of radioactivity from 1-^{14}C-D-glucosamine, N-acetyl-1-^{14}C-D-glucosamine and 1-^{14}C-N-acetyl-D-glucosamine in growing cultures of Bacillus subtilis NCTC 1379 has been investigated.
2. The radioactivity from N-acetyl-1-^{14}C-D-glucosamine is incorporated rapidly and the rate of incorporation is almost independent of the external concentration. The radioactivity from 1-^{14}C-D-glucosamine is incorporated more slowly and the rate of incorporation is proportional to the external concentration over a wide range. Both compounds are incorporated more rapidly in cells pregrown in the presence of amino sugars than in cells pregrown without amino sugar.
3. The incorporation of 1-^{14}C-D-glucosamine is greatly reduced by D-glucose, but very little affected by N-acetyl-D-glucosamine, N-propionyl-D-glucosamine or N-formyl-D-glucosamine in the growth medium. The incorporation of N-acetyl-1-^{14}C-D-glucosamine is not greatly reduced by D-glucose, but severely affected by N-propionyl-D-glucosamine and N-formyl-D-glucosamine.
4. Radioactivity from 1-^{14}C-D-glucosamine and N-acetyl-1-^{14}C-D-glucosamine is incorporated mainly into the hot trichloracetic

acid fraction and residue fraction of Bacillus subtilis, whereas that from 1-^{14}C—N-acetyl-D-glucosamine is incorporated into all the fractions. Evidence is presented that the acetyl group of N-acetyl-D-glucosamine is liberated or exchanged during incorporation of the glucosamine moiety. The distribution of radioactivity from labelled amino sugars in Escherichia coli is somewhat different from that obtained in Bacillus subtilis.

5. When the fractions obtained from Bacillus subtilis grown with 1-^{14}C-D-glucosamine or N-acetyl-D-^{14}C-D-glucosamine are subjected to acid hydrolysis, a high proportion of the radioactivity can be recovered in compounds behaving like glucosamine, galactosamine and muramic acid; most of the muramic acid is present in the residue fraction. The overall ratios in terms of radioactivity are : glucosamine (14) : galactosamine (1) : muramic acid (4).
6. Addition of 6-azauracil to the growth medium results in a small increase in amino sugar-containing material in the acid-soluble fraction of the cells.
7. 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (deaminating), E.C.5.3.1.10. (Gm-6-P deaminase) and 2-acetylamino-2-deoxy-D-glucose-6-phosphate N-acetyl hydrolase (AcGm-6-P deacetylase) are induced by N-acetyl-D-glucosamine in Bacillus subtilis and in Escherichia coli, but not by many structurally related amino sugars in Bacillus subtilis.

8. L-glutamine-D-fructose-6-phosphate aminotransferase, E.C.2.6.1.16. (Gm-6-P synthetase) is repressed by N-acetyl-D-glucosamine in Bacillus subtilis and Escherichia coli and by D-glucosamine, N-propionyl-D-glucosamine and N-formyl-D-glucosamine in Bacillus subtilis. Other structurally related amino sugars were found not to repress this enzyme in Bacillus subtilis.

9. All the amino sugars which produce control effects are metabolised by the growing cells; a gratuitous inducer or repressor was not found.

10. If D-glucose is added to a culture of Bacillus subtilis growing in the presence of N-acetyl-D-glucosamine, the induction of Gm-6-P deaminase and that of AcGm-6-P deacetylase are reduced from 20-30 fold to 1-3 fold. Likewise the repression of Gm-6-P synthetase is reduced from tenfold to twofold. If D-glucose is added to a culture of Bacillus subtilis growing in the presence of D-glucosamine, N-formyl-D-glucosamine or N-propionyl-D-glucosamine, the repression of Gm-6-P synthetase is abolished.

11. Glucose has a much smaller effect on the induction of Gm-6-P deaminase and AcGm-6-P deacetylase by N-acetyl-D-glucosamine in Escherichia coli. It does, however, reduce the rate of disappearance of N-acetyl-D-glucosamine from the growth medium and the rate of growth of this organism on N-acetyl-D-glucosamine as sole nitrogen source.

12. Benzyl penicillin, 2,6 dimethoxybenzamido penicillin and 6-azauracil have no appreciable effect on the specific activity Gm-6-P deaminase, AcGm-6-P deacetylase or Gm-6-P synthetase in Bacillus subtilis.

13. When grown beyond the end of logarithmic phase in broth + glutamate medium, the specific activity of Gm-6-P deaminase in Bacillus subtilis is increased. If glucose is present in the growth medium, this increase is not observed, but instead some material behaving like an acylated amino sugar accumulates in the growth medium.

14. Methods for the partial purification of Gm-6-P deaminase from Bacillus subtilis are described.

15. L-valine-sRNA ligase (AMP), E.C.6.1.1.9. was shown to be present in extracts of Bacillus subtilis. Attempts to demonstrate incorporation of radioactivity from 1-^{14}C-DL-valine and synthesis of Gm-6-P deaminase in a cell-free system were unsuccessful.

16. Kinases responsible for the phosphorylation of D-glucosamine and of N-acetyl-D-glucosamine were demonstrated in extracts of Bacillus subtilis and Escherichia coli. The products formed from radioactive amino sugars by Bacillus subtilis extracts were purified and their properties examined.

17. In Bacillus subtilis, both kinases are induced by N-acetyl-D-glucosamine and by D-glucosamine, whereas in Escherichia coli they are constitutive. In both organisms the kinase which phosphorylates D-glucosamine appears to be separate from that which phosphorylates N-acetyl-D-glucosamine.

18. The activity of acetyl CoA : 2-amino-2-deoxy-D-glucose-1-phosphate N-acetyl transferase is slightly lower in extracts of Escherichia coli grown on N-acetyl-D-glucosamine than in extracts of cells grown on D-glucose. The activity of D-glucose-6-phosphate ketol-isomerase E.C.5.3.1.9. is the same in both extracts.

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Abbreviations.

Amino sugars.

Gm	D-glucosamine HCl
AcGm	N-acetyl-D-glucosamine
PrGm	N-propionyl-D-glucosamine
n-BuGm	N-n-butyryl-D-glucosamine
iso-BuGm	N-iso-butyryl-D-glucosamine
succ-Gm	N-succinyl-D-glucosamine
FoGm	N-formyl-D-glucosamine
GalM	D-galactosamine HCl
AcGalm	N-acetyl-D-galactosamine
AcMm	N-acetyl-D-mannosamine
Gm-6-P	D-glucosamine-6-phosphate
AcGm-6-P	N-acetyl-D-glucosamine-6-phosphate
PrGm-6-P	N-propionyl-D-glucosamine-6-phosphate
n-BuGm-6-P	N-n-butyryl-D-glucosamine-6-phosphate
AcMm-6-P	N-acetyl-D-mannosamine-6-phosphate
Gm-1-P	α-D-glucosamine-1-phosphate
AcGm-1-P	N-acetyl-α-D-glucosamine-1-phosphate
UDPGm	Uridine diphosphate-α-D-glucosamine
UDPAcGm	Uridine diphosphate-N-acetyl-α-D-glucosamine
UDP PyGm	Uridine diphosphate-3-enol-pyruvyl-N-acetyl--D-glucosamine

UDPAcMur	Uridine diphosphate-N-acetyl muramic acid
UDPAcGalm	Uridine diphosphate-N-acetyl-α-D-galactosamine
TDPGm	Thymidine diphosphate-α-D-glucosamine
TDPAcGm	Thymidine diphosphate-N-acetyl-α-D-glucosamine
TDPAcGalm	Thymidine diphosphate-N-acetyl-α-D-galactosamine
NANA	N-acetyl neuraminic acid
NGNA	N-glycolyl neuraminic acid
NANA-9-P	N-acetyl neuraminic acid-9-phosphate
CMPNANA	Cytidine monophosphate-N-acetyl neuraminic acid
deAcGm	N-acetyl-6-deoxy-D-glucosamine
IAcGm	N-acetyl-6-iodo-6-deoxy-D-glucosamine

Sugars

G-6-P	D-glucose-6-phosphate
F-6-P	D-fructose-6-phosphate

Other chemicals.

AMP,ADP,ATP	Adenosine mono, di, tri-phosphate
GDP,GTP	Guanosine di, tri-phosphate
CMP, CDP,CTP	Cytidine mono, di, tri-phosphate
UMP,UDP,UTP	Uridine mono, di, tri-phosphate
TDP,TTP	Thymidine di, tri-phosphate
PEP	Phospho-enol-pyruvate

$NAD, NADH_2$	Nicotinamide adenine dinucleotide (oxidised and reduced)
$NADP, NADPH_2$	Nicotinamide adenine dinucleotide phosphate (oxidised and reduced)
CoA	Coenzyme A
AzU	6-azauracil
FU	5-fluorouracil
EDTA	Ethylene diamine tetra-acetic acid
Tris	Tris-(hydroxymethyl)-aminomethane

Radioactive compounds

^{14}Gm	1-^{14}C-D-glucosamine HCl
$Ac^{14}Gm$	N-acetyl-1-^{14}C-D-glucosamine
$^{14}AcGm$	1-^{14}C-N-acetyl-D-glucosamine
^{14}Ac	Sodium 1-^{14}C-acetate

Enzymes

(Reaction numbers refer to diagram I on page 22).

G-6-P dehydrogenase	D-glucose-6-phosphate-NADP oxidoreductase E.C.1.1.1.49
Hexokinase	ATP : D-hexose-6-phosphotransferase E.C.2.7.1.1. (Reaction 1)
Gm kinase	ATP : 2-amino-2-deoxy-D-glucose-6-phosphotransferase E.C.2.7.1.8. (Reaction 1)
AcGm kinase	ATP : 2-acetylamino-2-deoxy-D-glucose-6-phosphotransferase E.C.2.7.1.10. (Reaction 2)

Glucose phosphate isomerase	D-glucose-6-phosphate ketol isomerase E.C.5.3.1.9. (Reaction 3)
Gm-6-P synthetase	L-glutamine:D-fructose-6-phosphate aminotransferase E.C.2.6.1.16 Reaction 4)
Gm-6-P deaminase	2-amino-2-deoxy-D-glucose-6-phosphate ketol isomerase (deaminating) E.C.5.3.1.10 (Reaction 5)
Gm-6-P N-acetylase	Acetyl Co A:2-amino-2-deoxy-D-glucose-6-phosphate N-acetyl transferase E.C.2.3.1.4 (Reaction 6)
AcGm-6-P deacetylase	2-acetylamino-2-deoxy-D-glucose-6-phosphate N-acetyl hydrolase (Reaction7).
Gm-1-P N-acetylase	Acetyl Co A:2-amino-2-deoxy-D-glucose-1-phosphate N-acetyl transferase (Reaction 10)
Phosphoglucomutase	α-D-glucose-1,6-diphosphate : α D-glucose-1-phosphate phosphotransferase E.C.2.7.5.1 (Reaction 11)
Phosphoglucosamine mutase	2-amino-2-deoxy-α-D-glucose-1,6-diphosphate: 2-amino-2-deoxy-α-D-glucose-1-phosphate phosphotransferase (Reaction 11)
Phospho-N-acetyl-glucosamine mutase	2-acetylamino-2-deoxy-α-D-glucose-1,6-diphosphate : 2-acetylamino-2-deoxy-α-D-glucose-1-phosphate phosphotransferase E.C.2.7.5.2. (Reaction 12)
Valyl-sRNA synthetase	L-valine-sRNA ligase (AMP) E.C.6.1.1.9
Lysozyme	N-acetyl muramide glycanohydrolase E.C.3.2.1.17

INTRODUCTION

1. Nature and occurrence of amino sugars.

Amino sugars may be defined as sugars in which one or more hydroxyl groups are replaced by amino groups. The commonest naturally occurring amino sugars are D-glucosamine (2-amino-2-deoxy-D-glucose), D-galactosamine (2-amino-2-deoxy-D-galactose), D-mannosamine (2-amino-2-deoxy-D-mannose), muramic acid (2-amino-3-O-(2-carboxy-ethyl)-2-deoxy-D-glucose) and neuraminic acid (5-amino-3,5-dideoxy-D-glycero-α-D-ido-nonulopyranos-1-onic acid). The following are less widely distributed : D-fucosamine (2-amino-2,6-dideoxy-D-glucose) which occurs in a lipopolysaccharide of Chromobacterium violaceum (1) and in a polysaccharide produced by a strain of Bacillus subtilis (2) ; a 2,4-diamino-dideoxy-hexose which occurs in the same strain of B. subtilis (3) ; talosamine (2-amino-2-deoxy-talose) in a hydrolysate of chondroitin sulphate from tracheal cartilage (4,5,6)* ; 2-amino-2,6-dideoxy-L-galactose and 2-amino-2,6-dideoxy-talose in pneumococcal polysaccharides (7), a 4-amino-4,6-dideoxy sugar (as the TDP derivative) in Escherichia coli (8), and a variety of unusual 2,3,4, and 6-amino-6-deoxy sugars, frequently N-methylated, in bacterial and fungal antibiotics (see reviews by Foster and

* This may be an artifact of the isolation procedure.

Horton, (9) and Dutcher (10)).

2-amino-2-deoxy-D-hexuronic acids have recently been found as components of bacterial polysaccharides : 2-amino-2-deoxy-glucuronic acid in SPA polysaccharide of Staphylococcus aureus (11,12) and in the type-specific substance of Haemophilus influenzae type D (13) ; 2-amino-2-deoxy-D-galacturonic acid has been identified in the Vi antigen of E. coli (14) and 2-amino-2-deoxy-D-mannuronic acid has been found in a cell wall polysaccharide of Micrococcus lysodeikticus (15).

In animals, amino sugars occur in the structural elements of connective tissue and in polymers present in extracellular fluids (16,17,18). In bacteria, amino sugars occur in polymers of the cell wall, spore coats and capsules (19-27). They do not generally occur in storage polymers like starch and glycogen, and the polymers which do contain them usually contain other sugars, amino acids and lipids also, although two homopolymers of amino sugars (chitin and colominic acid) are known. Polymers containing both amino sugars and amino acids are known as muco-polysaccharides or mucoproteins, and those containing lipid are known as lipopolysaccharides. The amino sugars of most of these polymers have substituents on the nitrogen atom ; the commonest is acetyl, but N-glycolyl and N-sulphate groups also occur, in sialic acids and heparin respectively. In certain cases the hydroxyls bear acetyl groups (28).

The polymers of bacterial cell walls which contain amino sugars may be divided into four main types :

(a) Polysaccharides with no amino acids, lipids or phosphate groups attached.

(b) Teichoic acids, consisting of chains of glycerol or ribitol molecules linked through the terminal hydroxyls by phosphate groups, and bearing sugars, amino sugars and D-alanine residues on the free hydroxyl groups of the ribitol and glycerol (29-32).

(c) Mucopeptide, which consists of a backbone of polysaccharide (alternating N-acetyl-D-glucosamine and N-acetyl muramic acid residues) with short peptide chains in ester linkage with the carboxyl of the muramic acid. These may link two mucopeptide chains together, or they may link a mucopeptide chain with another polysaccharide (23).

(d) Complex protein-polysaccharide-lipid polymers and lipopolysaccharides occurring in Gram-negative bacteria (26).

The nature and relative amounts of these constituents varies considerably between different species of bacteria, between different strains of the same species, and even in a given species grown under different conditions. All bacteria appear, however, to contain a basic network of muramic peptide which is responsible for the rigidity of the cell wall and which is broken down by the

bacteriolytic enzyme, lysozyme (N-acetyl muramide glycano hydrolase E.C.3.2.1.17.). The other constituents containing amino sugars are generally found associated with cell wall material when this is prepared by mechanical disruption (19). However, they can usually be extracted into solvents such as trichloracetic acid, formamide or phenol under relatively mild conditions, which suggests that they are linked to mucopeptide by ionic attraction and hydrogen bonding only (23) although in one instance there is some evidence for covalent linkages (33,34).

The work described in this thesis is mainly concerned with the Gram-positive bacterium, B. subtilis, although some experiments have been performed with the Gram-negative E. coli. B. subtilis contains the following polymers in the cell wall of vegetative cells (35) :

(a) Mucopeptide, containing D-glucosamine, muramic acid, D- and L-alanine, D-glutamic acid and meso-diaminopimelic acid (20).

(b) Ribitol teichoic acid, containing D-ribitol, D-glucose and D-alanine (36-38).

(c) Teichuronic acid, a polysaccharide containing equimolar amounts of N-acetyl-D-glucosamine and D-glucuronic acid (39).

(d) A protein fraction (39) comprising 7-10 % of the cell wall.

(e) A polysaccharide containing D-galactose, D-glucosamine

and D-galactosamine in the ratio 2:1:1, and small amounts of a diamino sugar (40,41).

Teichuronic acid has not been found in all strains of B.subtilis (42,43) and the amount of the other major components is variable. B.subtilis is a spore-forming organism and the spore walls contain a much higher proportion of protein than the vegetative ones, although all the components of mucopeptide are present (42). The following table shows the percentage of the major amino sugar components in vegetative walls and the spore coats of B.subtilis :

Table I. Amino sugars occurring in the cell walls and spore coats of various strains of B.subtilis.

	Percentage(by weight) in the cell walls.			Percentage(by weight) in the spore coat.
Amino sugar	Ref.42	Ref.44 (four strains)	Ref.45	Ref.42
D-glucosamine	7.9% *	7.3,7.2,7.0,8.7%	-	1.5% *
D-galactosamine		4.7,4.6,4.2,2.7%	-	
Muramic acid	4.1%	7.1,7.2,7.6,8.7%	11.6%	1.1%

* Gm and Gaim not separated.

II. Metabolism of amino sugars.

Diagram 1 (page 22) summarises the known pathways of amino sugar metabolism with special reference to micro-organisms.

Reaction 1 (R1 on the diagram) is catalysed by a kinase specific for D-glucosamine (ATP ; 2-amino-2-deoxy-D-glucose phosphotransferase, E.C.2.7.1.8.) in certain animal tissues (46,47) and by the more common but less specific enzyme ATP: D-hexose 6-phosphotransferase, E.C.2.7.1.1. in bacteria (48-50) and other tissues.

Reaction 2 (R2) is catalysed by ATP: 2-acetylamino-2-deoxy-D-glucose 6-phosphotransferase, E.C.2.7.1.9. and has been studied in extracts of E.coli (50), Lactobacillus casei (51) and higher animals (52).

Reaction 3 (R3) is catalysed by D-glucose-6-phosphate ketol-isomerase, E.C.5.3.1.9., which is widely distributed in living tissues.

Reaction 4 (R4) is catalysed by L-glutamine:D-fructose-6-phosphate aminotransferase E.C.2.6.1.16. It has been purified from E.coli,Neurospora crassa and rat liver(53), and has been demonstrated in Bacillus cereus, M. lysodeikticus and Aerobacter aerogenes (54) ; in Streptococcus haemolyticus (55) and B.subtilis (56) and in the tissues of many higher organisms. D-glucose-6-phosphate will not replace D-fructose-6-phosphate with the purified enzyme (57), although crude extracts frequently

give more activity with G-6-P than with F-6-P (58,59). There is some evidence that the amino group is transferred from the amide group of L-glutamine and that the products of the reaction are Gn-6-P and glutamic acid (53,55). Neither L-asparagine nor ammonia can replace L-glutamine, and no cofactor requirements could be detected (57). Gram-positive organisms usually contain larger amounts of the enzyme than Gram-negative ones (54).

Reaction 5 (R5), is catalysed by 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (deaminating) E.C.5.3.1.10. and has been purified from E.coli and hog kidney (60) and human brain (61). It also occurs in B.subtilis (56). The reaction is reversible, with the equilibrium lying on the side of deamination ; nevertheless a significant rate of conversion of F-6-P to amino sugar can be obtained by coupling the synthetic reaction to reaction 6 (N-acetylation). The bacterial enzyme has no absolute requirement for cofactors, although N-acetyl-D-glucosamine-6-phosphate stimulates it by decreasing the K_m. The mechanism of this stimulatory effect has been discussed (60,62) ; it appears that AcGn-6-P is not an intermediate in the reaction as originally suggested (63).

Reaction 6 (R6) has been demonstrated in animal tissues(68,69) and in Clostridium kluyveri (70). It is catalysed by acetyl Co A: aryl-amine N-acetyl transferase E.C.2.3.1.4. and by acetyl Co A: 2-amino-2-deoxy-D-glucose N-acetyl transferase E.C.2.3.1.3.

Reaction 7 (R7) is catalysed by acetyl Co A: 2-amino-deoxy-D-glucose-6-phosphate N-acetyl transferase E.C.2.3.1.5. It has been demonstrated in extracts of yeast, N.crassa, group A haemolytic streptococci, E.coli, and higher animals (61-64). In some cases the true substrate may be α-D-glucosamine-1-phosphate formed from the 6-phosphate by Reaction 12 . Attempts to demonstrate N-acetylation of D-glucosamine-6-phosphate in extracts of B.subtilis were unsuccessful (56).

Reaction 8 (R8) and Reaction 9 (R9) have been demonstrated with extracts of Pseudomonas aeruginosa, Streptococcus griseus and E.coli (71). These may be catalysed by a single enzyme which will be referred to as acetyl Co A:2-amino-2-deoxy-α-D-glucose-1-phosphate N-acetyl transferase (abbrev. Gm-1-P N-acetylase).

Reaction 10 (R10) has been demonstrated in extracts of various bacteria including B.cadaveris, Streptococcus faecalis and several strains of E.coli. The enzyme which catalyses this reaction will be referred to as 2-acetylamino-2-deoxy-D-glucose-N-acetyl hydrolase (abbrev. AcGm deacetylase).

Reaction 11 (R11) has been demonstrated in crude extracts of pig kidney (63) and B.subtilis (56). The enzyme will be referred to as 2-acetylamino-2-deoxy-D-glucose-6-phosphate N-acetyl hydrolase (abbrev. AcGm-6-P deacetylase).
Studies on the dissimilation of N-acetyl-D-glucosamine in vivo suggest that it is degraded to glycolytic intermediates, ammonia and acetic acid (73-75).

Reaction 12 (R12) has been studied in extracts of rabbit muscle (76). It is catalysed by an enzyme which appears to be α-D-glucose-1,6-diphosphate:α-D-glucose-1-phosphate phosphotransferase E.C.2.7.5.1., but the activity is several hundred times less with D-glucosamine-6-phosphate than with D-glucose-6-phosphate as the substrate. α-D-glucose-1,6-diphosphate stimulates the reaction at low concentrations.

Reaction 13 (R13) is catalysed by 2-acetylamino-2-deoxy-α-D-glucose-1,6-diphosphate phosphotransferase E.C.2.7.5.2., which requires α-D-glucose-1,6-diphosphate or N-acetyl-α-D-glucosamine-1,6-diphosphate as a cofactor. It has been studied in extracts of N.crassa (77).

Reaction 14 (R14) is catalysed by an enzyme which appears to be UTP:α-D-glucose-1-phosphate uridylyl transferase E.C.2.7.7.9.. It has been studied in extracts of rat liver nuclei (78,79), and a mast cell tumour (80).

Reaction 15 (R15) has been demonstrated in extracts of Ps. aeruginosa, E.coli and S.griseus (68). N-acetyl-α-D-glucosamine-1-phosphate will not replace α-D-glucosamine-1-phosphate and the substitution of uridine triphosphate for thymidine triphosphate results in a ten-fold reduction in the rate of reaction.

Reaction 16 (R16) has been studied in extracts of calf liver and S.aureus (81), yeast (82), sheep brain and the desert locust (67). In the case of calf liver and S.aureus it was partly separated from UTP:α-D-glucose-1-phosphate uridylyl transferase. N-acetyl-α-D-galactosamine-1-phosphate was a much poorer substrate.

Reaction 17 (R17) has been demonstrated in extracts of B.subtilis and rat liver (84) and Reaction 18 (R18) is catalysed by extracts of Ps.aeruginosa (71).

Reaction 19 (R19). The reaction of uridine diphosphate N-acetyl-α-D-glucosamine with phospho-enol-pyruvate to give uridine diphosphate 3-enol-pyruvyl N-acetyl-α-D-glucosamine and Reaction 20 (R20) to uridine diphosphate N-acetyl muramic acid have been studied in extracts of S.aureus, E.coli and A.aerogenes (85). N-acetyl-α-D-glucosamine-1-phosphate

will not replace uridine diphosphate N-acetyl-α-D-glucosamine in Reaction 19.

Reaction 21 (R21). The addition of amino acids to uridine diphosphate N-acetyl muramic acid has been studied in extracts of S.aureus (88,89). The resulting peptide chain differs from normal proteins by the presence of D-amino acids, the fact that D-glutamic acid is joined by the γ-carboxyl to the neighbouring L-lysine and the fact that two D-alanines are added as a dipeptide unit. In distinction from protein synthesis this reaction involves the conversion of adenosine triphosphate to adenosine diphosphate and inorganic phosphate.

Reaction 22 (R22). The incorporation of N-acetyl muramic peptide from uridine diphosphate N-acetyl muramic peptide into a particulate fraction from S.aureus has recently been reported (91,92). It requires uridine diphosphate N-acetyl-D-glucosamine and appears to represent the addition of N-acetyl-glucosamine and N-acetyl muramic peptide units to pre-existing mucopeptide in the particles.

Reaction 23A (R23A). The synthesis of chitin from uridine diphosphate N-acetyl-α-D-glucosamine has been demonstrated in extracts of N.crassa (93,94). The enzyme is particulate and requires a primer (chitin or chitodextrin), and N-acetyl-D-glucosamine stimulates the reaction.

Reaction 23B (R23B). The synthesis of teichoic acids has been studied in a cell-free extract of S.aureus (95). An enzyme which catalyses the synthesis of polyribitol phosphate from cytidine diphosphate ribitol, and enzymes which add AcGm units from UDP AcGm to give N-acetyl glucosamine-polyribitol phosphate containing both α and β linked AcGm units, were demonstrated.

Reaction 23C (R23C). The synthesis of hyaluronic acid has been demonstrated with particles from group A haemolytic streptococci (96-99), Diplococcus pneumoniae (100) and Rous chicken sarcoma (101). An unidentified polysaccharide (possibly a heparin precursor) is synthesised from uridine diphosphate-N-acetyl α-D-glucosamine and uridine diphosphate-α-D-glucuronic acid in a mast cell tumour (102).

Reaction 24 (R24), the formation of free N-acetyl mannosamine from uridine diphosphate-N-acetyl-α-D-mannosamine occurs in animal tissues (113), and its phosphorylation (Reaction 25(R25)) is catalysed by an enzyme different from those which catalyse Reactions 1 and 2 (106).

Reaction 26 (R26) has been demonstrated in extracts of Aerobacter cloacae, Clostridium perfringens and E.coli (97,103-105). N-acetyl-D-mannosamine-6-phosphate is a key intermediate in the synthesis of sialic acids in mammals (106-108), and the enzyme which catalyses Reaction 27 (R27) has been purified from extracts of liver and submaxillary gland (108,109).

This enzyme has not been reported in bacteria, but Reaction 28 (R28), the reversible reaction between N-acetyl- or N-glycolyl-D-mannosamine and pyruvic acid, is catalysed by an enzyme found in extracts of E.coli and Cl.perfringens (107,110,111). Neisseria meningitides contains an enzyme which synthesises N-acetyl neuraminic acid from N-acetyl-D-mannosamine and phospho-enol-pyruvate (112).

Reaction 29 (R29), the conversion of the sialic acids to their cytidine monophosphate derivatives, and Reaction 30 (R30), their incorporaion into the polymer colominic acid have both been demonstrated in extracts of E.coli K 235 (105).

Little is known about the early reactions involving D-galactosamine. There is one report of the phosphorylation of N-acetyl-D-galactosamine in rat liver extracts (52) and extracts of L.casei grown on galactose contain an enzyme which converts D-galactosamine-6-phosphate to tagatose-6-phosphate and ammonia (114).

Reaction 31 (R31) has been demonstrated in extracts of Pseudomonas fluorescens (115,116), and an unidentified organism has been isolated which degrades D-glucosaminic acid to 2-keto-3-depxy-D-gluconic acid and ammonia and can thus

grow on D-glucosaminic acid as sole carbon source (117).

Reaction 32 (R32) is catalysed by extracts of M.lysodeiktious (86). The significance of this reaction is not clear but muramic acid-6-phosphate and a compound believed to be uridine diphosphate-N-acetyl-muramic acid-6-phosphate have been isolated from L.casei (87).

The synthesis of a thymidine diphosphate-4-acetylamino-4, 6-dideoxy sugars from thymidine diphosphate-α-D-glucose has been demonstrated with extracts of E.coli and Pasteurella pseudotuberculosis (118,119). The significance of these reactions is not yet understood.

Some evidence about the nature of the metabolic pathways operating in vivo has been obtained by labelling experiments. The glucosamine of hyaluronic acid appears to arise from labelled glucose without scission of the carbon chain (120-122). Muramic acid in the cell wall of Lactobacillus bifidus var. pennsylvanicus becomes labelled when the cells are grown in the presence of 1-^{14}C-1-methyl-N-acetyl glucosaminide (123), and there is some evidence from work on staphylococci that the lactyl side chain can arise from substances like alanine, pyruvic acid, aspartic acid and lactic acid, which may give rise to phospho-enol-pyruvate inside the cell (124). There are several reports of the incorporation of labelled glucose and glucosamine into mucosubstances in animal tissues (125-129). Incorporation of

glucose was stimulated by L-glutamine in one case, which suggests that Reaction 4 may be operating. When 1-^{14}C-D-glucosamine and N-acetyl-1-^{14}C-D-glucosamine were injected intraperitoneally into rats, labelled AcGm6P, UDPAcGm, and UDPAcGalm were formed but there was little or no labelling of hexoses, hexose phosphates or UDPG (130).

III. The control of enzyme synthesis.

The rate of many enzyme-catalysed reactions is specifically controlled by the metabolite on which the enzyme acts, or by the product of the reaction. This control may be achieved in two ways : by alteration of enzyme activity, or by changes in the rate of enzyme synthesis (131). A model for the latter effect has been proposed (132); it is suggested that the controlling metabolite interacts with a macromolecular cytoplasmic "repressor" whose function is to alter the rate of transcription of the enzyme-coding genes into messenger ribonucleic acid. This in turn alters the rate of synthesis of the enzyme. If the result of the interaction is to deactivate the cytoplasmic repressor, the rate of transcription into messenger ribonucleic acid and hence of enzyme synthesis is increased : in this case the metabolite is termed an "inducer".

If the result is to activate the cytoplasmic repressor, the rate of enzyme synthesis is decreased, in this case the metabolite is termed a "corepressor".

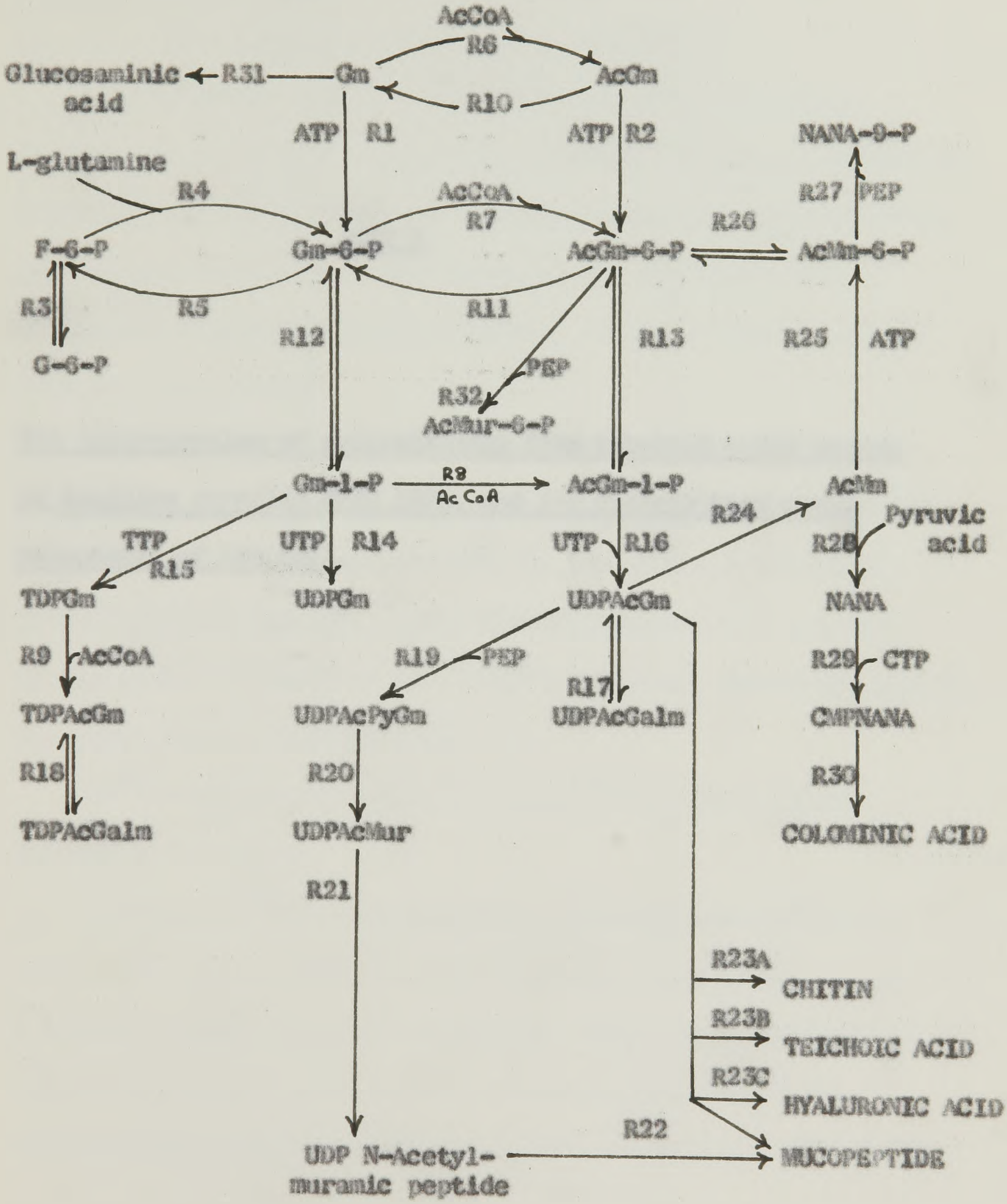
Certain degradative pathways are also controlled by the concentration of catabolic intermediates ; if these are present in abundance, the enzymes which degrade more complex metabolites (like amino acids, purines, pyrimidines and amino sugars) are repressed. This phenomenon has been termed "catabolite repression"(133), and its relation to the specific control effects described in the previous paragraph is not yet clear. In certain cases degradative pathways are controlled by specific end-products, which happen to be easily interconvertible with other carbon and energy sources (134,135).
In the case of β-galactosidase synthesis in E.coli there is evidence that catabolite repression does not act on the parts of the genome which are responsible for the structure and induction of the enzyme (136). Catabolite repression does, however, appear to resemble theother form of repression in-so-far as it acts by reducing the rate of messenger ribonucleic acid production(137,138).

Only a few instances of the control of amino sugar metabolism in micro-organisms have been described. AcGm kinase (Reaction 2) is induced by N-acetyl-D-glucosamine in Aspergillus

parasiticus (139). Gn-6-P synthetase (Reaction 4) is repressed by N-acetyl-D-glucosamine in B.subtilis and Gn-6-P deaminase (Reaction 5) is induced (56) ; the latter enzyme is also induced in E.coli grown on D-glucosamine (140). AcGn deacetylase (Reaction 10) is induced by N-acetyl-D-glucosamine in E.coli(72). AcGn-6-P deacetylase is induced by N-acetyl-D-glucosamine in B.subtilis (139).

The investigations described in this thesis are a continuation of the work on B.subtilis. Part I describes the incorporation of radioactivity from labelled amino sugars into components of the cell, and Part II describes some further investigations on the enzymes concerned with amino sugar metabolism.

Diagram 1. Known pathways of amino sugar metabolism with special reference to micro-organisms.



PART I

The incorporation of radioactivity from labelled amino sugars by Bacillus subtilis NCTC 1379, and its distribution among components of the cell.

Materials and Methods.

(a). Source and synthesis of chemicals.

D-glucosamine HCl, N-acetyl-D-glucosamine, D-glucose, D-fructose, D-mannose, D-galactose, sucrose, lactose, D-arabinose, D-ribose, acetic anhydride and sodium acetate were obtained from the British Drug Houses Ltd. D-lyxose was obtained from Pfanstiehl Chemical Company, Illinois. Muramic acid was a gift from Dr. R.E. Strange.

N-propionyl-D-glucosamine, N-n-butyryl-D-glucosamine, and N-iso-butyryl-D-glucosamine were prepared from D-glucosamine HCl and the appropriate acid anhydride by the method of Kuhn and Bister(143)(see page 65). N-formyl-D-glucosamine was prepared by the method of Meyer zu Reckendorf and Bonner, from D-glucosamine and ethyl formate (144)(see Page 66).

1-^{14}C-D-glucosamine HCl was obtained from New England Nuclear Corporation.

N-acetyl-1-^{14}C-D-glucosamine was prepared from 1-^{14}C-D-glucosamine HCl by the method of Kuhn and Bister (143), using a ten-fold excess of acetic anhydride. When chromatographed in solvent A it gave a radioactive peak containing 90% of the radioactivity coincident with N-acetyl-D-glucosamine and several

minor peaks nearer the solvent front which may have been O-acetylated derivatives. The product was treated with methanol saturated with ammonia and kept at room temperature overnight; it then gave a single radioactive peak coincident with N-acetyl-D-glucosamine (when chromatographed in solvent A). The ammonia and methanol were removed by rotary evaporation.

1-^{14}C-N-acetyl-D-glucosamine was similarly prepared from D-glucosamine HCl and 1-^{14}C-acetic anhydride (obtained from the Radiochemical Centre, Amersham); in this case the glucosamine was present in excess. The product was purified by :

i) adding a tenfold excess of water, and heating 2 min. at 100°C to decompose the acetic anhydride.

ii) passing through a column of Amberlite IR 120 (hydrogen form) to remove glucosamine.

iii) passing through a column of Dowex I (chloride form) to remove labelled acetic acid.

The product gave a single radioactive peak coincident with authentic N-acetyl-D-glucosamine when chromatographed in Solvent A.

1-^{14}C-sodium acetate was obtained from the Radiochemical Centre, Amersahm.

(b). Paper chromatography: methods and solvents.

Whatman no.1 paper was used, with the following solvents (descending) :

A). n-butanol/pyridine/water, 6/4/3, v/v/v (145).

B). n-butanol/acetic acid/water, 4/1/5, v/v/v (146)

C). Pyridine/ethyl acetate/acetic acid/water, 5/3/5/1, v/v/v (147).

D). n-butanol/ethanol/water, 4/1/1, v/v/v (148).

E). n-butanol/n-propanol/acetone/formic acid/trichloracetic acid/water, 8/4/5/4/1/3, v/v/v/v/w/v. (149).

Solvents A-D were used to separate sugars and amino sugars, and solvent E was used to separate sugar phosphates and nucleotides. All these solvents required 20-24 hrs. to obtain optimum separation of the relevant compounds, usingpapers 45cm. long.

Reducing compounds were detected by staining with silver nitrate (150) and aniline hydrogen phthalate (151), and amino sugars were detected by staining with Ehrlich's reagent (152). Radioactivity was assayed with a Radioactive Chromatogram Counter (Baird and Tatlock Ltd.). In certain cases the coincidence of the radioactive and stained areas was confirmed by radioautography on Kodirex X-ray film.

(c). Growth and harvesting of organisms.

Bacillus subtilis NCTC 1379 was grown in a medium containing 1.3% w/v Oxoid nutrient broth, grade CM1, plus 25 mM sodium glutamate, sterilised by autoclaving at 15 lb./sq.in. for 15 min. Other chemicals were sterilised separately by autoclaving, except for D-glucosamine and the radioactive amino sugars which were sterilised by filtration through Oxoid membrane filters.

The cultures were grown in Erlenmeyer flasks, shaken on a reciprocal shaker at 37°C for 10 hrs. Growth was measured on 5ml. samples with an EEL Unigalvo Type 20 nephelometer, the red filter (OR2) being in position (to allow for the colour of the broth) and the perspex standard adjusted to give a reading of 25. The bacteria were harvested when the EEL reading was between 50 and 80 : this corresponds to a range of dry weights of 20-50 mg./100 ml. of growth medium. Readings of 90-100 were obtained when the culture had reached stationary phase. The cells were harvested by centrifuging at 5,000 r.p.m. for 15 min. in an M.S.E. refrigerated centrifuge, washed twice by resuspending in 100 ml. of (chilled) 0.85% sodium chloride solution, and suspended in 5 ml. water. Aliquots were assayed for radioactivity (section(e)) and total protein (154).

(d). Fractionation of the cells.

The cells were fractionated by the method of Roberts et al. (153), slightly modified. To a suspension of the cells in water (5 ml.), 0.5 ml. of 50% trichloracetic acid was added. After standing 30 min. at 0-4°C, the insoluble material was centrifuged down and the supernatant ('cTCA fraction') decanted. The pellet was then extracted with 5 ml. portions of 75% ethanol/25% water ('alc$_1$ fraction'), followed by 50% ethanol/50% ether ('alc/ether fraction'), both kept at 40-50°C for 15 min. ; then with 5% trichloracetic acid : three successive 5 ml. portions kept in a boiling water bath for 15 min. each ('hTCA fraction'), and finally with 75% ethanol/25%water again ('alc$_2$ fraction'). The final residue was suspended in 5 ml. of water ('res fraction'), and part was removed for the assay of protein by the biuret method (154).

(e). Assay of radioactivity.

Samples of the growth medium, cells suspended in water, and the various fractions were evaporated on aluminium planchettes (2.2 cm.diam.), and the radioactivity assayed with a G.E.C. probe type Geiger-Müller tube* fitted to a Panax scaler, or with an automatic sample changer (Nuclear Chicago** or Isotopes Development Ltd.***). The samples of growth medium had a large self absorption,

* counting efficiency c. 2%; background 15 counts/min.
** counting efficiency c.10%; background 7 counts/min.
*** counting efficiency c. 2%; background 2 counts/min.

and it was difficult to obtain an accurate measure of the radioactivity in this material, but the self absorption of the other samples was negligible.

(f). Hydrolysis of the fractions and investigation of their composition.

Before hydrolysis the trichloracetic acid fractions were extracted with ether until neutral (pH 6-7). The amount of radioactive material lost in this process was negligible. The other fractions were evaporated to dryness on a rotary evaporator.

In one experiment the fractions were hydrolysed with 1N H_2SO_4 for 6 hrs. at 105°C in sealed tubes. They were neutralised with barium hydroxide, the barium sulphate centrifuged down, washed with water and the combined supernatants reduced to a small volume by rotary evaporation.

In another experiment the cTCA, hTCA and res. fractions were hydrolysed with 1N HCl at 100°C for 6.5 hrs., and the residue fraction was hydrolysed under the same conditions for 30 hrs. A small amount of material remained undissolved at the end of this time, but it contained no radioactivity, and was discarded. The hydrochloric acid was removed by repeated evaporation and addition of water.

The hydrolysed fractions were analysed for total amino sugars (155) using glucosamine as both internal and external standards, and for total phosphate (156). Part was subjected to paper chromatography, using solvents A and B.

The major part of each hydrolysate was fractionated on a column (59cm. X 0.8 cm. diameter) of Dowex 50Wx8 by the method of Gardell (157). Approximately 2 ml. fractions were collected at a flow rate of 4 ml./hr. by drop counting, using an LKB fraction collector. The eluates were assayed for amino sugars (155) and for radioactivity, the (HCl-containing) samples being evaporated on glass cover slips embedded in silicone grease on aluminium planchettes. When 240 ml. of 0.3 N HCl had been passed through, the column was washed with 200 ml. of 6 N HCl.

Results.

1. Incorporation of labelled amin o sugars from the growth medium.

a). Incorporation of 1-^{14}C-D-glucosamine.

In some initial experiments the bacteria were grown with D-glucose (5mM) and 1-^{14}C-D-glucosamine HCl (0.01mM, 0.2-0.5 µc. in 100 ml. growth medium). Incorporation was negligible under these conditions, but when the glucose was omitted, an appreciable incorporation was obtained (up to 13% when the amino sugar was present for the whole of the growth period. and the cells were grown to 43mg. dry weight).

Table II shows the effect of variations in concentration on the rate of incorporation of 1-^{14}C-D-glucosamine from the growth medium.

Table II. Incorporation of 1-^{14}C-D-glucosamine at various initial concentrations in the growth medium.

100ml. portions of broth + glutamate medium containing 0.5 μc. of 1-^{14}C-D-glucosamine HCl and uniabelled D-glucosamine HCl to give the desired concentration were inoculated with B.subtilis and grown to 40-50 mg. dry wt.. They were harvested, washed and assayed for radioactivity and protein as described under Materials and Methods.

Concentration of Gm in growth medium (mM)	Radioactivity incorporated (μc.)	Percentage of total Gm incorporated	Protein (mg.)	μ moles Gm incorporated per mg. protein
0.0025	0.035	7.0	14.7	0.0012
0.01	0.067	13.3	14.5	0.0092
0.025	0.039	7.8	15.5	0.0126
0,1	0.036	7.2	13.3	0.047
0,25	0.036	7.1	13.5	0,132
1.0	0.039	7.8	12.4	0.63
5.0	0.032	6.3	16.8	1.87

Evidently the addition of unlabelled D-glucosamine does not dilute the incorporation of radioactivity : in fact the percentage incorporated was constant over a 500-fold concentration range, apart from a small peak at 0.01 mM. In order to test whether D-glucosamine affects the levels of enzymes concerned in amino sugar metabolism, the rate of incorporation of 1-^{14}C-D-glucosamine and N-acetyl-1-^{14}C-D-glucosamine was measured in cells pregrown on glucosamine- or glucose-containing media. The results are given in Table III.

Table III. The effect of pregrowth on D-glucosamine on the rate of incorporation of labelled amino-sugars.

400 ml. of broth + glutamate containing 5 mM D-glucose and 400 ml. of broth + glutamate containing 5 mM D-glucosamine HCl were each inoculated with B.subtilis and grown to 9 mg. dry wt. /100 ml. They were each harvested, washed with 100 ml. broth + glutamate and resuspended in 200 ml. portions of broth + glutamate. 100 ml. portions of each were treated with 1mM 1-^{14}C-D-glucosamine HCl (0.4 μc)and the remaining 100 ml. portions with 1 mM 1-^{14}C-D-glucosamine HCl (0.13 μc.). They were incubated at 37°C (shaking) for a further 60 min., harvested, washed and the radioactivity and protein assayed as described under Materials and Methods.

Radioactive amino sugar	Cells pregrown on :	Radioactivity incorporated (μc)	Percentage of total amino sugar incorporated	Protein (mg)	μmoles amino sugar incorporated per mg. protein
^{14}Gm	D-glucose	0.0028	0.7	12.0	0.058
^{14}Gm	D-glucosamine	0.019	4.8	12.9	0.40
Ac^{14}Gm	D-glucose	0.0006	0.5	10.3	0.046
Ac^{14}Gm	D-glucosamine	0.0060	5.0	11.0	0.46

It therefore appears that pregrowth on D-glucosamine does stimulate the subsequent incorporation of amino sugars. A similar experiment was performed with chloramphenicol in the growth medium (at 50 µg/ml.) during the incubation with radioactive amino sugars (1mM) to prevent the synthesis of protein and hence any change in enzyme levels during the second incubation (the duration of which was increased to 150 min.). The incorporation of ^{14}Gm by the glucosamine-grown cells was 5.5 times higher than that of the glucose-grown cells,and the incorporation of Ac^{14}Gm by the glucosamine-grown cells was 4.7 times higher than that of the glucose grown cells.

b). Incorporation of N-acetyl-1-^{14}C-D-glucosamine.

At a concentration of 0.01 mM., the incorporation of N-acetyl-1-^{14}C-D-glucosamine was much more rapid than the incorporation of 1-^{14}C-D-glucosamine, and the majority was incorporated in 15 min. by a culture in mid-logarithmic phase. Table IV shows the effect of increasing concentrations of unlabelled AcGm on the rate of incorporation of Ac^{14}Gm :

Table IV. Incorporation of N-acetyl-1-^{14}C-D-glucosamine at various initial concentrations in the growth medium.

1 litre of broth glutamate was inoculated and grown to 14 mg. dry wt./100 ml.. It was divided into 100 ml. portions ; five of these were treated with N-acetyl-1-^{14}C-D-glucosamine (0.4 µc.) and unlabelled N-acetyl-D-glucosamine to give the final concentrations shown in column 1. They were incubated for the periods shown in column 2 and harvested, washed and radioactivity assayed as described under Materials and Methods.

Final Concentration of AcGm in growth medium (mM)	Period of incubation (min.)	Radioactivity incorporated (µc.)	µmoles of AcGm incorporated per min.
0.0085	15	0.063	0.0089
0.029	15	0.025	0.0122
0.10	60	0.069	0.029
0.50	90	0.044	0.060
2.50	90	0.0084	0.059

There is evidently some increase in the incorporation of Ac^{14}Gm as the total concentration is raised, but the increase is only 6.6 fold over a 300 fold range of concentration, whereas in the case of ^{14}Gm it was 200-300 fold over a similar concentration range.

Pregrowth of the cells on N-acetyl-D-glucosamine increases the rate of incorporation of labelled amino sugars (Both ^{14}Gm and Ac^{14}Gm) about ten-fold.

c). Incorporation of 1-^{14}C-N-acetyl-D-glucosamine.

The incorporation of label from 1-^{14}C-N-acetyl-D-glucosamine (0.005 mM in the growth medium) was much slower and less complete than that of a similar amount of N-acetyl-1-^{14}C-D-glucosamine ; during 12-14 hrs. growth only about 10% of the label was incorporated. After a period of contact with growing cells, the radioactive material remaining in the medium no longer behaved like N-acetyl-D-glucosamine but was volatile and could be adsorbed on Dowex 1 (chloride form). After only 1 hour's incubation with a growing culture (initially at a density of 7.5 mg./ml.), more than 90% of the radioactivity in the medium could be adsorbed by Dowex 1. 12% of the radioactivity was incorporated by the cells in this instance.

2. Competition between labelled amino sugars and unlabelled carbon sources during incorporation by growing cells.

Cells grown in the presence of D-glucose plus N-acetyl-D-glucosamine have smaller amounts of deaminase than cells grown in the presence of N-acetyl-D-glucosamine alone (56). It was

therefore of interest to examine the effect of glucose on the rate of incorporation of the amino sugars. Table V (which is an extension of the experiment described in Table IV) shows the effect of glucose on the rate of incorporation of various concentrations of N-acetyl-1-^{14}C-D-glucosamine.

Table V. Competition between N-acetyl-1-^{14}C-D-glucosamine and D-glucose.

For experimental details, see table IV. In addition to the five flasks containing N-acetyl-1-^{14}C-D-glucosamine, another five were incubated with N-acetyl-1-^{14}C-D-glucosamine plus 1 mM glucose.

Concentration of N-acetyl glucosamine in the growth medium (mM)	(a)Radioactivity incorporated in the absence of glucose (μc.)	(b)Radioactivity incorporated in the presence of glucose (μc.)	(b) expressed as a percentage of (a)
0.0085	0.063	0.034	54
0.029	0.025	0.013	52
0.10	0.069	0.028	41
0.50	0.044	0.011	25
2.50	0.0084	0.0022	26

Evidently glucose reduces the rate of incorporation of N-acetyl-1-^{14}C-D-glucosamine by about 50% at low concentrations of AcGm, and this is not overcome, but is actually increased, by increasing the concentration of AcGm. This increase in the glucose effect at high concentrations of AcGm may be because, in addition to competing for an enzyme, glucose reverses the control effects exerted by AcGm, and thus prevents it from facilitating its own incorporation at high concentrations (see table XIV page 86).

In view of the fact that glucose was found to have some effect on the rate of incorporation of N-acetyl-1-^{14}C-D-glucosamine, a variety of other carbon sources were tested, in the hope of finding one which produced the 'catabolite repression' effect of glucose without affecting the rate of incorporation of N-acetyl-1-^{14}C-D-glucosamine (at a concentration too low to induce or repress the enzymes concerned in amino sugar metabolism). The results are given in Table VI.

Table VI. Effect of various carbon sources on the rate of incorporation of N-acetyl-1-^{14}C-D-glucosamine.

This table summarises the results of several different experiments : in each experiment the cultures were grown to about 25 mg./ 100 ml., divided into 100 ml. portions and N-acetyl-1-^{14}C-D-glucosamine (0.005 mM ; 0.25 µc.) and carbon sources (5mM) were added. The cultures were incubated at 37°C for 15-20 min. (which was sufficient to permit the incorporation of 10-40 % of the labelled amino sugar), harvested and assayed for radioactivity as described under Materials and Methods. The results are expressed as a percentage of the incorporation obtained in a control flask with no added carbon source.

Carbon source	Incorporation compared with that in the absence of competitors.
none	100 %
D-glucose	40
D-fructose	40
D-mannose	70
D-galactose	70
Sucrose	50
Lactose	90

Table VI cont.

Carbon source	Incorporation compared with that in the absence of competitors.
D-Arabinose	90 %
D-Ribose	90
Sodium acetate	95
AcGm	1.0
Gm	60
PrGm	1.0
FoGm	3
n-BuGm	50
iso-BuGm	50

None of the carbon sources other than amino sugars thus had a very marked effect on the rate of incorporation of Ac^{14}Gm, although all those which produced catabolite repression, namely glucose, fructose and sucrose (see table XIV, page 86) also slightly reduced the rate of incorporation. Two of the amino sugars (PrGm and FoGm) on the other hand, reduced the rate of incorporation almost as effectively as unlabelled AcGm itself.

Table VII shows the effect of varying the concentration ratio of PrGm to Ac^{14}Gm.

Table VII. Effect of N-propionyl-D-glucosamine on the rate of incorporation of N-acetyl-1-^{14}C-D-glucosamine.

A 500 ml. culture was grown to 5 mg./ 100 ml., divided into 100 ml. portions, and N-acetyl-1-^{14}C-D-glucosamine (0.01mM, 0.15µc.) and N-propionyl-D-glucosamine at the stated concentrations were added. The cultures were incubated at 37°C for 30 min., harvested, washed and assayed for radioactivity as described under Materials and Methods.

PrGm (mM)	Radioactivity incorporated (µc.)	Incorporation compared with that in the absence of PrGm
0	13.6	100 %
0.005	10.5	77
0.05	4.1	33
0.5	0.8	6
5.0	0.2	1.5

It can be seen from this table that at the concentrations tested, an increase in N-propionyl-D-glucosamine concentration produces a comparable decrease in the incorporation of N-acetyl-1-^{14}C-D-glucosamine : that is, the effect is closer to a simple competitive relationship then with glucose (see Table V).

The effect of N-propionyl-D-glucosamine, N-formyl-D-glucosamine and N-butyryl-D-glucosamine on the rate of incorporation of label from 1-^{14}C-N-acetyl-D-glucosamine was also tested. The results are shown in table VIII.

Table VIII. Effect of amino sugars on the rate of incorporation of label from 1-^{14}C-N-acetyl-D-glucosamine.

A 400 ml. culture was grown to 7.5mg./100 ml., divided into 100 ml. portions and 1-^{14}C-N-acetyl-D-glucosamine (0.005 mM, 0.5μc.) and amino sugars (5mM) were added. The cultures were incubated for 60 min. at 37°C and harvested, washed and assayed for radioactivity as described under Materials and Methods. The radioactivity in the medium (after incubation) was assayed before and after passage through a column of Dowex I (chloride form) ; KOH was added to the planchettes to prevent the loss of acetate.

Competitor	Radioactivity incorporated (μc.)	Incorporation compared with that in the absence of competitors.	Radioactivity in growth medium (μc.)	
			Before Dowex I	After Dowex I
none	0.0063	100 %	0.46	0.05
PrGm	0.0006	10	0.51	0.30
FoGm	0.0007	9	0.49	0.27
n-BuGm	0.0061	97	0.41	0.05

Evidently N-propionyl-D-glucosamine and N-formyl-D-glucosamine reduce both the rate of incorporation of radioactivity, and its rate of conversion to acidic material ; N-n-butyryl-D-glucosamine has no appreciable effect on either process.

Since the incorporation of N-acetyl-1-^{14}C-D-glucosamine is not reduced to the same extent by glucosamine as by N-propionyl-D-glucosamine and N-formyl-D-glucosamine, it seemed possible that D-glucosamine might be incorporated by a different pathway and therefore show a different response to added carbon sources from N-acetyl-D-glucosamine.
Table IX shows the result of an experiment designed to test this prediction.

Table IX. Effect of various carbon sources on the rate of incorporation of 1-^{14}C-D-glucosamine

An 800 ml. culture was grown to 22 mg./100 ml., divided into 100 ml. portions and 1-^{14}C-D-glucosamine (0.005 mM, 0.25 µc.) and carbon sources (5mM) added. The cultures were incubated at 37°C for 60 min., harvested, washed and assayed for radioactivity as described under Materials and Methods. The incorporation obtained in the control flask containing ^{14}Gm alone was 2.7 % of the total added.

Competitor	Incorporation compared with that in the absence of competitors.
none	100 %
D-glucose	5
D-fructose	95
AcGm	100
Gm	170
PrGm	100
FoGm	100
n-BuGm	100

In other experiments, where the ^{14}Gm and competitors were present throughout the growth period, glucose reduced the incorporation of label to less than 1 % of the control value, and fructose, mannose and sucrose also had appreciable effects,

while the N-acylated amino sugars had comparatively small effects (less than 50 % reduction). Even when equal concentrations of glucose and ^{14}Gm (5mM) were used, the reduction of incorporation by glucose was greater than 95 %.

In an attempt to locate the site of competition between glucose and glucosamine more precisely, the following experiment was performed. Cells grown on broth + glutamate were harvested and resuspended in 3 ml. broth + glutamate (about 6 mg/ml.) and treated with 1-^{14}C-D-glucosamine (1.2 mM, 1.5 µc.). To 1 ml. of this, glucose was added immediately (final conc. 25 mM.) and it was incubated 30 min. at 37°C. The other 2 ml. were incubated 5 min. at 37°C ; 1 ml. was washed and harvested immediately ; the other was treated with glucose (25mM) and incubated for a further 25 min. at 37°C. All three were washed with 100 ml. 0.85 % sodium chloride and assayed for radioactivity and protein. There was at least four times as much radioactivity in the sample incubated 5 min. without glucose and 25 min. with glucose than in either of the others. This suggests that in the first 5 min. 1-^{14}C-D-glucosamine gave rise mainly to diffusible products which could be washed out of the cell by NaCl solution, and glucose prevented the accumulation, but not the further metabolism, of these products.

3. Fractionation of B.subtilis after growth with labelled amino sugars and labelled sodium acetate.

In order to investigate the nature of the labelled compounds incorporated by growing cultures of B.subtilis, the cells were fractionated by the method of Roberts et al.(153). The results are given in Table X :

Table X. Distribution of radioactivity in fractions obtained after growth on labelled amino sugars and labelled sodium acetate.

Labelled compounds ($Ac^{14}Gm$, ^{14}Gm, $^{14}AcGm$ and ^{14}Ac), plus a small inoculum, were added to 100 ml. medium ; after 10 hrs. growth at 37°C the cultures were harvested, washed and fractionated as described under Materials and Methods. In the case of N-acetyl-1-^{14}C-D-glucosamine, about 80 % of the added radioactivity was incorporated ; in the case of the other compounds, only about 10 % was incorporated. The radioactivity in each fraction was expressed as a percentage of the total incorporated, and the dry weights of the individual fractions are expressed as a percentage of the total dry weight (determined in a separate experiment).

Fraction	Dry weight %	Incorporation $Ac^{14}Gm$	^{14}Gm	$^{14}AcGm$	^{14}Ac
Whole cells	100 %	100 %	100 %	100 %	100 %
cTCA	15	10	12	3	5
Alc_1	15	5	2.5	38	35
Alc/Ether	5	1	1	14	12
hTCA	34	65	60	14	10
Alc_2	5	1	1	13	11
Res	36	19	25	18	27

The proportion of radioactivity in the cTCA fraction could not be increased appreciably by increasing the extraction time to 12 hrs. or by increasing the temperature to 37°C. The majority of the radioactivity from N-acetyl-1-^{14}C-D-glucosamine and 1-^{14}C-D-glucosamine HCl is thus incorporated into the hTCA fraction, with smaller quantities in the res. and cTCA fractions ; the pattern of incorporation was very similar for these two amino sugars. 1-^{14}C-N-acetyl-D-glucosamine and 1-^{14}C sodium acetate, on the other hand, show a different distribution, with the highest incorporation in the alc. fraction, which suggests that the radioactivity may be incorporated into lipids (153).

In one experiment the distribution of radioactivity between the particulate and supernatant fractions of sonicated cells grown in the presence of 1-^{14}C-D-glucosamine was examined. The cells were grown to 17.5 mg./100ml., treated with 1-^{14}C-D-glucosamine (0.25mM.10μc.) and incubated for a further 30 min. at 37°C. They were harvested, washed twice with cysteine/EDTA medium and sonicated (see Page 73). The sonicate was centrifuged at 105,000g for 60 min., and the pellet and supernatant assayed for radioactivity, 46 % of the radioactivity was present in the supernatant fraction. The supernatant was then centrifuged again at 105,000g for a

further 60 min. : only 17.5 % of the total radioactivity was now present in the supernatant. At least 80 % of the radioactivity incorporated into the cells under these conditions is therefore present in, or attached to, particulate material.

Since N-acetyl-D-glucosamine induces the degradative enzymes Gm-6-P deaminase and AcGm-6-P deacetylase, it might be expected that cells pregrown with N-acetyl-D-glucosamine in the growth medium would show a different pattern of incorporation from those pregrown with glucose. In the case of ^{14}Gm incorporation, however, this was not so : the distribution of radioactivity was identical in cells pregrown on either 5mM glucose or 5mM N-acetyl-D-glucosamine (although the rate of incorporation was about ten times greater in the cells grown on AcGm (see page 37).

Attempts were made to increase the proportion of radioactivity in the cTCA fraction by reducing the period of contact of the cells with labelled amino sugars, but it was not possible to obtain any large accumulation or to detect intermediates such as amino sugar phosphates by the analytical techniques described on page 57.

4. Fractionation of E.coli grown with labelled amino sugars.

Since Gram negative organisms are known to differ from Gram positive ones in the structure and components of their cell walls, the distribution of radioactivity from amino sugars in E.coli was measured. The results are given in Table XI, which includes those of Table X for comparison.

Table XI. Distribution of radioactivity from 1-^{14}C-D-glucosamine and N-acetyl-1-^{14}C-D-glucosamine in B.subtilis and E.coli.

E.coli 518 was grown to 38 mg./100ml. in 200 ml. of Davis minimal medium containing 5 mM. glucose. It was divided into two 100 ml. portions ; one received 1-^{14}C-D-glucosamine (0.005 mM, 0.25µc.) and the other N-acetyl-1-^{14}C-D-glucosamine (0.005 mM.,0.25µc.). Both cultures were shaken for 30 min. at 37°C, harvested, washed and assayed for radioactivity as described under Materials and Methods.

Fraction	Incorporation of radioactivity			
	Ac^{14}Gm (B.subtilis)	Ac^{14}Gm (E.coli)	^{14}Gm (B.subtilis)	^{14}Gm (E.coli)
Whole cells	100 %	100 %	100 %	100 %
CTCA	10	34	12	31
Alc_1	5	11	2.5	13
Alc/Ether	1	1	1	1
HTCA	65	19	60	18
Alc_2	1	1	1	1
Res	19	33	25	36

It can be seen that more radioactivity enters the cTCA, alc$_1$ and res. fractions with E.coli than with B.subtilis, and considerably less enters the hTCA fraction. As in the case of B.subtilis, the distribution obtained with ^{14}Gm was similar to that obtained with Ac^{14}Gm. The incorporation pattern obtained with E.coli grown in broth + glutamate medium was similar to that obtained in Davis minimal medium.

5. Further analysis of the fractions obtained from B.subtilis grown in the presence of labelled amino sugars.

The cells were pregrown on a glucosamine-containing medium in order to obtain maximal incorporation. A 200 ml. culture containing D-glucosamine (5mM) was inoculated with B.subtilis and grown to about 60 mg. total dry weight. The culture was harvested, washed once with 200 ml. of broth + glutamate and resuspended in 400 ml. of broth + glutamate containing 1-^{14}C-D-glucosamine (0.01mM., 10μc.). The culture was grown to about 260 mg. total dry weight, harvested, washed, fractionated, hydrolysed and analysed as described under Materials and Methods. The cTCA, hTCA and res. fractions together contained more than 90 % of the incorporated radioactivity.

Attempts to separate the components of the crude reaction mixture by paper chromatography using solvents A and B were

unsuccessful since the amino sugars frequently gave more than one spot, (cf.Crumpton(159)). The alternative method of separation by chromatography on Dowex 50 was therefore employed.

A preliminary investigation of the separation of D-glucosamine, D-galactosamine and muramic acid on Dowex 50 as described under Materials and Methods had shown that Gm and Galm are satisfactorily separated from each other, but that muramic acid and Galm are not. The samples of hydrolysate were therefore mixed with 2-5 µmoles of unlabelled Gm and Galm markers before chromatography. Graphs I-III* show the distribution of radioactivity and amino sugar in the effluent fractions obtained when the cTCA, hTCA and res. fractions were analysed by this method. It can be seen that the two main peaks of radioactivity in each fraction coincide with the marker amino sugar peaks. These main peaks were evaporated to dryness on a rotary evaporator, and re-evaporated several times from aqueous solution to remove HCl. Part was chromatographed on paper using solvents A, B and C ; part was acetylated by the method of Kuhn and Bister (143) and chromatographed in the same solvents. The chromatograms were stained with silver nitrate (150) and assayed for radioactivity as described under Materials and Methods. Solvents B and C were found to separate Galm and muramic acid satisfactorily and thus permitted resolution of the second peak (graphs I-III),* The results of the analysis are shown in table XII.

* pages 134-136.

Table XII. Distribution of radioactivity among cell fractions and their components.

Components of acid hydrolysate	Radioactivity (μc.)		
	cTCA fraction	hTCA fraction	Res fraction
Glucosamine *	0.095	0.42	0.20
Galactosamine *	0.016	0.038	0.005
Muramic acid	0.028	0.045	0.13
Material not adsorbed by column	0.005	0.070	0.011
Other radioactive peaks in 0.3N HCl eluate of column	0.019	0.16	0.025
6N HCl wash	0.045	0.60	0.24
Total radioactivity added to column	0.39	3.55	1.40
Total radioactivity recovered from column	0.21	1.33	0.61
	Other measurements on the acid hydrolysates		
Approx. weight (mg.)	39	89	94
Amino sugar (μmoles) calc. as Gm.	1.33	16.3	7.9
Phosphate (μmoles)	18	450	5

* Gm and Galm are not distinguished from mannosamine and talosamine respectively, by the analytical methods employed.

About 40–50 % of the radioactivity added to the column could thus be recovered, and of this 50–60 % was present in identifiable amino sugars. The cTCA and hTCA fractions contained mainly Gm, with smaller amounts of Galm and muramic acid, and the res. fracion contained approximately equal amounts of Gm and muramic acid, but practically no Galm. Very little radioactivity was present in the early fractions from the column where neutral substances might have moved, although these fractions did contain reducing material which moved in the region of D-galactose when chromatographed in solvents A and B.

A much larger proportion of the radioactivity was present in material which was more strongly adsorbed than the amino sugars, and eluted with 6N HCl. 80–90 % of this material was dialysable ; it was not adsorbed by Dowex I (chloride) at neutral pH and less than 25 % of the radioactivity was converted to a volatile form when it was treated with ninhydrin by the method of Stoffyn and Jeanloz (148). When chromatographed on paper in solvent A, the majority of the radioactivity remained at the origin, but if hydrolysed with 6N HCl at 100°C for 24 hrs. and rechromatographed in solvents A and B the majority of the radioactivity was found in the region of Gm, Galm and muramic acid markers. The material eluted by 6N HCl thus probably consists of small fragments of

the original polymers which are resistant to the conditions of hydrolysis originally used.

Samples of the amino sugar peaks from the column were treated with ninhydrin as above, chromatographed on paper using solvent D, stained with silver nitrate and assayed for radioactivity. Less than 10 % of the radioactivity originally present in the amino sugars was now present in the region of lyxose and arabinose markers, which indicates that randomization of the carbon chain has not occurred.

In another experiment in which N-acetyl-1-^{14}C-D-glucosamine was used in place of 1-^{14}C-D-glucosamine, and the fractions hydrolysed with H_2SO_4 in place of HCl (see Materials and Methods), a similar distribution of radioactivity was obtained.

6. Incorporation of labelled amino sugars in the presence of 6-azauracil.

An accumulation of amino sugar derivatives has been observed when E.coli is grown in the presence of 6-azauracil (160,161). This inhibitor was therefore used in an attempt to obtain accumulation of labelled intermediates in B.subtilis growing in the presence of radioactive amino sugars.

6-azauracil (5mM) reduced the growth rate of B.subtilis by more than 90 %. A culture of B.subtilis was grown to 7 mg. dry weight/100 ml. in broth + glutamate medium containing 6-azauracil (5mM). It was treated with N-acetyl-1-^{14}C-D-glucosamine (0.01 mM,0.6 µc), incubated for a further 60 min. at 37 C and harvested in the usual way. 25 % of the radioactivity added had been incorporated by the cells, and of this about 20 % was present in the cTCA fraction ; otherwise the distribution was normal. The cTCA fraction was extracted with ether to remove trichloracetic acid, evaporated to a small volume on a rotary evaporator and chromatographed on paper using solvent D ; it was also subjected to paper electrophoresis in 0.1M ammonium acetate buffer pH 5.6 for 3.5 hrs. at 500 volts. Amino sugar phosphate markers were added, and detected with aniline hydrogen phthalate spray. The majority of the radioactivity remained near the origin in both cases, but a small amount moved in the region of the amino sugar phosphates. The amount was too small to identify further however, and attempts to obtain a larger incorporation of radioactivity in the cTCA fraction were not successful.

Discussion.

Ring labelled D-glucosamine and N-acetyl-D-glucosamine are both taken up from the medium by growing cultures of B.subtilis. The reason why the amount of 1-^{14}C-D-glucosamine incorporated is proportional to the external concentration over a very wide range (table II) may be that :

a) the rate-limiting step in the uptake mechanism is saturated only at a high external concentration of glucosamine, or

b) that glucosamine facilitates its own incorporation at high concentrations, by induction or stimulation of the enzymes responsible of the uptake or by inhibition or repression of the enzymes which make amino sugars from other carbon sources (assuming that the 'salvage' pathway of incorporation from free amino sugar competes with the pathway of synthesis from hexose phosphates).

The fact that cells pregrown with glucosamine incorporate ^{14}Gm more rapidly than cells pregrown with glucose (table III) tends to suggest that the second alternative (induction or repression) may be operative. Evidence is presented in part II that glucosamine induces a kinase which phosphorylates glucosamine and that it represses Gm-6-P synthetase. The incorporation of N-acetyl-1-^{14}C-D-glucosamine (table IV) is

much less dependent on the external concentration of the amino sugar, but since in this experiment the incorporation was measured for a short period only, using cells in the middle of log. phase, any control mechanism such as repression of Gm-6-P synthetase would not have taken effect.

The behaviour of acetyl-labelled N-acetyl-D-glucosamine (^{14}AcGm) is consistent with the hypothesis that the acetyl group is liberated into the growth medium during incorporation of the glucosamine moiety, and the fractionation experiments suggest that the distribution of the radioactivity which is incorporated resembles that obtained with ^{14}Ac rather than that obtained with Ac^{14}Gm. Similar observations have been made with streptococci (162). It is unlikely that AcGm is simply deacetylated at the surface of the cell and the glucosamine moiety subsequently incorporated, since the label from Ac^{14}Gm is incorporated more rapidly than that from ^{14}Gm and glucose inhibits the incorporation of ^{14}Gm more than that of Ac^{14}Gm. It is not possible to decide from the available data whether actual deacetylation occurs, or whether the acetyl group is simply exchanged with free acetate or the acetyl of other acetylated compounds.

The competition experiments (tables V-IX) indicate that glucose greatly reduces the incorporation of ^{14}Gm but not that

of Ac^{14}Gm ; PrGm and FoGm greatly reduce the incorporation of Ac^{14}Gm but not that of ^{14}Gm and Gm does not compete with Ac^{14}Gm. These observations are consistent with the hypothesis that Gm is incorporated by a pathway different from that used by AcGm, but PrGm and FoGm may be incorporated by the same pathway as AcGm. The kinase which catalyses the phosphorylation of glucosamine is different from that which catalyses the phosphorylation of AcGm in several micro-organisms (48-51), but it appears to be identical with a non-specific hexokinase which also acts on glucose. If glucose and glucosamine are phosphorylated by the same kinase in B.subtilis while AcGm, PrGm and FoGm are all phosphorylated by another kinase, then the competition effects would be explained. Alternatively, there may be competition for permeases, since sugar permeases in bacteria are active systems capable of accumulating sugars against large concentration differences and are stereospecific and subject to inhibition by structurally similar compounds (163-165). Whereas the effect of PrGm on the incorporation of Ac^{14}Gm appears to be competitive (table VII), the effect of glucose on Ac^{14}Gm incorporation is not (table V), which suggests that glucose may not act at the same site as PrGm and FoGm. It may act later in the incorporation pathway, at a stage where the concentration of the metabolites derived from glucose and from AcGm is independent, or nearly independent, of their external

concentration. The fact that PrGm and FoGm reduce the rate of incorporation of label from ^{14}AcGm and the rate at which acidic material, probably free acetate, is released into the medium (table VIII) suggests that the competition between these compounds occurs before the removal or exchange of the acetyl group.

The distribution of radioactivity in B.subtilis grown on N-acetyl-1-^{14}C-D-glucosamine and 1-^{14}C-D-glucosamine (table X and page 49) suggests that the majority of the radioactivity enters compounds of high molecular weight. At least 50 % could be recovered in identifiable amino sugars ; a better recovery might have been obtained if more drastic conditions of hydrolysis had been used (see (35) and page 55). The presence of glucosamine and galactosamine in hydrolysates of the cTCA and hTCA fractions suggests that a polysaccharide such as teichuronic acid (39) or that described by Sharon (40) may be present. The reason why most of this material appeared in the cTCA rather than the hTCA fraction may be that prior removal of protein (39) was not carried out. The residue fractions, which contained approximately equal amounts of labelled glucosamine and muramic acid, presumably contain mucopeptide (166). A comparison with the distribution of amino sugars in the cell walls of other strains of B.subtilis (table I) indicates that B.subtilis NCTC 1379 may contain more

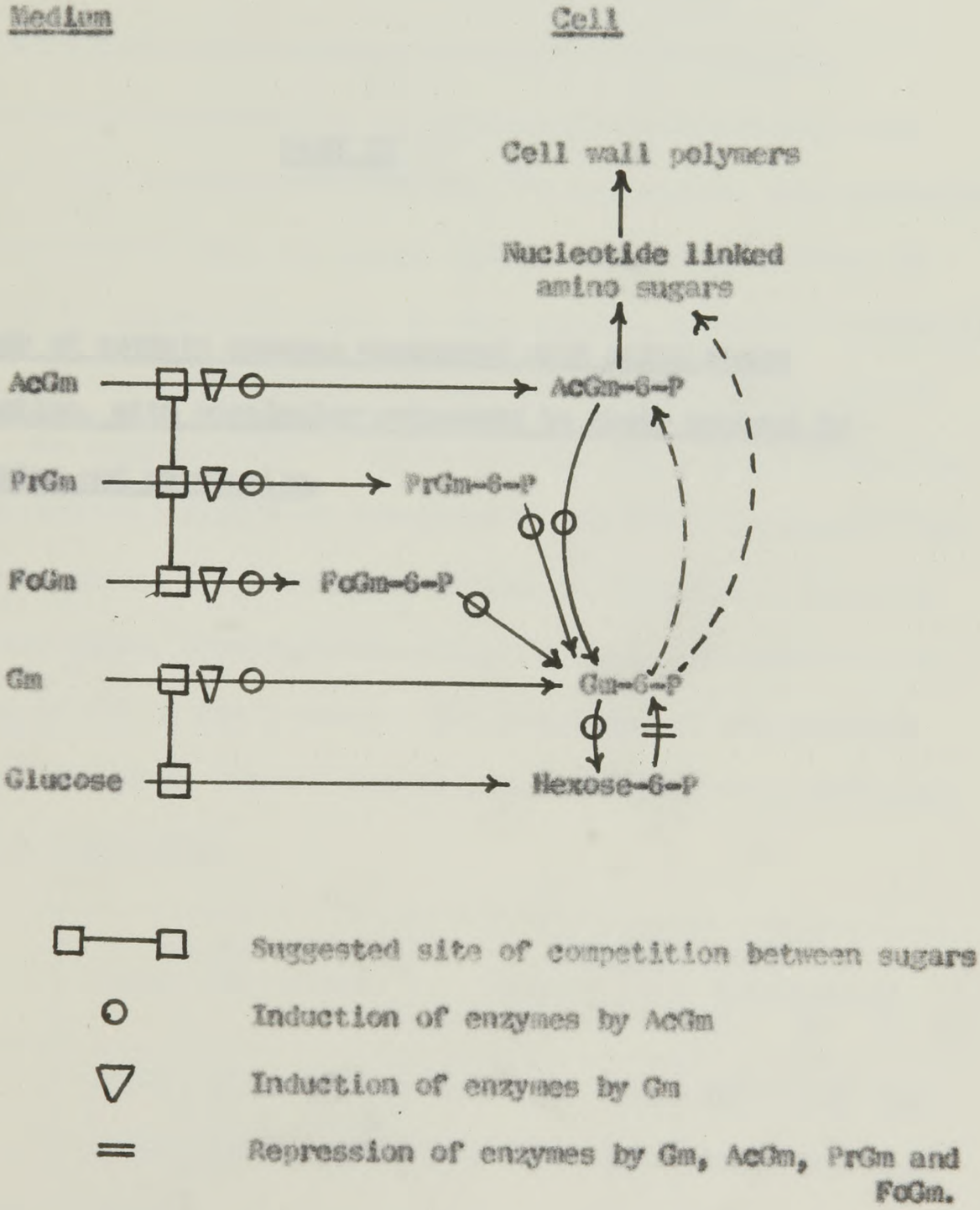
glucosamine and less galactosamine than usual (assuming that all the galactosamine in the cell is synthesised from the same early intermediates as glucosamine, so that both amino sugars have the same specific activity, when derived from ^{14}Gm in the growth medium).

Attempts to obtain an accumulation of amino sugar phosphates and nucleotide-linked amino sugars by reducing the period of incubation with the labelled amino sugar or by inhibiting the later stages by addition of azauracil were only partly successful (page 56).

Comparison of the distribution of radioactivity from ^{14}Gm and $Ac^{14}Gm$ in E.coli with that obtained in B.subtilis suggests that the fate of the incorporated amino sugars in the two organisms is probably different, and this is consistent with the fact that Gram positive cell walls differ considerably from Gram negative ones (19-26).

A tentative scheme for the metabolism of amino sugars in B.subtilis, including the enzyme control mechanisms discussed in part II is presented in Diagram II.

Diagram II. A tentative scheme for the early stages of amino sugar metabolism in B.subtilis NCTC 1379.



PART II

A study of certain enzymes concerned with amino sugar metabolism, with particular reference to their control by induction and repression.

1. Materials and Methods.

(a) Source and preparation of materials.

(see also part I page 24).

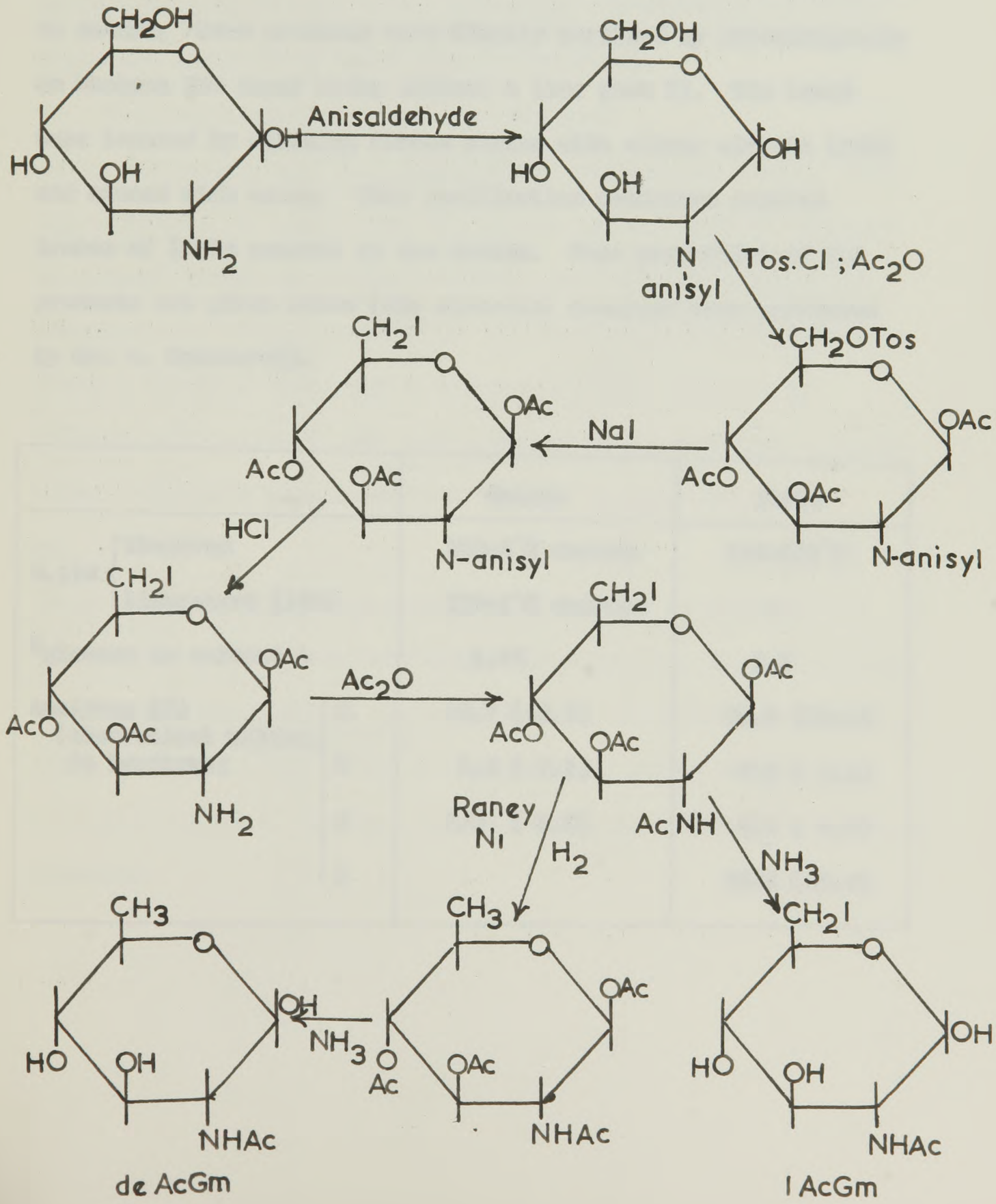
N-propionyl-Dglucosamine, N-n-butyryl-D-glucosamine, N-iso-butyryl-D-glucosamine and N-succinyl-D-glucosamine were prepared from D-glucosamine HCl and the appropriate acid anhydride by the method of Kuhn and Bister (143). They were purified by addition of acetone (10 vols) to the reaction mixture in methanol/water, and the precipitated material was recrystallized several times from acetone-water or methanol-water and , where necessary, passed through a column of Amberlite IR 120 resin (hydrogen form) to remove traces of glucosamine (which was detected by spotting on filter paper, spraying with ninhydrin (0.2 % in acetone) and heating at 100 C for 5 min.). The properties of the purified products are given below (the elemental analyses were performed by Dr. A. Bernhardt).

	m.pt.		Analyses (%)					
	found	literature	Found			Theoretical		
			C	H	N	C	H	N
PrGm	195 C	177-8 C(169) (for form)	45.9	7.2	5.9	46.2	6.8	6.0
n-BuGm	206 C	209 C (168)	48.2	7.7	5.7	48.4	7.2	5.6
iso-BuGm	228 C							

The succGm was difficult to crystallize and was still yellowish in colour after repeated precipitation with acetone and passage through Amberlite. All four products gave about the same colour yield as AcGm in the amino sugar assay of Reissig, Strominger and Leloir (167), and no appreciable increase in colour yield was obtained by acetylation (155).

N-formyl-D-glucosamine was prepared from D-glucosamine and ethyl formate by the method of zu Reckendorf and Bonner (144). It was passed through Amberlite resin to remove free glucosamine as described above and recrystallized from methanol. The melting point of the product was 154-8 C. It gave the same colour yield as AcGm in the amino sugar assay, and this was not increased by acetylation.

6-deoxy-N-acetyl-D-glucosamine(N-acetyl-D-chinovosamine) and 6-iodo-6-deoxy-N-acetyl-D-glucosamine were prepared by the method of Morel (170) as summarised below :



Tri-O-acetyl-N-acetyl-6-iodo-6-deoxy-D-glucosamine was converted to IAcGm by treatment with methanol-ammonia as described for the conversion of tri-O-acetyl-N-acetyl-6-deoxy-D-glucosamine to deAcGm. Both products were finally purified by chromatography on Whatman 3MM paper using solvent A (see part I). The bands were located by staining narrow strips with silver nitrate (150) and eluted with water. This purification procedure removed traces of IAcGm present in the deAcGm. Some properties of the products are given below (the elemental analyses were performed by Dr. A. Bernhardt).

		deAcGm	IAcGm
m.pts.	observed	202-3°C decomp.	169-171°C
	literature (169)	210-1°C decomp.	-
$R_{glucose}$ in solvent A		1.75	2.1
Analyses (%) (theoretical values in brackets)	C	46.7 (46.8)	28.9 (29.0)
	H	7.3 (7.1)	4.5 (4.3)
	N	7.0 (6.8)	4.2 (4.4)
	I		38.2 (38.4)

Both amino sugars gave about the same colour yield as AcGn in the amino sugar assay (167) although the colour developed at different rates for the three compounds. No increase in colour yield was obtained by prior acetylation (155).

D-glucosamine-6-phosphate was obtained :

a) as the barium salt from Nutritional Biochemicals Corpn. This was dissolved in 0.01 N HCl, the barium precipitated by addition of a small excess of sodium sulphate, and in certain cases the material was further purified by chromatography on a column of Dowex I (acetate form) (191), using a gradient obtained by feeding 0.05 N-acetic acid into a mixing vessel containing 200 ml water. Coloured impurities were removed in this way.

or

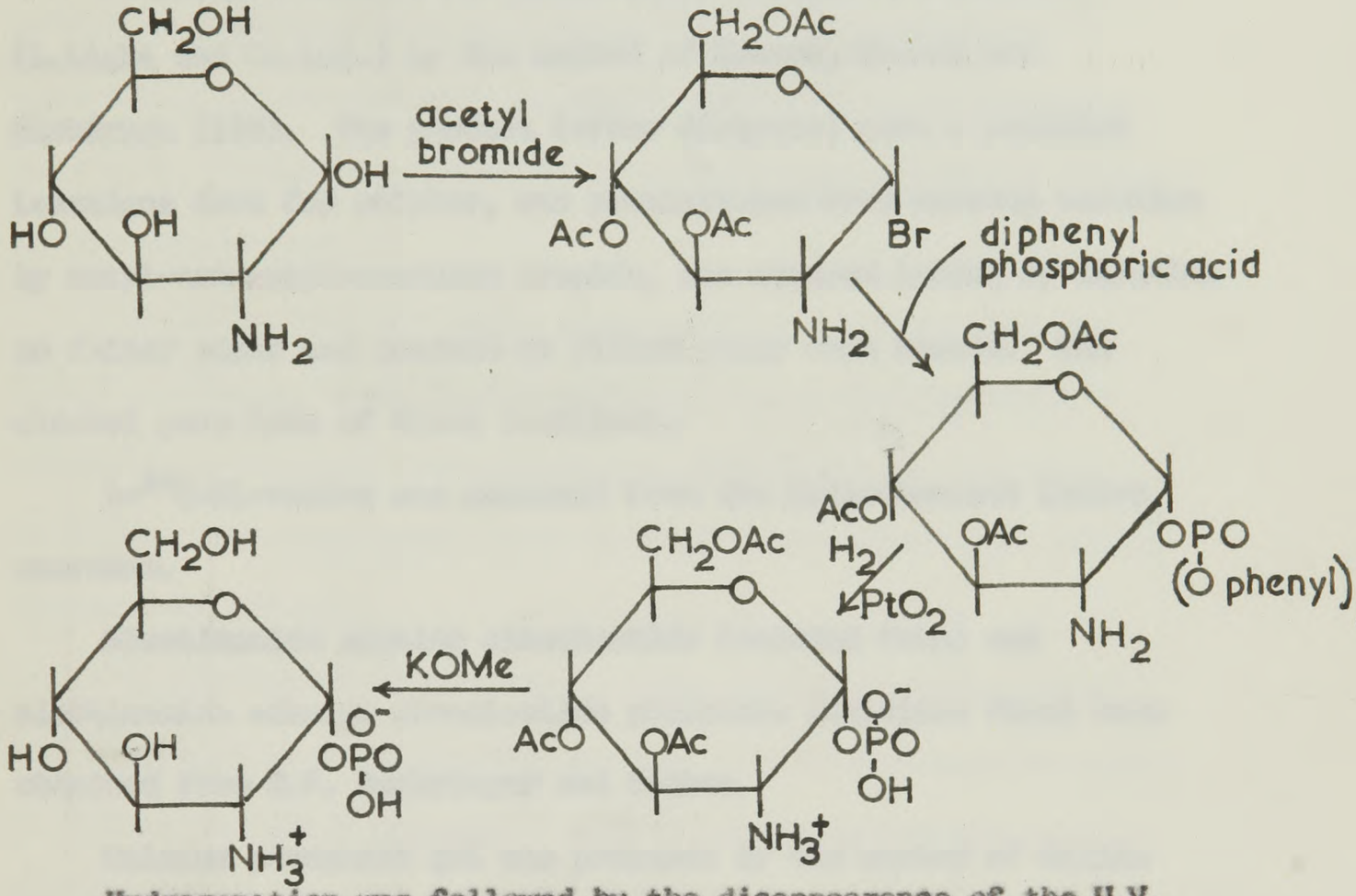
b) by synthesis from D-glucosamine HCl and ATP in the presence of purified yeast hexokinase (Sigma Chemical Co., Type III)(171). The product was purified by chromatography on Whatman 3 MM paper, using isobutyric acid / 0.5 N ammonia, 5:3, and by passage through a column of Norite A to remove the remaining U.V. absorbing material.

N-acetyl-D-glucosamine-6-phosphate was prepared by acetylation of D-glucosamine-6-phosphate by the method of Kuhn and Bister (143) and purified by adsorption to Dowex I (chloride form), from which the product was eluted with 0.15 N HCl.

N-propionyl-D-glucosamine-6-phosphate and N-n-butyryl-D-glucosamine-6-phosphate were prepared from D-glucosamine-6-phosphate and the appropriate acid anhydride in the same way.

The purity of these amino sugar phosphates was checked by amino sugar analysis (155, 167). In the case of the N-acetylated amino sugar phosphates the colour yield in the presence of acetic anhydride was equal to that obtained in its absence. The phosphate content of the Gm-6-P and AcGm-6-P preparations was measured before and after ashing (156); before ashing it was negligible and after ashing it was equivalent to the amino sugar content.

α-D-glucosamine-1-phosphate was prepared from D-glucosamine by the method of Maley, Maley and Lardy (172) as summarised below :



Hydrogenation was followed by the disappearance of the U.V. absorbing peak at 265 mμ (which is presumably due to the presence of phenyl groups) and the identity of the product checked

(a) by appearance of free phosphate and material giving a positive amino sugar reaction (155) in the theoretical amounts after hydrolysis with 1 N HCl at 100°C for 40 min. ;

(b)by the optical rotation :

(found) = +90°(c.1.98 H_2O).

(literature =+100°(c.1.98 H_2O). (172))

(c) by acetylation in the presence of AcCoA and E.coli extract (see page 119).

Polyvinyl sulphate was prepared from polyvinyl alcohol (L.Light and Co.Ltd.) by the method of Nomura, Hosada and Nishimura (173). The product (after dialysis) gave a positive Lassaigne test for sulphur, was precipitated from aqueous solution by cetyl-trimethyl-ammonium bromide, was stained (pink) by thionine on filter paper and charred on filter paper when heated. The alcohol gave none of these reactions.

1-^{14}C-DL-valine was obtained from the Radiochemical Centre, Amersham.

Nicotinamide adenine dinucleotide (reduced form) and nicotinamide adenine dinucleotide phosphate (oxidised form) were obtained from C.F. Boehringer and Soehne.

Calcium phosphate gel was prepared by the method of Keilin and Hartree (185) and C_γ alumina gel was prepared by the method of Dawson and Magee (186).

Polidase was a gift from Dr. P. Johnson. Glucose phosphate isomerase ('crude') was obtained from Sigma Chemical Co.. G-6-P dehydrogenase, ATP:pyruvate phosphotransferase E.C.2.7.1.40. and L-lactate-NAD oxidoreductase E.C.1.1.1.27. were obtained from C.F. Boehringer and Soehne.

(b) Growth of bacteria and preparation of extracts.

B.subtilis was grown in flask culture on broth glutamate medium as described in part I (page 27). The cultures were harvested, washed with 100 ml. buffer (see below), resuspended in 2-5 ml. buffer and disrupted by sonication, the French Press or the Hughes Press.

For sonication the cells were washed and resuspended in 0.01 M cysteine / 0.01 M EDTA buffer pH 7.5 ('cysteine-EDTA buffer'). A Mullard 500W ultrasonic generator at a frequency of 19 kc / sec. was used ; more than 90 % disruption was usually obtained in 1-2 min..

For disruption by the French Press the cells were washed and suspended in cysteine-EDTA buffer, in 0.07 M sodium phosphate ph 7.0 or in 0.01 M Tris-HCl / 0.01 M 2-mercapto-ethanol pH 7.6 (Tris-mercapto-ethanol buffer). The pressure cell (American Instrument Co. Inc.) was precooled in iced water, the suspension inserted and the majority of the air removed by adjusting the piston with the needle valve open and the pressure cell inverted : this is essential to minimise frothing. The needle valve was closed, a pressure of 9,000 p.s.i. applied with a Wabash hydraulic press, and the valve and pressure adjusted

so that the cells were forced through between 6,000 and 9,000 p.s.i..

For disruption in the Hughes Press the cells were washed and resuspended in Tris-mercapto-ethanol buffer, and the block was cooled to -25°C before inserting the cell suspension.

After disintegration, the extracts were centrifuged at 105,000 x g in a refrigerated Spinco ultracentrifuge, and except where otherwise stated, the supernatant only was used for the enzyme assays.

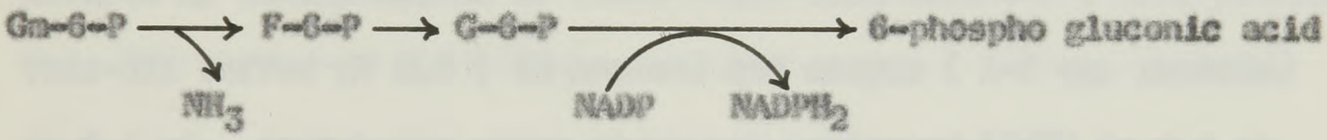
Gm-6-P synthetase was separated from Gm-6-P deaminase where necessary by the method of Clarke and Pasternak (56),(Gm-6-P synthetase is selectively precipitated with protamine sulphate and then re-extracted with pyrophosphate buffer).

(c) Assay of enzymes.

(i) Gm-6-P synthetase (R4 on diagram I) was assayed by measuring the formation of amino sugar from D-glucose-6-phosphate (2μmoles), L-glutamine (1μmole), sodium phosphate buffer pH 7.6 (50 μmoles) and enzyme (1-2 mg. protein) in 0.45 ml. (56). After incubation, protein was precipitated by addition of 50 % trichloracetic acid (0.05 ml.) and amino sugar in the supernatant was assayed (155).

(ii) Gm-6-P deaminase (R5 on diagram I) was normally assayed by measuring the disappearance of amino sugar from D-glucosamine-6-phosphate (0.25 μmoles), Tris-HCl buffer pH 7.5 (50μmoles) and enzyme (1-2 mg. protein) in 0.45 ml. (56). Protein was precipitated and amino sugar assayed as described above.

In some cases Gm-6-P deaminase was measured spectrophotometrically by coupling to G-6-P dehydrogenase (174) :



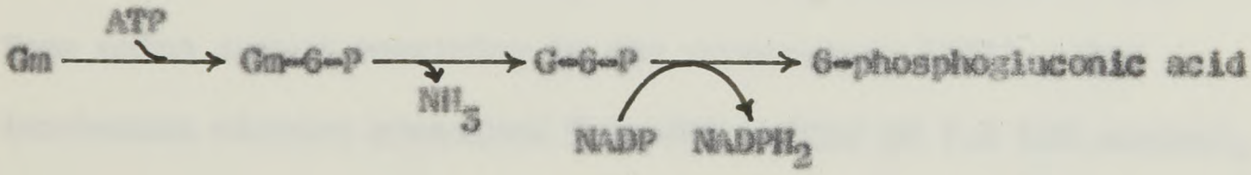
The incubation mixture was adapted from that used by Kornberg (175) and contained, in 1 ml. : Tris-HCl buffer pH 7.6 (37μmoles), $MgCl_2$ (10 μmoles), NADP (0.14 μmoles), G-6-P dehydrogenase (5 μg), Gm-6-P (0.5 μmoles) and enzyme. $NADPH_2$ formation was measured by the change in optical density at 340 mμ, read at 0.5-1.0 min. intervals on a Unicam SP 500 U.V. spectrophotometer.
Crude extracts reduced NADP to a small extent in the absence of added Gm-6-P (possibly due to traces of G-6-P or other oxidisable compound in the extract). The incubation mixture was therefore made up without Gm-6-P, mixed with the enzyme, and incubated until the optical density was constant. Gm-6-P was then added

and the rate measured. In the case of extracts with very low activities, there was an appreciable lag before the rate became constant : this presumably represents the time required for the level of intermediates to build up. When purified enzyme was used, it was necessary to add glucose phosphate isomerase (0.5 mg.) to the incubation mixture.

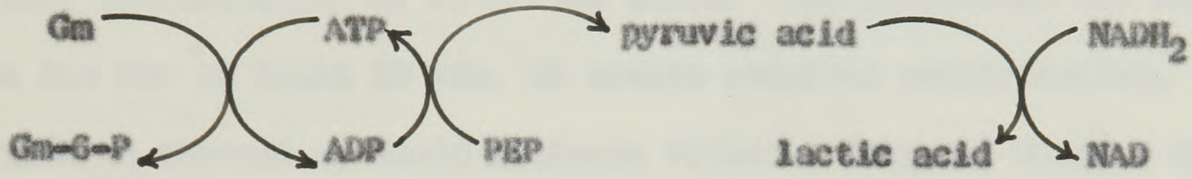
(iii) AcGm-6-P deacetylase (R11 on diagram I) was assayed by disappearance of amino sugar from AcGm-6-P (0.25 µmoles), Tris-HCl buffer pH 8.5 (50 µmoles) and enzyme (1-2 mg. protein) in 0.5 ml. Acetylated amino sugar was estimated (167) in 0.2 ml. aliquots after removal of protein as described above.

(iv) The following methods were used in an attempt to measure amino sugar kinases (R1,R2 on diagram I) :

(iv a) Oxidation of NADP by coupling to G-6-P dehydrogenase :



(ivb) Oxidation of NAD by coupling to ATP:pyruvate phosphotransferase E.C.2.7.1.40. and L-lactate : NAD oxidoreductase E.C.1.1.1.27 (176) :



The incubation mixture contained : Tris pH 7.6 (50 umoles), $MgCl_2$ (10 µmoles), K_2SO_4 (10µmoles), ATP (2µmoles), PEP (1 µmole), $NADH_2$ (0.1 µmole), ATP:pyruvate phosphotransferase (10 µg), L-lactate:NAD oxidoreductase (10 µg), Gm (1 µmole) and enzyme in 1ml. NAD formation was measured by the change in optical density at 340 mµ.

(iv c) Precipitation of amino sugar phosphates with zinc sulphate and barium hydroxide, followed by estimation of the free amino sugars remaining in the supernatant (177). The incubation mixture contained Tris-HCl buffer pH 7.5 (50 µmoles), $MgCl_2$ (5 µmoles), ATP (5 µmoles), amino sugar (0.3-1.2 µmoles) and enzyme in 0.5 ml. After incubation, the solution was treated with $ZnSO_4$ (50 µmoles) followed by $Ba(OH)_2$ (50 µmoles).

(iv d) Precipitation of radioactive amino sugar phosphates with barium acetate in ethanol followed by estimation of the radioactivity in the precipitate (modified from (178)).

The incubation mixture contained : Tris-HCl buffer pH7.9 (50 µmoles), $MgCl_2$ (5 µmoles), ATP (5 µmoles), 1-^{14}C-D-glucosamine or N-acetyl-1-^{14}C-D-glucosamine (0.05 µmoles, 0.25 µc.) and enzyme in 0.5 ml. After incubation, barium acetate (250 µmoles) and ethanol to a final concentration of 80 % were added. The suspension was kept on ice for at least 10 min. to ensure complete precipitation, filtered through an Oxoid membrane filter (internal diam. 2 cm.) and washed (by filtration) with 5 successive 5 ml. portions of 80 % ethanol and 5 ml. absolute ethanol. After drying, the radioactivity on the filter was assayed with a G.E.C. end window Geiger-Muller tube connected to a Panax scaler. In some cases the samples were treated with 0.2 µmoles of Gm-6-P or AcGm-6-P carrier, before the addition of barium acetate, but this was not found to increase the yield of precipitable material. In the case of ^{14}Gm, the zero time controls frequently gave finite amounts of precipitable radioactivity : this is probably due to adsorption of ^{14}Gm to the precipitated protein and adenosine phosphates. Unlabelled glucosamine (about 5mM) was therefore added to the 80 % ethanol washes in these cases.

(v) Gm-1-P N-acetylase (R8 in diagram I) was assayed with the following incubation mixture (adapted from 71) :

Tris-HCl buffer pH 8.5 (50 μmoles), $MgCl_2$,(0.5 μmoles), 2-mercaptoethanol (2 μmoles), Gm-1-P (0.2 μmoles), acetyl CoA (0.2 μmoles) and enzyme, in 0.6 ml. Amino sugar in 0.2 ml. samples was assayed (155) after hydrolysing the 1-phosphate with 1 N HCl at 100 C for 6 min. and adding trichloracetic acid to precipitate protein.

(vi) In an attempt to measure the interconversion of Gm-6-P and Gm-1-P the following incubation mixture was used (adapted from 71) : Tris-HCl buffer pH 7.5 (50 μmoles), $MgCl_2$ (0.5 μmoles), 2-mercaptoethanol (2 μmoles), Gm-1-P (0.2 μmoles) and enzyme in 0.5 ml. Amino sugar in 0.2 ml. samples was assayed (155) after removal of protein with trichloracetic acid. Gm-1-P gives no colour in this assay.

(vii) Valyl-sRNA synthetase was measured by the method of Loftfield and Eigner (187) using the following incubation mixture : Salt-free NH_2OH pH 7.4 (1000 μmoles), ATP (20 μmoles), $MgCl_2$ (20 μmoles), 1-^{14}C-DL-valine (0.21 μmoles, 1μc.) and enzyme (3 mg.) in 1 ml. After incubation at 37°C for 0 min., 15 min. and 60 min., 0.2 ml. samples were removed and applied to a strip (1.8 x 22 cm.) of Amberlite anion exchange paper, grade SA-2, (sodium form), and eluted with 0.05 M sodium phosphate buffer pH 7.2 (ascending). Radioactivity remaining at the

origin (valine hydroxamate, formed by the reaction of hydroxylamine with the intermediate valyl AMP) and radioactivity at the solvent front (free valine) were measured.

(viii) In attempts to measure Gm-6-P deaminase formation in a cell-free system, the following incubation mixture adapted from Novelli (184) was used : Tris-HCl buffer pH 7.5 (100 µmoles), magnesium acetate (4 µmoles), manganese chloride (2 µmoles), ATP (10 µmoles), PEP (20 µmoles), ATP:pyruvate phosphotransferase (20 µg.), CTP, GTP, UTP, CDP, GDP, UDP (0.035 µg. each), 2-mercaptoethanol (10 µmoles), Oxoid casein hydrolysate grade L 41 (20 µg.), L-tryptophan (2 µg.), in 1 ml.

(ix) The amount of protein used in the enzyme assays was measured either (after precipitation with 5 % trichloracetic acid) by the Biuret method (154) or (for dilute solutions) by the measurement of optical density at 280 mµ. (with a correction for the absorption due to nucleic acids, estimated from the optical density at 260 mµ. (189)).

Results.

1. The effect of amino sugars on Gm-6-P deaminase, AcGm-6-P deacetylase and on Gm-6-P synthetase in B.subtilis.

N-acetyl-D-glucosamine induces enzymes responsible for the deamination of Gm-6-P and the deacetylation of AcGm-6-P and represses the enzyme responsible for the synthesis of Gm-6-P in B.subtilis (see page 21). These observations pose the following questions :

(i) What is the specificity of the inducer and repressor ?

(ii) Can the control effects be separated by using different amino sugars ?

(iii) Are any further stages of amino sugar metabolism controlled in a similar way ?

(iv) Does induction or repression have any effect on the metabolism of amino sugars in vivo ?

Question (iv) has already been partly answered by the results given in Table III

Questions (i) and (ii) were investigated by measuring the specific activity of the enzymes in extracts of B.subtilis which had been grow in media containing various amino sugars. The results are given in Table XIII :

Table XIII. Induction of Gm-6-P deaminase and AcGm-6-P deacetylase and repression of Gm-6-P synthetase by amino sugars in B.subtilis.

Amino sugars were added to the growth medium together with a small inoculum of B.subtilis and the cultures were grown to 20-50 mg. dry wt./100 ml., harvested, washed, disrupted by sonication or the French Press, and the 105,000 x g supernatant used for enzyme assays as described on pages 74-6; Gm-6-P deaminase was measured by the disappearance method. The results are expressed as a ratio of the specific activity in cells grown with amino sugar to the specific activity in cells grown with broth + glutamate alone (± standard error where applicable ; no. of determinations in brackets).

Amino sugar in growth medium	Gm-6-P deaminase	AcGm-6-P deacetylase	Gm-6-P synthetase
none*	1.0	1.0	1.0
AcGm (5-10 mM)	26.9 ± 7.0 (19)	22.0 ± 6.8 (3)	0.097± 0.046 (9)
Gm (")	2.09 ± 0.76 (17)	1.31 ± 0.55(3)	0.112± 0.052 (12)
PrGm (")	2.02 ± 1.11 (16)	1.34 ± 0.71(3)	0.23 ± 0.11 (13)
FoGm (")	1.50 ± 0.42 (9)		0.38 ± 0.14 (7)
n-BuGm(")	1.77 ± 0.36 (5)		1.01 ± 0.15 (5)
iso-BuGm(")	1.30 ± 0.33 (6)		1.00 ± 0.32 (7)
SuccGm(")	1.03 ± 0.33 (3)		1.36 ± 0.47 (3)
GalM (")	0.90 (1)		0.95 (1)
AcGalM(")	0,72 ± 0.20 (2)		0.87 ± 0.14 (2)
AcMn (")	1.55 ± 0.46 (3)		1.06 ± 0.36 (3)
AcGm (0.5 mM)	13.0 (1)		
Gm-6-P(")	0.95 (1)		1.27 (1)
AcGm-6-P(")	1.52 (1)		1.13 (1)
deAcGm(0.9 mM)	0.90 ± 0.15 (2)		1.13 ± 0.13 (2)
IAcGm (0.8 mM)	1.05 ± 0.07 (2)		1.02 ± 0.07 (2)
*Av. sp. act. (mμmoles/mg.protein/min.)	7.0	0.61	10.4

Amino sugar in the medium was measured before and after growth to check that a significant amount remained at the end of the growth period. When initially present at a concentration of 5 mM, about 50 % of the AcGm, about 20 % of the Gm, PrGm and FoGm, and negligible quantities of the other amino sugars were used. Variations in initial concentration over the range 5-10 mM did not significantly affect the extent of induction or repression produced by any of the amino sugars tested.

The only amino sugar which was found to give more than two-fold induction of Gm-6-P deaminase or AcGm-6-P deacetylase was AcGm. More than 50 % repression of Gm-6-P synthetase, on the other hand, occurred in the presence of AcGm, PrGm and FoGm. Gm was almost as efficient a repressor as AcGm.

Graphs IV* and V** show the variation in specific activity of Gm-6-P deaminase and Gm-6-P synthetase in extracts of B. subtilis grown in the presence of a range of concentrations of AcGm, Gm, PrGm and FoGm. Gm-6-P synthetase was measured in unfractionated supernatant except in the case of AcGm-grown cells, which were fractionated by the method of Clarke and Pasternak (56). The results indicate that in each case there is a range of amino sugar concentrations over which the magnitude of the control effects varies with the concentration, but that at high concentrations

* page 137 ** page 138

of amino sugar the enzyme specific activities level out to a constant value. At the lowest concentrations of amino sugar, all that was added was used up during growth, and in each case, the lowest concentration at which repression of Gm-6-P synthetase was observed coincided with the lowest concentration at which amino sugar was still detectable in the growth medium after harvesting.

The reduction of Gm-6-P synthetase activity by Gm, PrGm and FoGm is unlikely to be due to inhibition rather repression of the enzyme for the following reasons :

(i) Activity is as low in fractionated extracts, in which all the material soluble in 2 % protamine sulphate has been removed, as it is in whole supernatant.

(ii) When the supernatant from cells grown in the absence of amino sugar is mixed with supernatant from cells grown in its presence, the activities are additive.

Likewise the increase in Gm-6-P deaminase activity in cells grown in the presence of AcGm is not due to traces of N-acetyl-D-glucosamine-6-phosphate or other heat-stable activators of deaminase since the addition of boiled supernatant from cells grown on AcGm to supernatant from cells grown without amino sugars produced less than 25 % stimulation of activity. Addition of

0.05 µmoles of AcGm-6-P to the reaction mixture did not increase the activity of Gm-6-P deaminase in non-induced extracts (but see page 96). The induced enzyme could be purified considerably (see pages 94-6) without appreciable loss of activity.

Extracts of cells induced with AcGm were found to deacylate PrGm-6-P at 71 % and n-BuGm-6-P at 43 % of the rate of AcGm-6-P.

2. The effect of glucose and other carbon sources on the control effects exerted by amino sugars in B.subtilis.

Addition of glucose to growth media containing AcGm resulted in a considerable decrease in the induction of Gm-6-P deaminase (56). Glucose also reduced the rate of incorporation of radioactivity from Ac^{14}Gm (see page 38), which means that it may affect induction by reducing the rate at which AcGm is taken up or converted to the inducer inside the cell. A search was therefore made for carbon sources which would repress Gm-6-P deaminase without affecting the rate of incorporation of radioactivity form Ac^{14}Gm. At the same time the effect of glucose and other carbon sources on induction of AcGm-6-P deacetylase and repression of Gm-6-P synthetase by AcGm was examined. The results of the enzyme assays are given in table XIV (and the results of the radioactive work are given in table VI).

Table XIV Effect of glucose and other carbon sources on induction of Gm-6-P deaminase and AcGm-6-P deacetylase and on repression of Gm-6-P synthetase.

Both amino sugars and carbon sources were present at 5-10 mM ; for other details see Table XIII.

Addition to growth medium	Gm-6-P deaminase	AcGm-6-P deacetylase	Gm-6-P synthetase
none	1.0	1.0	1.0
glucose	0.83 ± 0.29(5)	0.89 ± 0.15 (2)	0.96 ± 0.08 (3)
AcGm	26.9 ± 7.0 (19)	22.0 ± 6.8 (3)	0.097 ± 0.046(9)
AcGm + D-glucose	2.40 ± 0.72(12)	1.64 ± 0.03 (2)	0.48 ± 0.21 (7)
AcGm + D-fructose	2.15 ± 0.89(5)	2.17 (1)	0.55 ± 0.25 (5)
AcGm + D-galactose	25.7 ± 9.1 (2)		0.07 ± 0.05 (2)
AcGm + D-mannose	15.5 (1)		0.14 ± 0.10 (2)
AcGm + sucrose	7.0 (1)		0.32 ± 0.03 (2)
AcGm + lactose	27.2 (1)		
AcGm + D-arabinose	21.2 (1)		0.12 (1)
AcGm + D-ribose	16.4 (1)		0.06 (1)
AcGm + sodium succinate	27 (1)		
AcGm + sodium DL-lactate	35 (1)		0.08 (1)
AcGm + sodium acetate	24 (1)	16.2 (1)	
AcGm + glycerol	18.4 (1)		0.10 (1)

It can be seen that glucose reduced the induction of AcGm-6-P deacetylase and the repression of Gm-6-P synthetase by AcGm. Of the other compounds tested, only fructose and sucrose had an appreciable effect, and they both reduced the incorporation of Ac^{14}Gm to about the same extent as glucose.

Graph VI* shows the variation in specific activity of Gm-6-P deaminase and Gm-6-P synthetase in cells grown in the presence of 100 mM glucose and various concentrations of AcGm, and Graph VII** shows the variation in specific activity of these enzymes in cells grown in the presence of 100 mM fructose and various concentrations of AcGm. It can be seen that, as the concentration of AcGm was increased, the specific activity of Gm-6-P deaminase and of Gm-6-P synthetase in the glucose-grown cells, and that of Gm-6-P synthetase in the fructose-grown cells approached a constant limiting value ; which suggests that these effects of glucose and fructose cannot be overcome simply by increasing the concentration of AcGm in the growth medium.

The induction of Gm-6-P deaminase by AcGm in the presence of PrGm was also tested : PrGm (25mM) did not reduce the induction produced by AcGm (2.5 mM).

Table XV shows the effect of glucose and other carbon sources on the repression of Gm-6-P synthetase by Gm, PrGm and FoGm :

* page 139 ** page 140

Table XV. Effect of carbon sources on the repression of Gm-6-P synthetase by Gm, PrGm and FoGm.

For details of growth, disruption of the cells and enzyme assays, see Table XIII ; both amino sugars and carbon sources were present at 5-10 mM. The results are expressed as in Table XIII.

Addition to growth medium	Gm-6-P synthetase
none	1.0
Gm	0.112 ± 0.052 (12)
Gm + D-glucose	1.07 ± 0.26 (5)
Gm + D-fructose	0.73 ± 0.11 (2)
Gm + D-galactose	0.22 ± 0.14 (2)
PrGm	0.23 ± 0.11 (13)
PrGm + D-glucose	1.04 (1)
FoGm	0.38 ± 0.14 (7)
FoGm + D-glucose	1.02 (1)

It can be seen that the repression of Gm-6-P synthetase by Gm, PrGm and FoGm was abolished by glucose ; fructose also reduced the repression by Gm while galactose had little effect.

In order to show that the effect of glucose and fructose on Gm-6-P deaminase induction was not due to the production of an inhibitor, an extract from cells grown on AcGm alone was mixed with an extract from cells grown on AcGm+glucose : the Gm-6-P deaminase activities were additive.

3. The effect of penicillin and 6-azauracil on Gm-6-P deaminase and Gm-6-P synthetase.

It has been shown that soluble amino sugar derivatives accumulate in S.aureus grown in the presence of penicillin (179) and in E.coli grown in the presence of 6-azauracil (160,161). These compounds were therefore added to growing cultures of B.subtilis in the hope that they would produce an accumulation of amino sugar intermediates in the absence of added amino sugars and thus cause induction of Gm-6-P deaminase and repression of Gm-6-P synthetase indirectly. This would permit the investigation of the effects of glucose under conditions where no competition for entry of inducer or repressor could occur.

Some initial experiments with benzyl penicillin indicated that B.subtilis NCTC 1379 is highly resistant, and concentrations up to 500 ug.(83.5 i.u.) per ml. could be tolerated. Since a test for the presence of penicillinase (180) in extracts of the organism gave a positive result, it is possible that the high degree of

resistance is due to destruction of the penicillin. 2,6-dimethoxy-benzamido penicillin (Celbenin, or Methicillin) was therefore tested, since strains of S.aureus which are resistant to benzyl penicillin are sensitive to this derivative (181). It could be tolerated at concentrations up to 10 µg. per ml.. Neither of these compounds was found to affect the specific activity of Gm-6-P deaminase or Gm-6-P synthetase in the cells at any concentration up to the maximum tolerated by the bacteria. It therefore appears that these compounds do not cause the inducer or repressor to accumulate in significant amounts.

Likewise 6-azauracil at concentrations which severly depress the growth rate had no measurable effect on the enzymes, which is consistent with the observation that it did not cause a large accumulation of labelled amino sugar derivatives in the cTCA fraction (page 57).

4. The effect of incubation in stationary phase on the specific activity of Gm-6-P deaminase and the release of amino sugar-containing material into the growth medium.

The specific activity of many enzymes in bacterial spores differs from those found in vegetative cells of the same species (182,183). It was therefore of interest to examine the variation in specific activity of the enzymes concerned in amino sugar

metabolism in B.subtilis in stationary phase, where spore formation might be expected to occur.

In a preliminary experiment, the specific activity of Gm-6-P deaminase was found to rise after 14 hrs. growth on broth + glutamate + glucose (100mM). It was noted, however, that the pH of the medium rose sharply in the cultures grown without glucose after about 10 hrs. growth, while in the cultures grown with glucose it fell. In order to rule out the possibility that the observed effects were due to variations in pH of the medium, the medium was neutralised at frequent intervals in subsequent experiments.

Graph VIII* shows the variation in specific activity of Gm-6-P deaminase in the cells and in the optical density at 585mμ given by samples of the growth medium in the acetylated amino sugar assay (167) when the cells are grown on broth + glutamate alone.

Graph IX** shows the same measurements made on cells grown on broth + glutamate + 100mM glucose. In the absence of glucose, the specific activity of Gm-6-P deaminase rose steadily from 3 mμmoles/mg./min. after 8 hrs. incubation to 11mμmoles/mg./min. after 36 hrs. The O.D. in the acetylated amino sugar assay (167) given by 0.2 ml. aliquots of the growth medium at harvesting did not increase with time, and the spectrum did not show the characteristic

* page 141 ** page 142

double peak, with maxima at 550 and 585 mμ, which is obtained with amino sugars. In the presence of glucose the specific activity of Gm-6-P deaminase increased from 2 mμmoles/mg./min. after 8 hrs. incubation to 5 mμmoles/mg./min. after 36 hrs. The O.D. in the acetylated amino sugar assay given by 0.2 ml. aliquots of the growth medium at harvesting increased with time between 13 and 28 hrs. growth, and the spectrum of this material showed the characteristic double peak given by the amino sugars. In two other experiments the results were similar except that the specific activity of Gm-6-P deaminase in cells grown with glucose showed no increase at all during 36 hrs. incubation. In one of these the specific activity of Gm-6-P deaminase in cells grown on broth + glutamate increased to nearly ten times the basal level and the specific activity of AcGm-6-P deacetylase also showed a small increase (to about 2.5 times the basal level) ; this was not observed in the presence of glucose.

5. Partial purification of Gm-6-P deaminase and attempts to obtain cell-free synthesis of this enzyme by extracts of B. subtilis.

Since B. subtilis does not appear to metabolise Gm-6-P or AcGm-6-P when these are added to the growth medium (see page 83) it is possible that these amino sugars do not produce induction or repression (Table XIII) because they cannot penetrate the cell membrane. In order to study the action of these compounds under

conditions where the problem of penetration is eliminated, an attempt was made to synthesize Gm-6-P deaminase in cell free extracts of B.subtilis, using the technique developed by Novelli et.al.(184) for the synthesis of β-galactosidase by extracts of E.coli.

Some preliminary work was necessary :

(a) to develop a sensitive and reproducible assay for Gm-6-P deaminase which would not be affected by the presence of amino sugars in the incubation medium (the assay based on the disappearance of material giving the amino sugar colour reaction obviously could not be used).

(b) to try to develop a procedure for the partial purification of Gm-6-P deaminase with good recovery of the enzyme.

(c) to try to show preferential incorporation of radioactivity into the fractions containing Gm-6-P deaminase from whole cells which had been incubated with 1-^{14}C-DL-valine and inducer (AcGm).

(d) to demonstrate amino acid activation, which is a pre-requisite for protein synthesis, in extracts of B.subtilis.

5. (a) Assay of Gm-6-P deaminase.

The method chosen was the spectophotometric method based on the reduction of NADP (see page 75). Unlike the disappearance assay this could be performed in the presence of AcGm and AcGm-6-P (on incubation with extracts of B.subtilis, AcGm-6-P did not give rise to G-6-P at a rate sufficient to affect the assay). It was also

possible to obtain a measure of the initial rate more easily and reproducibility was better. Even at room temperature,, using extracts from non-induced cells, the rate was easily measurable, and extracts from induced (AcGm grown) cells gave about thirty times this rate, which is in reasonable agreement with the result of the disappearance assay (Table XIII).

5 (b) Purification of Gm-6-P deaminase.

Cells grown (with forced aeration) on broth + glutamate medium (16 litres) containing 1.5 mM AcGm were harvested, washed, disrupted in the French Press and the 105,000 x g supernatant obtained as described on page 73 . Part was fractionated with ammonium sulphate (a saturated solution containing 0.001 M EDTA at 0°C) ; the precipitates obtained over a range of concentrations from 0-90 % saturation were redissolved in cysteine-EDTA buffer and Gm-6-P deaminase was assayed by the spectrophotometric method. No large increase in the specific activity was obtained in any fraction.

A further portion of the supernatant was treated with an equal volume of protamine sulphate (2 %, pH 6.7) (56). This was found to give 5-7 fold purification of Gm-6-P deaminase, with more than 90 % recovery of the enzyme.

Further samples of the supernatant were treated with calcium phosphate gel and with C_γ alumina gel. Both adsorbed the enzyme, which could be recovered in an active form by extraction with 0.05 M K_2SO_4 or with 0.01 M Gm-6-P. When the supernatant after protamine fractionation was treated with either of these gels and eluted with 0.05 M K_2SO_4, the specific activity of the Gm-6-P deaminase was increased about four-fold, and 70-90 % of the enzyme was recovered.

Enzyme already partly purified by protamine sulphate fractionation and elution from C_γ alumina gel was further purified by elution from a column of DEAE cellulose. A preliminary experiment indicated that at least 80 % of the enzyme could be adsorbed and recovered by elution with 0.2 M KCl. A column (12 cm. x 0.8 cm. diam.) was washed and equilibrated with 0.005 M Tris-HCl buffer pH 7.5, and the extract applied ; more than 90 % of the enzyme was adsorbed. The column was washed with 30 ml. buffer and eluted with a gradient formed by feeding 0.2 M NaCl (in 0.005 M Tris-HCl buffer pH 7.5) into a mixing vessel containing 100 ml. 0.005 M Tris-HCl buffer pH 7.5. About 80 % of the enzyme activity was recovered in a single peak, giving 5-fold purification. The glucose phosphate isomerase activity was very low, but AcGm-6-P deacetylase activity was recovered in the same fractions as Gm-6-P

deaminase (although the ratio of their activities was not constant, which indicates that partial separation had occurred). The pH optimum in Tris-HCl buffer of Gm-6-P deaminase in these fractions was 7.5 ; that of AcGm-6-P deacetylase was between 7.5 and 9.5 (very broad). When present at a concentration of 0.2 µmole/ml., AcGm-6-P increased the activity of Gm-6-P deaminase two-fold in this purified preparation.

5 (c) Incorporation of 1-^{14}C-DL-valine by cells induced with AcGm.

5 litres of broth + glutamate were inoculated with B. subtilis and grown (with forced aeration) to 500 mg. total dry weight. They were harvested, washed and resuspended in 500ml. of buffer containing KH_2PO_4 (4g.), NaCl (4g.), $MgSO_4$ (0.2 g.), Oxoid casein hydrolysate grade L 41 (5.6 mg.) and D-L tryptophan (0.5 mg.) per litre. Half was treated with AcGm (10mM) and half with glucose (10mM) and each received 1-^{14}C-D-L-valine (0.016 mM, 5 µc.). The flasks were shaken at 37°C for 2 hrs., and the cells harvested and washed. The culture grown with glucose had incorporated 26 % of the radioactivity added and that grown with AcGm, 16 % ; the cells were disrupted by sonication and centrifuged at 105,000 x g for 60 min.. About 60 % ofthe total radioactivity incorporated was in the 105,000 x g supernatant in both cases ; the specific

activity of Gm-6-P deaminase from the Gm-grown cells was four times greater than that from the glucose-grown cells. The supernatants were then fractionated with protamine sulphate as described in 5 (b) ; about 20 % of the radioactivity remained in the soluble fraction in both cases. The soluble fractions were then fractionated on a column of DEAE cellulose as described in 5 (b). 85 % of the radioactivity was adsorbed initially, and on elution several peaks of radioactivity were obtained, but the large peak of deaminase activity in the induced extract did not contain more radioactivity than the small peak in the non-induced one. It therefore appears that this simple two-stage purification process (which gave an overall recovery of 70 % of the enzyme and about 50 times purification in the most active fractions) is not sufficient to distinguish between Gm-6-P deaminase and other proteins not specifically induced by AcGm. In view of the failure to obtain a significant incorporation of radioactivity from ^{14}C valine in cell-free extracts (see page 99), the purification procedure was not developed further.

5 (d) Assay of valyl-sRNA synthetase.

Cells were disrupted with the French Press , and valyl-sRNA synthetase was assayed in the 105,000 x g supernatant as described on page 79 . About three times as much radioactivity was present at the origin of the chromatograms after 60 min. as after 15 min.,

and omission of ATP from the reaction mixture reduced the rate of reaction to less than 10 %. The specific activities of the enzymes (calculated from the 15 min. values) were : 0.605 mμmoles/mg.protein/min. for an extract of cells grown in broth + glutamate alone and 0.57 mμmoles/mg.protein/min. for an extract of cells grown on broth + glutamate + AcGm (5mM). As expected, the presence of AcGm in the growth medium therefore does not affect the specific activity of valyl-sRNA synthetase.

Having completed these preliminary investigations, an attempt was made to show cell-free synthesis of Gm-6-P deaminase. 8 litres of broth + glutamate were inoculated with B.subtilis, and grown (with forced aeration). Early in log phase they were chilled rapidly by addition of lumps of ice, harvested in a Lourdes refrigerated centrifuge and disrupted by sonication or the French Press in a buffer (pH 7.0) containing Tris (0.01 M), $MgCl_2$ (0.01 M) and cysteine (0.01 M). The resulting suspension was centrifuged for 10 min. at 3,000 x g, to remove whole cells, then for 30 min. at 20,000 x g to give a 'heavy particle' fraction and finally at 105,000 x g for 90 min. to give a 'Light particle' fraction and a 'supernatant' fraction. Portions of these fractions (containing 1-2 mg. protein) were added to the incubation mixture (described

on page 80) in a total volume of 0.5 ml.. To certain tubes 2.5 µmoles AcGm and 2.5 µmoles of AcGm-6-P were added (as inducer) and the tubes were incubated at 37°C for 2-3 hrs.. Gm-6-P deaminase activity was assayed in aliquots before and after incubation. No increase could be detected (within the error of the assay, which is about 10 %) with mixtures of 'supernatant' (from cells grown with or without AcGm) and 'light' particles or 'heavy' particles (from cells grown with or without AcGm). In a parallel set of tubes containing the same incubation mixture and 1-^{14}C-DL-valine (0.21 µmoles, 1.0 µc.) the incorporation of radioactivity into material insoluble in 5 % trichloracetic acid was measured. No incorporation could be detected except in the tubes containing 'heavy' particles, and this may have been due to contamination with whole cells. In further attempts to demonstrate synthesis of enzyme and incorporation of label, the incubation mixture was modified to include per ml. : KCl (100 µmoles), glycerol (250 µmoles) and polyvinyl sulphate to inhibit ribonuclease (see page 72)(1.5 mg.), since these had been found to be required for the synthesis of α-amylase by cell-free extracts of B.subtilis (188). None of these modifications was successful, however. The failure to demonstrate synthesis may be due to destruction of the enzymes or polynucleotides concerned with

protein synthesis, or to the fact that neither of the 'inducers' added is identical with, or can be converted to, the true inducer.

6. Assay of amino sugar kinases and analysis of the products.

(a) Attempts to measure glucosamine kinase activity in extracts of B.subtilis by coupling to G-6-P dehydrogenase (method iv a on page 76) were not successful, although phosphorylation of Gm could be shown with purified yeast hexokinase and with E.coli extracts.

(b) No increase in ADP formation (method iv b on page 77) could be detected with extracts of B.subtilis in the presence of Gm or AcGm ; the ATP-ase activity of these extracts was considerable, but even when the latter was reduced by addition of sodium fluoride, there was no detectable kinase activity. E.coli extracts showed both glucokinase and AcGm kinase activity when tested by this method.

(c) Disappearance of amino sugar from the supernatant after treatment with $ZnSO_4$ and $Ba(OH)_2$ (method iv c on page 77) gave somewhat erratic results especially with glucosamine which itself tends to be adsorbed to the precipitate (190). Using the 105,000 x g supernatant obtained from a culture of B.subtilis grown with AcGm and disrupted in Tris-mercaptoethanol buffer by the French

Press, it was possible to show kinase activity with the following amino sugars : AcGm, PrGm, FoGm and n-BuGm, (but not iso-BuGm).

(d) Incubation with labelled amino sugar followed by precipitation of the amino sugar phosphates by barium acetate in 80 % ethanol (method iv d on page 77) was the most reproducible and sensitive of the methods tried. Using ^{14}Gm and Ac^{14}Gm (specific activity 1.4 to 5.0 μc./μmole) it was possible to detect the formation of 1 mμmole of amino sugar phosphate. Of the extraction media tested, the most successful was Tris-mercaptoethanol. Most of the enzyme was in the 105,000 x g supernatant, and the extracts were most active when the cell density was kept as high as possible.

A linear time course was obtained with either ^{14}Gm or Ac^{14}Gm as substrate (Graphs X and XI)*, and the yield of precipitable material reached a stable maximum when more than 50 % of the starting material had been converted to compounds insoluble in barium-ethanol. At this point the total nonvolatile radioactivity in the incubation mixture started to fall. Since this only occurred when the yield of barium-ethanol insoluble material was high, it probably represents degradation of the products rather than degradation of the starting material.

* page 143

Cells grown on AcGm consistently gave more activity in the assays for both kinases than cells grown on broth + glutamate alone, although the ratio of the activities was rather variable. For AcGm kinase the average ratio was 23.5 and for Gm kinase the average ratio was 10.4. The specific activity of AcGm kinase from AcGm-grown extracts was between 0.04 and 0.3 mμmoles/mg. protein/min. and that of Gm kinase from AcGm grown cells was between 0.02 and 0.06 mμmoles/mg.protein/min..

Cells grown on glucose or glucosamine gave higher kinase activities than cells grown on broth + glutamate alone and lower activities than cells grown on AcGm, but the measurements were too variable to permit a quantitative estimate of the ratios. The ratio of the activity obtained with Gm as the substrate to that obtained with AcGm as the substrate was not constant for the different extracts : this may tentatively be taken as evidence that the two amino sugars are phosphorylated by different enzymes.

Iodoacetic acid (0.01 M) reduced the activity by 90 % ; sodium cyanide (0.01 M) had no detectable effect. Omission of magnesium from the assay mixture reduced the activity by more than 90 %, and if the enzyme was dialysed against Tris-mercapto-ethanol buffer before assaying, there was no detectable incorporation in the absence of magnesium. Omission of ATP on

the other hand had less effect ; the initial rate in the absence of ATP was almost as high as in its presence, although the reaction proceeded further in the presence of ATP than in its absence. Preincubation of the enzyme at 37°C or addition of sodium fluoride (0.01 M) abolished activity in the absence of added ATP and reduced activity in the presence of ATP to about half. Dialysis did not affect the ratio of activities in the presence and absence of ATP.

In view of the fact that considerable activity was obtained in the absence of ATP some attempts were made to identify the products formed in its presence and absence. Preliminary experiments indicated that they could be adsorbed to Amberlite SB-2 paper (hydrogen form) which suggests that they are anionic. When subjected to paper electrophoresis in pyridinium acetate buffer (pyridine/acetic acid/water, 10/1/89 v/v/v pH 5.9) for 6 hrs. at 14 volts/cm., several radioactive bands moving towards the anode were obtained, one in the region of Gm-6-P marker (near the origin) and others in the region of G-6-P, F-6-P and AcGm-6-P markers which were not separated completely from each other. The products formed in the absence of ATP behaved similarly to those formed in its presence. In neither case were they

converted to free amino sugars by heating to 100°C in 2 N HCl for 4 hrs., which suggests that acid labile 1-phosphates are not present in large amounts (see page 109). Some degradation of the initial products appears to occur during the incubation, (there was always material behaving like Gm-6-P after incubation with Ac^{14}Gm and material behaving like hexose phosphates after incubation with ^{14}Gm) so an attempt was made to minimise this by using Gm rather than AcGm as the inducer, and by adding ammonium acetate to the incubation mixture at a concentration (0.5 M) at which Gm-6-P deaminase is inhibited by more than 90 %, and the kinases are inhibited by only 50 %. A large scale incubation was set up as follows :

Ten 600 ml. portions of broth + glutamate + 5mM Gm were inoculated with B.subtilis, grown to 42 mg. dry wt./100ml., harvested, washed and extracted by the French Press in Tris-mercaptoethanol buffer. The resulting suspension was centrifuged at 105,000 x g for 60 min..

Four tubes were prepared, containing the following incubation mixture : Tris pH 7.6 (500 µmoles), $MgCl_2$ (100 µmoles), ammonium acetate (5mmoles) and supernatant enzyme (40 mg. protein) ; final volume 5 ml.. Tubes 1 and 2 contained 1-^{14}C-D-glucosamine (1.8 µmoles;1 µc.); tubes 3 and 4 contained N-acetyl-1-^{14}C-D-glucosamine (0.9 µmoles;2 µc.), and tubes 2 and 4 contained ATP (100 µmoles). The tubes were incubated at 37°C for 80 min. and the

amount of radioactive product insoluble in barium-ethanol was measured on a small sample. The remainder was treated with 2 ml. of 50 % trichloracetic acid, the precipitate removed by centrifugation and the supernatant extracted with ether until the pH was neutral (no radioactivity could be detected in the precipitate or the ether extracts). The supernatants were lyophilised and extracted with 3 x 10 ml. portions of absolute ethanol to remove ammonium acetate (this also removed a large propoetion of the unchanged radioactive amino sugars). 5 % trichloracetic acid (2 ml.) was added to each and the small amount of undissolved material was removed by centrifugation.

The supernatants were again extracted with ether to remove trichloracetic acid, and diluted to 50 ml. with water and chromatographed on a column (40 x 0.8 cm.) of Dowex I (chloride form) 200-400 mesh)(191), using 2 µmoles of Gm-6-P and 2 µmoles of AcGm-6-P as internal markers. In each case the column was washed with 100 ml. water and eluted with a gradient obtained by feeding 0.05 N acetic acid into a mixing vessel containing 150 ml. water. Approx. 2 ml. fractions were collected by drop counting, and when 150 ml. eluate had been collected, the gradient was replaced by 0.15 N HCl. Amino sugar (155) and radioactivity were assayed in each fraction ; for the radioactive assay 0.2 ml. aliquots were evaporated on aluminium planchettes, (or on glass cover slips embedded in silicone grease on aluminium planchettes

in the case of the HCl-containing samples) and counted on the Nuclear Chicago automatic counter. The results are given in Table XVI.

Table XVI Analysis of the products formed when B.subtilis extracts are incubated with ^{14}Gm and Ac^{14}Gm in the presence and absence of ATP.

	Tube 1 ^{14}Gm (-ATP) (μc.)	Tube 2 ^{14}Gm (+ATP) (μc.)	Tube 3 Ac^{14}Gm (-ATP) (μc.)	Tube 4 Ac^{14}Gm (+ATP) (μc.)
Total radioactivity added to incubation mixture	1.0	1.0	2.0	2.0
Radioactivity insoluble in barium-ethanol	0.078	0.33	0.045	0.192
Radioactivity added to column	0.155	0.38	0.076	0.32
Unadsorbed material (free amino sugar)	0.033	0.075	0.0093	0.005
Peak 1 (eluted with the 0.05 N acetic acid gradient	0.033*	0.166*	0.011*	0.013*
Peak 2 (eluted with 0.15 N HCl)	0.064	0.055	0.046**	0.167**
Peak 3 (")		0.024		0.0044
Peak 4 (")		0.011		

* Coincident with Gm-6-P marker peak
** Coincident with AcGm-6-P marker peak

The contents of the tubes corresponding to each radioactive peak were combined, evaporated in a rotary evaporator and re-evaporated several times from aqueous solution to remove acetic acid and hydrochloric acid. They were further analysed as follows :

a) by degradation with the phosphatase 'polidase' which is specific for sugar phosphates (192). The following incubation mixture was used : Sodium acetate buffer pH 4.6 (μmoles), polidase (25 μg.) in a buffer (pH 4.6) 0.05 ml.) containing sodium acetate (0.01 M) and $MgCl_2$ (0.01M), toluene (0.05 ml.) and sugar phosphate sample, in 0.25 ml. total volume. It was incubated at 37°C for 60 hrs. in stoppered tubes. Samples were removed for paper chromatography in solvent A (page 26) with D-glucose, D-fructose, D-glucosamine and N-acetyl-D-glucosamine markers (located with silver nitrate (150). Authentic samples of G-6-P, F-6-P, Gm-6-P and AcGm-6-P were converted in high yield to the free sugars by this method. The distribution of radioactivity on the chromatograms of the degraded kinase products was measured with a B.T.L. radioactive chromatogram counter. Peak I from the column in each case was converted only to material having the same R_f as free glucosamine, and this material was completely adsorbed by Amberlite IR 120 resin (Hydrogen form). The other peaks from the column all gave some radioactivity near the origin, where sugar phosphates move, and some in the region where glucose, fructose

and AcGm move, The percentage of counts in the latter region in each case was as follows :

Tube 1 (^{14}Gm-ATP) , Peak 2 : 66 %

Tube 2 (^{14}Gm+ATP) , Peaks 2-4 : 10 %

Tube 3 (Ac^{14}Gm - ATP), Peak 2 : 90 %

Tube 4 (Ac^{14}Gm + ATP), Peak 2 : 26 %

This material was not adsorbed by Amberlite IR 120 resin.

b) by hydrolysis with 6N HCl for 8 hrs. at 100°C.

Portions were analysed by passage through columns of Amberlite IR 120 (hydrogen form) before and after hydrolysis, the results are given in Table XVII :

Table XVII. Analysis of products of kinase reaction before and after hydrolysis with 6N HCl.

	% radioactivity adsorbed by resin before hydrolysis	% radioactivity adsorbed by resin after hydrolysis
Tube 1 (^{14}Gm - ATP), Peak 2	6.5	47
Tube 2 (^{14}Gm + ATP), Peak 2	15	69
Peak 3	1.4	12
Tube 3 (Ac^{14}Gm-ATP), Peak 2	5.8	68
Tube 4 (Ac^{14}Gm+ATP), Peak 2	2.9	63

It thus appears that (apart from the third peak from tube 2), 50-70 % of the material present in the hydrolysed material contained a basic group, which presumably was, or was derived from, the amino group of glucosamine. Hydrolysis under milder conditions (0.5 N HCl for 15 min. at 100 C) did not give rise to compounds running in the same region as free sugars in solvent A, which suggests that none of them contained a significant amount of acid-labile 1-phosphates.

In summary, it appears that glucosamine was converted partly to Gm-6-P and partly to unidentified anionic compounds of which 50-70 % appear to retain the amino group of glucosamine. The proportion of Gm-6-P was about two thirds of the total for the extract incubated with ATP and about one third for the extract incubated without ATP. N-acetyl glucosamine was converted partly to Gm-6-P and partly to other anionic compounds which may have included a high proportion of AcGm-6-P, although this was not definitely identified. 60-70 % of the radioactivity was associated with material which still appeared to contain the amino group of glucosamine (probably acetylated in view of its behaviour on Dowex I) and behaving like a 6-phosphate rather than a 1-phosphate on acid hydrolysis. The proportion of Gm-6-P was one fifth of

the total in the absence of ATP and one fifteenth of the total in its presence. Since the material formed in the absence of ATP in both cases appeared to include the same components as that formed in its presence, it seems likely that the 105,000 x g supernatant from B.subtilis extracts contains small amounts of ATP or some other phosphate donor (0.008 µmoles per mg. of protein would be required to account for the amount of phosphorylated product formed from ^{14}Gm). This hypothesis is supported by the observation that preincubation at 37°C abolished the activity observed in the absence of ATP : any labile phosphates would tend to be broken down by enzymes such as ATP-ase under these conditions.

In view of the fact that a complex series of products was formed in these experiments, and attempt was made to purify the enzymes. The following processes were tried :

a) Protamine sulphate precipitation. 105,000 x g supernatant (30 mg. protein/ml.;20 ml.) from cells grown in the presence of glucosamine was treated with 2 % of protamine sulphate in successive 5 ml. portions. Most of the kinase activity disappeared from the supernatant when 35-40 ml. of protamine sulphate had been added. Attempts to re-extract it with 0.14 M sodium pyrophosphate buffer pH 6.7, with 1.0 M Tris-HCl buffer

pH 7.5 or with 1.0 M KCl in Tris-mercaptoethanol buffer were unsuccessful.

b) Ammonium sulphate precipitation. It was possible to precipitate the enzyme at 53-60 % saturation and some kinase activity was recovered by re-dissolving the precipitate in Tris-mercaptoethanol buffer. Recoveries were low, however, and deaminase was still present in the preparations.

c) Calcium phosphate gel. The enzyme was adsorbed, but could not be re-eluted with 1 M KCl in Tris-mercaptoethanol buffer.

d) Heating to 60°C for 5 min.. A single attempt to inactivate glucosamine kinase selectively by this method (see(50) and page 117 was unsuccessful.

7. Assay of Gm-6-P acetylase, Gm-1-P acetylase and phospho glucosamine mutase in extracts of B.subtilis.

Gm-6-P acetylase was assayed by incubating the 105,000 x g supernatant from cells extracted with the French Press and from cells extracted with the Hughes Press, with phosphate buffer pH 7.2 (50 µmoles), Gm-1-P (1µmole) and acetyl CoA (0.2-0.5 µmoles) in 0.5 ml.(or with sodium acetate (1 µmole), CoA (0.2 µmoles) and ATP (10 µmoles) in place of acetyl CoA). No acetylated amino sugar could be detected after incubation under a variety

of conditions with extracts prepared from cells grown on broth + glutamate alone, broth + glutamate + AcGm or broth + glutamate +Gm (cf. Clarke and Pasternak (56)).

Gm-1-P acetylase was assayed by incubating the 105,000 x g supernatant from cells (grown with and without AcGm) extracted with the French Press, with the assay mixture described on page 78. No acetylated amino sugar could be detected before or after heating to 100°C for 3 min. at a pH below 1.0 (to hydrolyse the 1-phosphate group.). Gm-1-P was not degraded by the B. subtilis extracts, and a similar experiment in which E.coli extracts were used was successful (see page 119).

Phospho-glucosamine mutase was assayed by incubating the 105,000 x g supernatant from cells (grown with and without AcGm) extracted with the French Press, with the assay mixture described on page 79. No conversion to material giving a positive amino sugar reaction before hydrolysis of the 1-phosphate was detected, which indicates that phosphoglucosamine mutase and Gm-1-P phosphatase activities were both negligible under the conditions of the experiment. In certain cases, fructose-1, 6-diphosphate (1 µmole) was added in the hope that it contained sufficient glucose-1,6-diphosphate to act as a cofactor in this reaction, but no mutase activity could be detected in its presence.

8. Enzymes concerned with amino sugar metabolism, and their control, in E.coli.

Some of the enzymes of amino sugar metabolism, and the control mechanisms associated with them, were examined in E.coli, in order

a) to compare a Gram negative organism with the Gram positive B.subtilis,

b) to test the assay methods which failed to give positive results with B.subtilis,

c) to make use of the fact that E.coli will grow well on simple defined media,

and d) to make use of the fact that mutants of E.coli can be isolated by the penicillin technique (193).

8 (i) Assay of Gm-6-P deaminase, AcGm-6-P deacetylase and Gm-6-P synthetase in E.coli ATCC 9723.

These enzymes were assayed in extracts of E.coli ATCC 9723 by the same techniques as were used with B.subtilis (see pages 74-6). The effect of adding AcGm to the medium and the effect of glucose were tested, and since AcGm can act as a nitrogen source as well as a carbon source, the effect of omission of nitrogen from the growth medium on induction and repression was examined. The results are given in Table XVIII.

Table XVIII Induction of Gm-6-P deaminase and AcGm-6-P deacetylase and repression of Gm-6-P synthetase by AcGm, in E.coli 9723.

E.coli 9723 was grown on Davis minimal medium (158) with the additions shown. All the cultures, except Nos. 4 and 6, were harvested after 14 hrs. growth at the dry weights shown ; No. 4 was harvested after 18 hrs. growth when it had reached a dry wt. of 85 mg./100 ml. and No. 6 was harvested after 36 hrs. when it had reached a dry wt. of 60 mg./100 ml.. They were washed, extracted with the French Press, and the 105,000 x g supernatant used for the enzyme assays (see pages 74-6) ; Gm-6-P deaminase was assayed by the disappearance method.

Addition to medium	Cell yield (mg/100 ml after 14hrs growth	Amino sugar remaining in medium (mM)	Enzyme activities (mμmoles/mg. protein/min. Gm-6-P deaminase	AcGm-6-P deacetylase	Gm-6-P synthetase
1 5mM AcGm	110	0.5	1.9	92	1.3
2 5mM AcGm + 5mM glucose	150	3.5	1.1	47	1.3
3 5mM glucose	85	-	0.19	17	8.6
4 5mM glycerol	10	-	0.08	8	14.0
5 5mM AcGm - NH_4^+ salts	100	0.5	2.0	63	1.0
6 5mM AcGm + 5mM glucose - NH_4^+ salts	3	4.0	2.2	49	0.86

Clearly AcGm induced Gm-6-P deaminase to 10-20 times the basal level obtained with glucose or glycerol as the carbon source and induced AcGm-6-P deacetylase to 5-10 times the basal level, in Davis medium. Glucose had only a small effect on the induction of these enzymes by the AcGm. Omission of ammonia from Davis minimal medium (so that AcGm is the only source of nitrogen) had practically no effect on the extent of induction of the enzymes, and had no effect on the rate of growth of the bacteria inthe absence of glucose. In the presence of glucose on the other hand, growth on AcGm as the sole nitrogen source was delayed by more than 22 hrs.. This is interesting in view of the observation that glucose reduced the rate of disappearance of AcGm from the medium. Since there was no substantial effect of glucose on the specific activity of Gm-6-P deaminase or AcGm-6-P deacetylase, and the amino sugar kinases appear to be constitutive in E.coli (50) and page 117), there must be an inhibitory effect of glucose or its products on the induction of some other inducible enzyme (e.g. a permease) or on the activity of one of the enzymes.

The apparent repression of Gm-6-P synthetase by AcGm in this experiment was of the same order of magnitude as that obtained in B.subtilis. The apparent activities obtained in

the presence of AcGm may have been lower than the true ones as a result of the presence of Gm-6-P deaminase in the (unfractionated) extracts but this factor would not completely account for the large difference between the activities obtained in the presence and absence of AcGm.

When incubated for brief periods with E.coli extracts, AcGm-6-P was converted to a compound or compounds which gave a higher colour yield in the amino sugar assay in the presence of acetic anhydride (155) than in its absence (167) : this is consistent with the hypothesis that AcGm-6-P is degraded to Gm-6-P (i.e. the acetylamino group is not removed as an intact unit.

8 (ii). Assay of amino sugar kinases in E.coli K 12.

Gm kinase and AcGm kinase were assayed by the barium-ethanol precipitation method described on page 77 ; the concentration of amino sugar was increased from 0.1 mM to 1.0mM since the activity of the enzyme was much greater in E.coli than in B.subtilis extracts. The assay gave a linear time course for at least 30 min. (15 % conversion) for both kinases. Omission of magnesium ion reduced the activity of Gm kinase by 95 % and of AcGm kinase by 75 %. Unlike the kinases from B.subtilis

those from E.coli showed negligible activity in the absence of added ATP (although the higher concentration of amino sugar and lower concentration of protein used in the case of E.coli would have obscured any phosphorylation produced by small amounts of high-energy phosphates in the extract). Brief incubation of the enzyme at 60°C was found to destroy Gm kinase without affecting AcGm kinase, as reported by Asensio (50).

Table XIX shows the specific activity of amino sugar kinases in extracts of E.coli K 12 grown on various carbon sources :

Table XIX. Specific activity of amino sugar kinases in E.coli K 12.

E.coli K 12 was grown on Davis minimal medium with the carbon sources shown, harvested, washed, disrupted with the French Press, and the 105,000 x g supernatant (0.5-0.7 mg.protein) used for the barium-ethanol precipitation assay.

Carbon source	Enzyme activities (mmoles/mg.protein/min.)	
	Gm kinase	AcGm kinase
Glucose (10mM)	1.55	4.38
AcGm (10mM)	1.68	5.35
AcGm (10mM) + glucose (10mM)	1.79	5.77
glycerol (20 mM)	2.09	4.35

Clearly AcGm does not induce either kinase to an appreciable extent in E.coli K 12, which agrees with the results obtained by Asensio (30).

8. (iii) Assay of glucose phosphate isomerase in E.coli 9723.

This enzyme was assayed by the spectrophotometric method used for Gm-6-P deaminase (see page 75) except that Gm-6-P in the incubation mixture was replaced by 0.2 umoles F-6-P. It was necessary to pre-incubate both the F-6-P and enzyme with portions of the assay mixture until a constant reading on the spectrophotometer was obtained. The two were then mixed, and the initial rate of reduction of NADP was measured on a Beckman DB spectrophotometer attached to a Sargent recorder. The rate was proportional to enzyme concentration, and the following specific activities were obtained (with the 105,000 x g supernatant from cultures extracted with the French Press) :

Cells grown with AcGm (10 mM) : 112 mumoles/mg.protein/min.

Cells grown with glucose (10 mM) :124 mumoles/mg.protein/min.

The enzyme is therefore not specifically induced by AcGm.

8. (iv) Assay of phosphoglucosamine mutase in E.coli 9723.

Attempts to detect this enzyme by coupling it to Gm-6-P deaminase in the spectrophotometric assay (using the 105,000 x g supernatant from cells grown with glucose or AcGm, extracted with the French Press) were unsuccessful. When the same extracts

were incubated with the assay mixture described on page 78 , Gm-1-P was slowly converted to material givng a positive amino sugar reaction without prior hydrolysis of the 1-phosphate, but the amounts which accumulated were too small for it to be possible to distinguish Gm-6-P formed by mutase action from Gm formed by phosphatase action. The specific activity was less than 0.5 mumoles/mg.protein/min., which is much lower than the specific activity of Gm-1-P acetylase (see below).

8,(v) Assay of Gm-1-P acetylase in E.coli 9723 (see ref.71).

The 105,000 x g supernatant of cells grown with glucose or AcGm and extracted with the French Press was assayed as described on page 77(iv d). A linear rate of reaction was obtained, and the specific activities in the two extracts were :

Cells grown with AcGm : 13.4 mumoles/mg.protein/min.

Cells grown with glucose : 21.8 mumoles/mg.protein/min.

Gm-1-P acetylase was therefore repressed to a small extent by AcGm, but the difference was scarcely significant.

The amount of acetylated product reached a plateau when 40 % of the theoretical yield had been obtained, and did not fall if the incubation was prolonged. The fact that the product

was not degraded suggests that AcGm-6-P deacetylase is much less active with AcGm-1-P than with AcGm-6-P as substrate, and that under the conditions of the acetylase assay, the activity of phospho-N-acetyl-glucosamine mutase is low.

8 (vi). An attempt to isolate mutants lacking AcGm kinase from E.coli K 12*

This attampt was made in the hope of using AcGm as a gratuitous inducer or repressor. The method used was based on the penicillin technique of Davis (193). A suspension of E.coli K 12 was irradiated for 12 min. at 8" below a Hanovia U.V. lamp with a 2" x 1.5" aperture (giving 1 in 5 x 10^5 survival), incubated in glucose-containing medium for 36 hrs. at 37°C to permit phenotypic expression of the mutations, harvested, washed and incubated for 32 hrs. at 37°C in a medium containing AcGm (10mM) and benzyl penicillin (10,000 i.u. per ml.). Samples were spread on agar plates containing glucose (10mM.). After 48 hrs. incubation about 300 colonies had appeared. Each plate was replicated on to two further agar plates, one of which contained glucose (10mM.) and the other AcGm (10mM.). These were incubated at 37°C and examined at intervals. All the colonies grew as rapidly on AcGm as on glucose, and are therefore penicillin-resistant mutants. It is evidently necessary to obtain and examine far more colonies than were obtained in this experiment.

* This experiment was performed in collaboration with Mr.R.J.White.

Discussion.

(i) The nature and specificity of the inducer of Gm-6-P deaminase and AcGm-6-P deacetylase and of the corepressor of Gm-6-P synthetase in E.coli and B.subtilis.

The results given in table XIII indicate that while AcGm is a good inducer of Gm-6-P deaminase and AcGm-6-P deacetylase, analagous amino sugars with the opposite configuration about carbons 2 and 4 of the ring, different substituents on carbon 6, and N-acyl side chains differing in size from the acetyl group are not. AcGm is also an efficient repressor of Gm-6-P synthetase; analogues with the opposite configuration about carbons 2 and 4 of the ring are not, but those with N-acyl side chains with three, one or no* carbon atoms are also repressors.

The fact that induction and repression have different specificities indicates that the two control mechanisms are not genetically linked in the same sense as the enzymes of a co-ordinate pathway (194,195). It also suggests that induction and repression are either controlled by different compounds within the cell, or by the same compound with different sensitivities, induction being less sensitive than repression (see question (ii) on page 81).

The observed specificity of induction and repression with regard

* i.e. glucosamine itself

to structural variations may, of course, reflect the specificity of the enzymes which convert the amino sugars to the inducer and corepressor rather than the specificity of the macromolecular repressor. Likewise the observation that Gm-6-P and AcGm-6-P do not significantly induce or repress does not rule out the possibility that they are identical with or convertible to the inducer or corepressor within the cell, since the cell membrane may effectively exclude them from the interior of the cell. Since these compounds do not disappear from the growth medium, this is a plausible explanation. If a gratuitous inducer or repressor could be found, the specificity of the macromolecular repressor might be defined more clearly, but attempts to achieve this by synthesis of analogues of AcGm (page 82) or by selecting mutants for which AcGm itself might be a gratuitous inducer or repressor (page 120) were unsuccessful.

The rate at which amino sugar is synthesised in non-repressed cells must be equivalent to the rate at which it is used for cell wall synthesis, since soluble intermediates do not accumulate (see page 50). When amino sugar is fed in from the medium at a rate lower than that at which it is normally synthesised de novo in non-repressed cells, the requirement for de novo synthesis is reduced, and the cell can afford to make less Gm-6-P synthetase. No amino sugar is available in excess of the requirement for cell

wall synthesis under these conditions however, and induction of the degradative enzymes; AcGm-6-P deacetylase and Gm-6-P deaminase, should not occur. This could explain the observation that Gm, PrGm and FoGm, which are slowly metabolised, repress Gm-6-P synthetase without inducing Gm-6-P deaminase (see Table XIII).

When amino sugar is fed in from the medium at a rate higher than that at which it is normally synthesised de novo by Gm-6-P synthetase in non-repressed cells, the cell can afford both to make less Gm-6-P synthetase and to degrade the 'extra' amino sugar to glycolytic intermediates. This could explain the observation that Ac Gm, which is rapidly metabolised, both represses Gm-6-P synthetase and induces AcGm-6-P deacetylase and Gm-6-P deaminase. The observation that AcGm induces Gm-6-P deaminase appreciably even when added at a concentration as low as 0.1 mM (Graph V) is consistent with the fact that the rate of incorporation of AcGm is nearly as great at 0.1 mM as it is at 2.5 mM (Table IV).

One observation which does not fit in with the hypothesis that induction is less sensitive than repression is that induction of Gm-6-P deaminase can occur (to a small extent) when the cells are grown with AcGm and glucose or with AcGm and fructose (Table XIV and graphs VI and VII) although the repression of Gm-6-P synthetase is substantially reduced under these conditions. The possibility that induction is linked to a specific structural property of AcGm rather than to its rapid rate of metabolism, cannot be definitely ruled out for this reason.

Since repression of Gm-6-P synthetase by PrGm and FoGm is never complete even at the highest concentrations tested (Graph IV), it is clear that if a common intermediate is responsible for repression there are rate-limiting steps in its formation from these amino sugars which are saturated at external concentrations in the region 0.5-1.0 mM. Repression by Gm falls continuously as the concentration in the growth medium is increased. This is consistent with the results given in Table II, which indicate that the incorporation of Gm is dependent on the external concentration over a very wide range. Induction of Gm-6-P deaminase by AcGm also reaches a maximum value when the concentration of AcGm in the medium is about 1 mM. This may represent saturation of the enzyme forming machinery, or of the rate of conversion of AcGm to the inducer ; there is at present no way of distinguishing between these possibilities.

Since AcGm induces Gm-6-P deaminase and represses Gm-6-P synthetase in B.subtilis and E.coli, it is probable that, in these organisms at least, Gm-6-P deaminase is concerned with the degradation of amino sugars and not, as suggested elsewhere (60,61) with their synthesis.

In view of the observation that E.coli can grow on AcGm as a sole nitrogen source, the nitrogen must be released from this compound in a form in which it can be used for other purposes, and this indicates that the degradative pathway can function in vivo.

The observation that PrGm-6-P is deacylated by extracts of B.subtilis is consistent with the metabolic pathway suggested in diagram II, on page 63 .

(ii). The nature of the glucose effect on induction and repression in E.coli and B.subtilis.

The effect of glucose, fructose and sucrose on the induction of Gm-6-P deaminase and AcGm-6-P deacetylase in B.subtilis (Table XIV) may be explicable in terms of catabolite repression (133) but since these carbon sources also have an effect on the rate of incorporation of $Ac^{14}Gm$ (Table VI), and on the repression of Gm-6-P synthetase (Table XIV), it is likely that they also reduce the rate at which AcGm is converted to the inducer and co-repressor. However, this is not a simple competitive effect in view of the results recorded in Table V, and those in graphs VI and VII which indicate that the repression of Gm-6-P synthetase by AcGm in the presence of glucose or fructose and the induction of Gm-6-P deaminase in the presence of glucose cannot be overcome by increasing the ratio of AcGm to glucose. A non-competitive effect of this type might arise if

glucose (or a derivative) were competing with AcGm (or a derivative) for an enzyme or a co-enzyme (such as ATP) at a site where its concentration is independent of that in the external medium. Since glucose delays the growth of E.coli on AcGm in the absence of a nitrogen source (Table XVIII) it may have an inhibitory effect on the release of nitrogen from AcGm in this organism.

The effect of glucose in abolishing repression of Gm-6-P synthetase by Gm (Table XV) is consistent with the observation that glucose severely inhibits the incorporation of glucosamine, possibly by competing for a kinase or permease (Table IX and page 60). It would obviously be disadvantageous for the cell to repress Gm-6-P synthetase under conditions where it is unable to use the amino sugar present in the medium. Glucose also abolishes repression by PrGm and FoGm which suggests that the rate of conversion of these amino sugars to the co-repressor is reduced by glucose.

(iii). The effects of inhibitors and of incubation in stationary phase on B.subtilis.

No induction of deaminase or repression of synthetase could be obtained by growing B.subtilis with high concentrations of penicillin or azauracil in the growth medium : it therefore appears that these inhibitors of amino sugar metabolism either

do not cause the accumulation of inducer or corepressor or do not permit their expression. In the case of penicillin this is in agreement with observation that B.subtilis has not been found to accumulate nucleotide derivatives in the presence of penicillin (196).

The observation that incubation in stationary phase results in an increase in specific activity of Gm-6-P deaminase in the absence of glucose, and an accumulation of amino sugar-containing material in its presence (Graphs VII and VIII) suggests that under these conditions the balance of amino sugar formation may be different from that observed in cells in log. phase. One possibility is that in stationary phase, B.subtilis produces enzymes which degrade the amino sugar-containing polymers of the cell wall to smaller units which are then able (in the absence of glucose) to induce enzymes such as Gm-6-P deaminase which can degrade the amino sugars to glycolytic intermediates. In the presence of glucose this may be prevented by catabolite repression, and the soluble fragments released into the growth medium. B.subtilis has been shown to produce various degradative enzymes (α-amylase, ribonuclease, alkaline phosphatase and proteases (173)) in stationary phase, and the germination of spores of B.subtilis and B.megatherium is accompanied by the release of a glycopeptide

containing AcGm and AcMur into the growth medium (197), which suggests that an enzyme degrading mucopeptide (such as lysozyme) is formed under these conditions. Lysozyme gives fragments in which the amino sugars are still N-acylated, and it may be significant that the amino sugar which accumulates in the medium in the presence of glucose appears to be N-acylated (page 92). These changes occurring at the end of log phase could explain the existence of Gm-6-P deaminase and AcGm-6-P deacetylase and the control mechanisms associated with them (see question (iv) on page 81). It seems probable that during log phase in the absence of exogenous amino sugar, the cell controls the flow of carbon into amino sugars by controlling the level of Gm-6-P synthetase (and possibly other anabolic enzymes which have not been investigated). Only under conditions where amino sugars are present in the growth medium or are produced internally by degradation of polysaccharides is it advantageous for the cell to degrade amino sugars by the Gm-6-P deaminase route. Although the induction and repression effects produced by AcGm may have the same net result (namely to control the distribution of carbon and nitrogen between amino sugars and other compounds) the two effects may be quite unconnected physiologically.

The nature of the inducer and repressor has not yet been determined. Repression of a synthetic enzyme is usually controlled by ' the last small molecule of a sequence before incorporation into macromolecules ' (131). According to this hypothesis, the repressor should be one of the nucleotide derivatives of the amino sugars. The inducer is probably one of the earlier intermediates such as AcGm or AcGm-6-P.

(iv). Purification of Gm-6-P deaminase from B.subtilis and attempts to synthesise it in a cell-free system.

Since Gm-6-P deaminase can be considerably purified without much loss of activity by methods which include protamine sulphate precipitation and re-extraction, adsorption on calcium phosphate gel and C_γ-alumina gel and re-extraction, and fractionation on DEAE cellulose, it is unlikely that an easily dissociated activator or cofactor is present in the 105,000 x g supernatant of induced extracts. The enzyme is stable under the condition of the assay used in the attempts at cell-free synthesis (page 98) although cell-free synthesis itself could not be demonstrated, possibly because of the high levels of degradative enzymes in B.subtilis (173). Since the ratio of induced to noninduced levels of Gm-6-P deaminase is not as great as it is in the case of other enzymes which have been studied in cell-free systems (173,184,198), it may be difficult to detect a small increase above the non-induced level.

(v). Assay of amino sugar kinases in E.coli and B.subtilis.

The results of the assay based on the barium-ethanol precipitation method indicate that kinases for glucosamine and N-acetyl glucosamine are present in extracts of E.coli and B.subtilis but that they are much weaker in the latter organism. They appear to be induced by AcGm and Gm in B.subtilis but not in E.coli (see question (iii) on page 81).
The fact that they are inducible in B.subtilis could explain the observation that pregrowth on amino sugars enhances the rate of incorporation of radioactivity from labelled amino sugars (see Table III and question (iv) on page 81).

Analysis of the products obtained when B.subtilis extracts are incubated with ^{14}Gm and Ac^{14}Gm with or without ATP indicates that a mixiture of anionic compounds are formed. In each case these include a compound behaving like Gm-6-P, and several other compounds behaving like the 6-phosphates of AcGm and other sugars, which have not been completely identified. This is consistent with the hypothesis that the initial products are Gm-6-P and AcGm-6-P which are subsequently degraded via the AcGm-6-P deacetylase-Gm-6-P deaminase catabolic pathway. Since some of the radioactivity is converted to a volatile form when the extracts are incubated for long periods with the radioactive amino sugars (see page 101), it is possible that some degradation

via the hexose monophosphate shunt may occur : this would result in the loss of the radioactive C-1 as CO_2. Likewise the presence of phosphatases acting on amino sugars might decrease the yield of products precipitable by barium and ethanol.. Neither of these interfering factors appears to affect the assay seriously however, since the yield of precipitable material increases at a rate which is linear with time in the early stages and reaches a stable maximum when more than 50 % of the labelled amino sugar has been phosphorylated (This maximum may of course represent the point at which the rates of the synthetic and degradative processes become equal).

(vi). Assay of glucose phosphate isomerase, amino sugar phosphate acetylases and amino sugar phosphate mutases in E.coli and B.subtilis.

Glucose phosphate isomerase is not specifically induced or repressed by AcGm in E.coli (see question iii on page 81) which is as expected, in view of the fact that this enzyme is not specifically concerned with amino sugar metabolism.

Amino sugar phosphate acetylases could not be demonstrated in extracts of B.subtilis, but the demonstration of Gm-1-P acetylase in extracts of E.coli was successfully repeated and the observation that more activity is obtained with Gm-1-P than

with Gm-6-P as substrate was confirmed. Growth on AcGm has only a small effect on the specific activity of the enzyme (see question (iii) on page 81).

If AcGm is fed into the pool of nucleotide-linked amino sugar without undergoing deacetylation, and thus replaces the cell's requirement for de novo amino sugar synthesis (see page 123), then the enzyme(s) responsible for acetylation of amino sugar ought to be repressed in its presence. Since Gm-1-P acetylase is repressed to only a small extent by AcGm, it is possible that AcGm (or a derivative) is deacetylated at an early stage and reacetylated later. It is significant that E.coli contains a highly active AcGm-6-P deacetylase, and that even in B.subtilis, where AcGm-6-P deacetylase appears to be much less active, the acetyl group of AcGm is partly liberated during incorporation of the glucosamine moiety (see Part I). Another reason why Gm-1-P acetylase is not repressed by AcGm could be that it has another function in the cell.

No evidence for phosphoglucosamine mutase activity could be obtained in extracts of B.subtilis and only slight activity could be detected in extracts of E.coli ; likewise indirect evidence suggested that phospho-N-acetyl-glucosamine mutase activity is very low in extracts of E.coli. The failure to

detect these enzymes may have been due to the absence of the co-factor, glucose-1,6-diphosphate from the assay mixture. The results do indicate, however, that the 6-phosphates are not converted to the 1-phosphates or *vice versa* under the conditions of the assays used for Gm-6-P deaminase, AcGm-6-P deacetylase and Gm-1-P acetylase.

(vii). Differences between *E.coli* and *B.subtilis*.

The principal ways in which *E.coli* differs from *B.subtilis* with respect to the enzymes examined are that :

(a) the ratio of Gm-6-P deaminase to AcGm-6-P deacetylase activity is much higher in *B.subtilis* than in *E.coli*.

(b) the amino sugar kinases are inducible in *B.subtilis* but are constitutive in *E.coli*.

(c) the glucose effect on induction of Gm-6-P deaminase and AcGm-6-P deacetylase is much greater in *B.subtilis* than in *E.coli*.

amino sugar [µmoles]
0.8
0.6
0.4
0.2
200
160
120
80
40
eluate [ml.]
2
1
radioactivity [counts / min. x 10^{-3}].

Graph I

Analysis of cTCA fraction on Dowex 50 resin.

Continuous line : radioactivity (counts/min. x 10^{-3}).

Dotted line : amino sugar markers (µmoles calculated in terms of glucosamine).

1st. peak = Gm

2nd. peak = Galm

amino sugar [μmoles]
1.6
1.2
0.8
0.4
radioactivity [counts / min. x 10^{-3}]
12
10
8
6
4
2
eluate [ml.]
40
80
120
160
200

Graph II

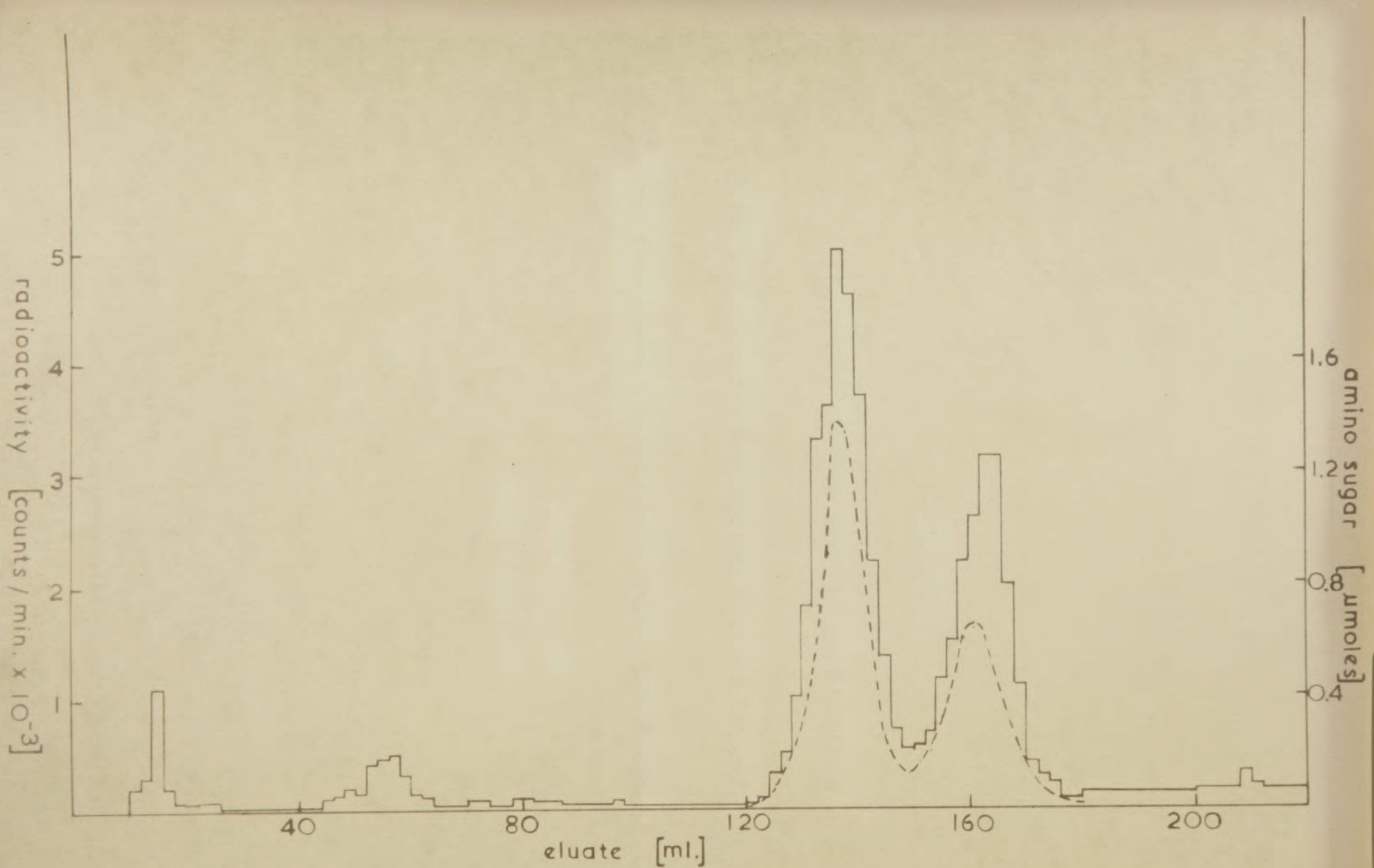
Analysis of HTCA fraction on Dowex 50 resin.

Continuous line : radioactivity (counts/min. x 10^{-3}).

Dotted line : amino sugar markers (µmoles calculated in terms of glucosamine).

1st. peak = Gm

2nd. peak = Galm


radioactivity [counts / min. x 10-3]
5
4
3
2
1
amino sugar [μmoles]
1.6
1.2
0.8
0.4
40
80
120
160
200
eluate [ml.]

Graph III

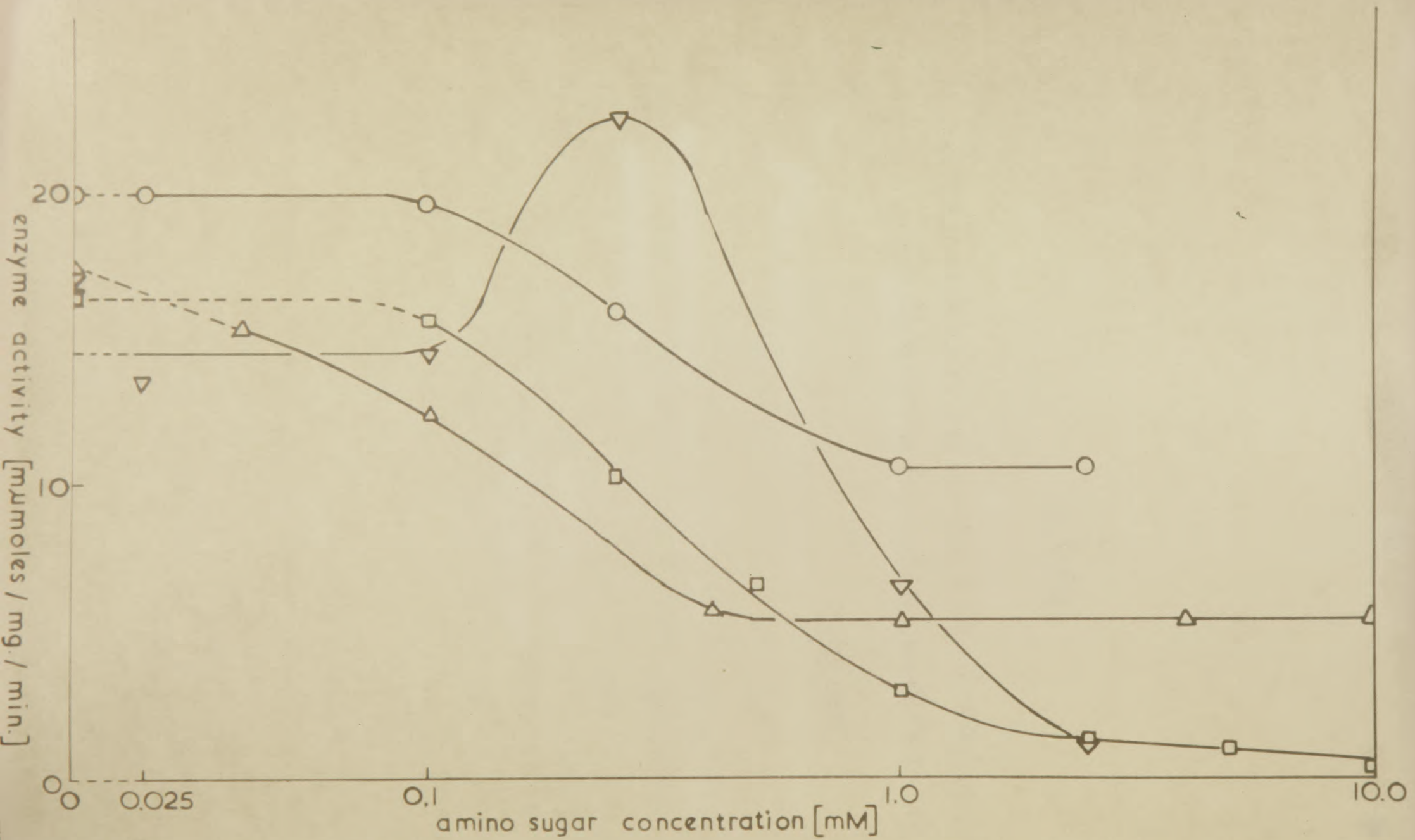
Analysis of res. fraction on Dowex 50 resin.

Continuous line : radioactivity (counts/min. x 10^{-3}).

Dotted line : amino sugar markers (μmoles calculated in terms of glucosamine).

1st. peak = Gm

2nd. peak = Galm


20
10
0
0
0.025
0.1
1.0
10.0
enzyme activity [mumoles / mg. / min.]
amino sugar concentration [mM]

Graph IV

Variation of Gm-6-P synthetase concentration in B. subtilis with amino sugar concentration in the growth medium.

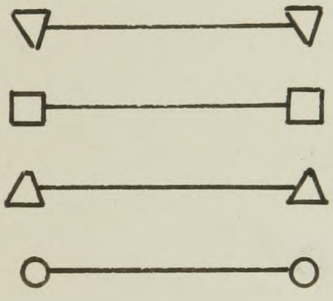
Amino sugar

AcGm

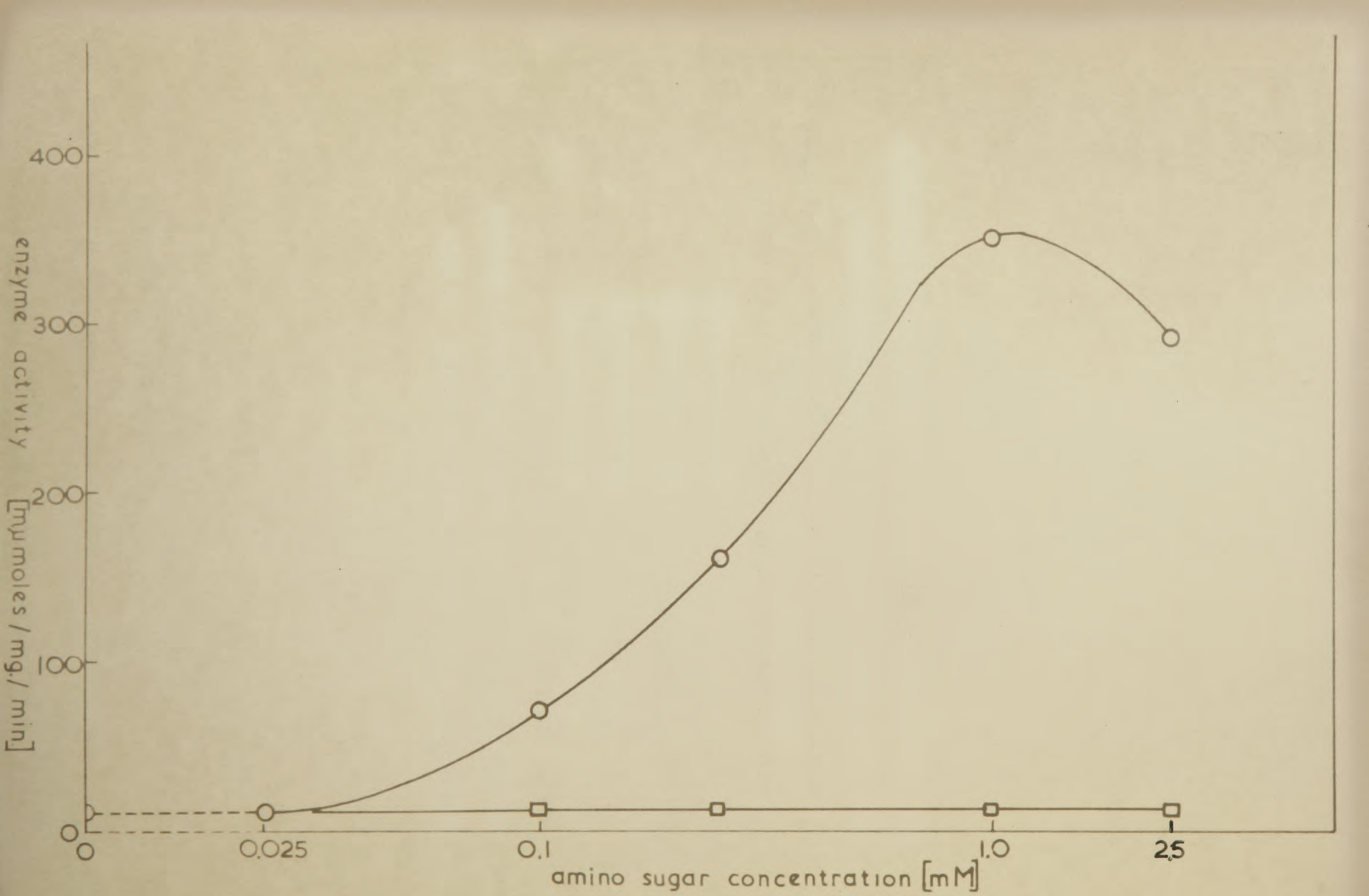
Gm

PrGm

FoGm



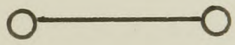
Specific activity of the enzyme expressed as mμmoles Gm-6-P formed per mg. supernatant protein per min.


400
300
200
100
0
enzyme activity [μmoles / mg. / min]
0
0.025
0.1
1.0
2.5
amino sugar concentration [mM]

Graph V

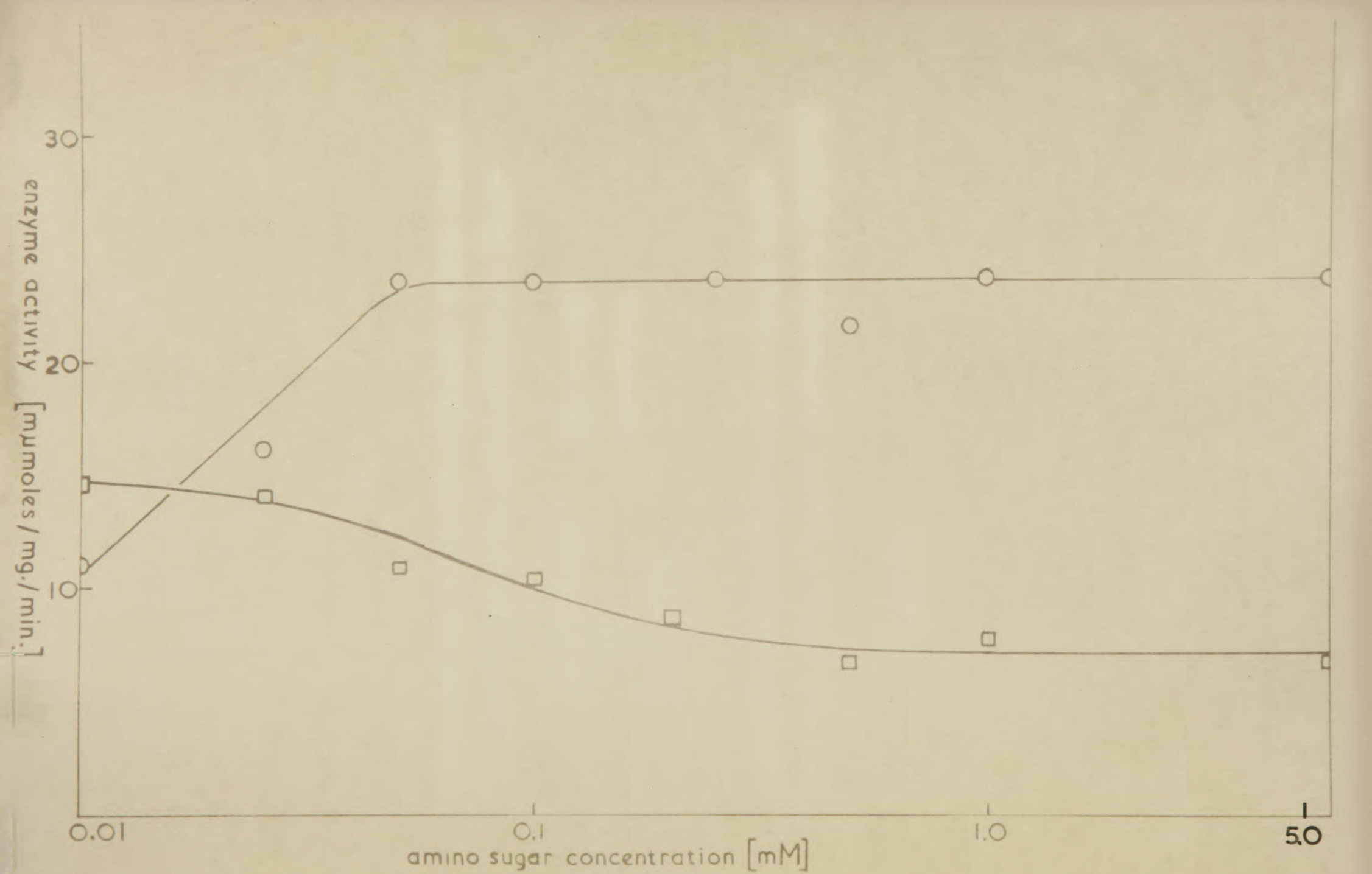
Variation of Gm-6-P deaminase concentration in B.subtilis with amino sugar concentration in the growth medium.

Amino sugar

AcGm 

Gm, PrGm, FoGm 

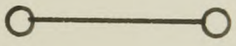
Specific activity of the enzyme expressed as mμmoles Gm-6-P disappearing per mg. supernatant protein per min.


30
20
10
enzyme activity [mumoles / mg. / min.]
0.01
0.1
1.0
5.0
amino sugar concentration [mM]

Graph VI

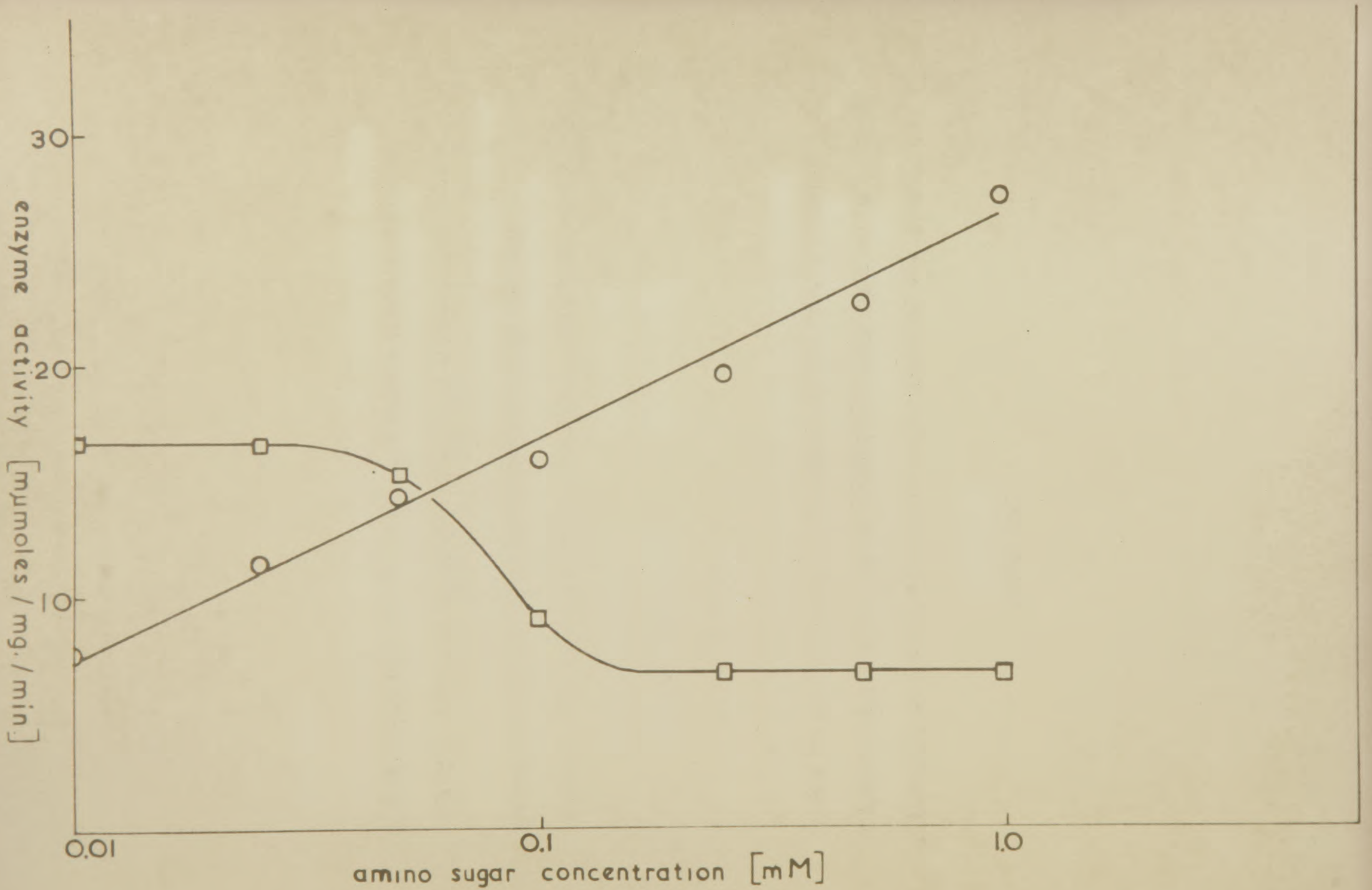
Variation of Gm-6-P synthetase concentration and Gm-6-P deaminase concentration in B.subtilis with AcGm concentration in the presence of 100mM glucose in the growth medium.

Gm-6-P synthetase 

Gm-6-P deaminase 

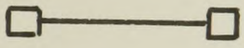
Specific activity of Gm-6-P synthetase expressed as μmoles Gm-6-P formed per mg. supernatant protein per min.

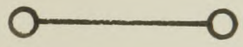
Specific activity of Gm-6-P deaminase expressed as μmoles Gm-6-P disappearing per mg. supernatant protein per min.


30
20
10
enzyme activity [μmoles / mg. / min.]
0.01
0.1
1.0
amino sugar concentration [mM]

Graph VII

Variation of Gm-6-P synthetase concentration and Gm-6-P deaminase concentration in B.subtilis with AcGm concentration in the presence of 100 mM fructose in the growth medium.

Gm-6-P synthetase 

Gm-6-P deaminase 

Specific activity of Gm-6-P synthetase expressed as mmoles Gm-6-P formed per mg. supernatant protein per min.

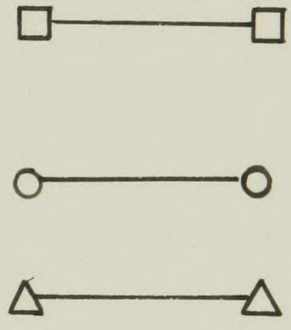
Specific activity of Gm-6-P deaminase expressed as mmoles Gm-6-P disappearing per mg. supernatant protein per min.

amino sugar in medium
[O.D. at 585 mμ]
0.10
0.05
40
30
20
10
0
hrs. incubation
10
5
Gm-6-P deaminase [mμmoles/mg/min]
0
50
100
cells [mg. dry wt./100 ml.]

Graph VIII

Variation of Gm-6-P deaminase concentration in *B. subtilis* and of the optical density given by samples of the growth medium in the amino sugar assay, with length of incubation :

a) Cells grown in broth + glutamate medium



Gm-6-P deaminase (mμmoles Gm-6-P disappearing per mg. supernatant protein per min.)

dry weight of cells (mg. per 100 ml. growth medium)

optical density at 585 mμ given by 0.2 ml. samples of the growth medium in the acetylated amino sugar assay (167)

amino sugar in medium
0.10
0.05
[O.D. at 585 mμ]
40
30
20
10
0
hrs. incubation
10
5
0
Gm-6-P deaminase [mμmoles/mg./min.]
cells [mg. dry wt. / 100 ml.]
100
50
0

Graph IX

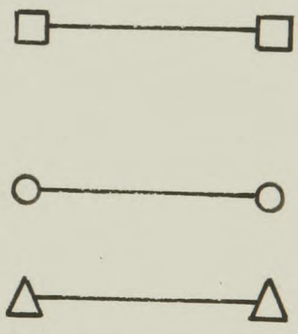
Variation of Gm-6-P deaminase concentration in B.subtilis and of the optical density given by samples of the growth medium in the amino sugar assay, with length of incubation :

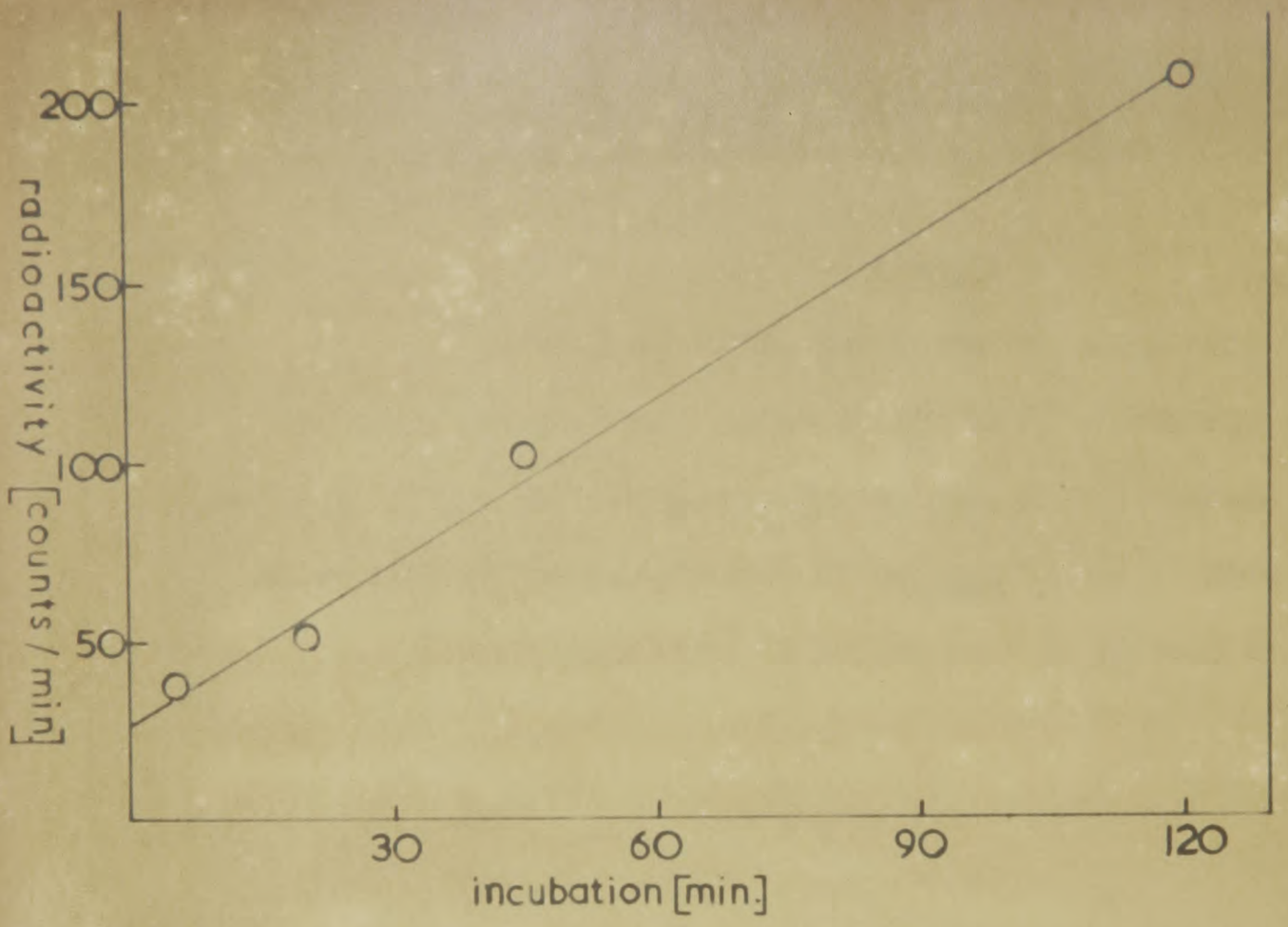
b) Cells grown in broth + glutamate medium containing 100 mM glucose

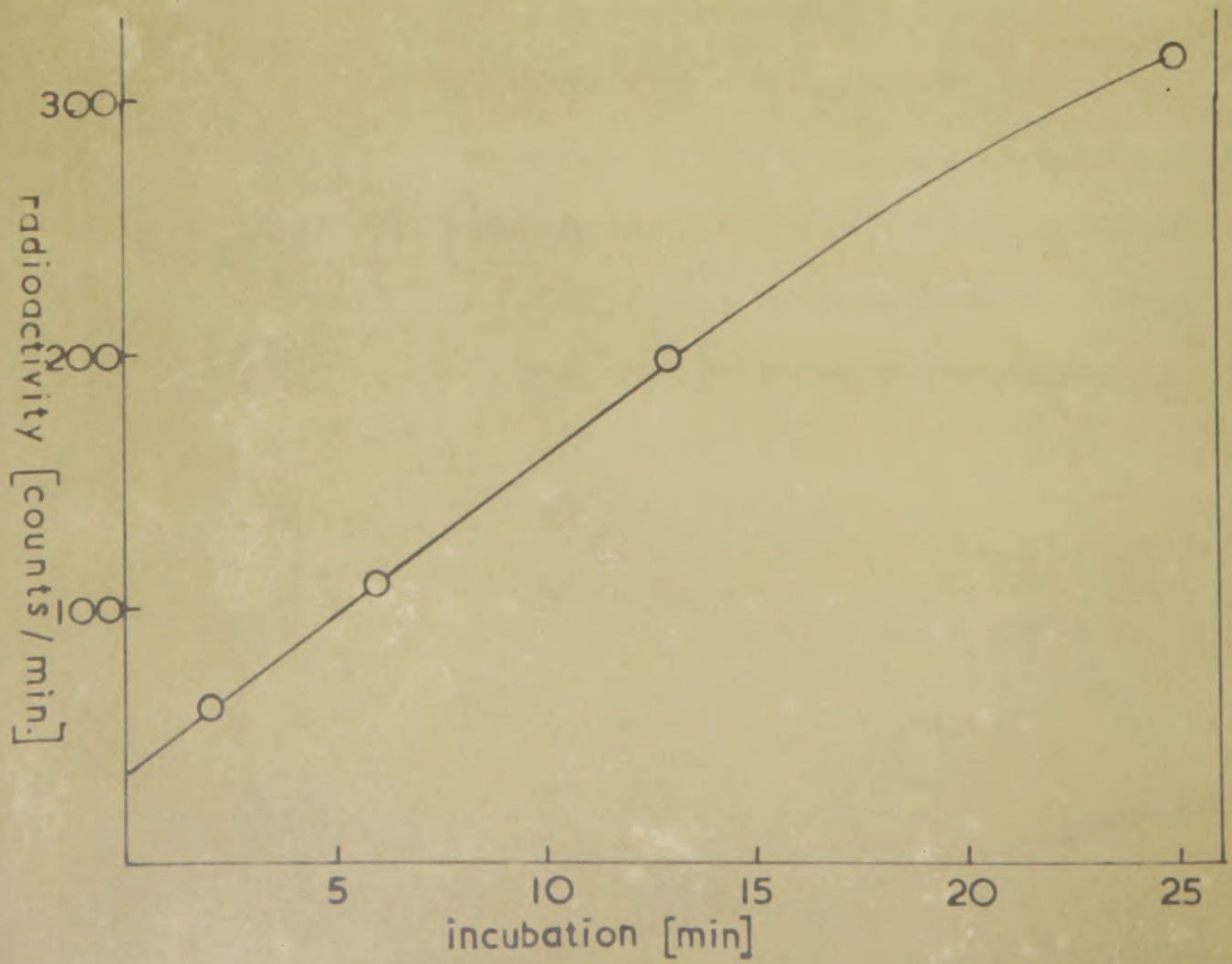
Gm-6-P deaminase (mμmoles Gm-6-P disappearing per mg. supernatant protein per min.)

dry weight of cells (mg. per 100 ml. growth medium)

optical density at 585 mμ given by 0.2 ml. samples of the growth medium in the acetylated amino sugar assay (167)




200
150
100
50
radioactivity [counts / min]
30
60
90
120
incubation [min.]


300
200
100
radioactivity [counts / min.]
5
10
15
20
25
incubation [min]

Graph X

Time course for Gm kinase from B.subtilis.

Supernatant (0.11 mg. protein) from Gm-grown cells was incubated with the standard assay mixture + ATP ((ivd) on page). Radioactivity (counts/min. above background *) is plotted against period of incubation (min.).

Graph XI

Time course for AcGm kinase from B.subtilis.

Supernatant (0.13 mg. protein) from Gm-grown cells was incubated with the standard assay mixture + ATP ((ivd) on page). Radioactivity (counts/min. above background *) is plotted against period of incubation (min.).

* 15 counts / min.

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The Effect of Amino Sugars and Glucose on Amino Sugar Metabolism in *Bacillus subtilis*

By C. J. Bates* and C. A. Pasternak. (*Department of Biochemistry, University of Oxford*)

D-Glucosamine 6-phosphate is a key intermediate in the biosynthesis and breakdown of amino sugars (Roseman, 1959). It is synthesized by the enzyme L-glutamine D-fructose 6-phosphate aminotransferase, EC 2.6.1.16 (Ghosh, Blumenthal, Davidson & Roseman, 1960) and is degraded by 2-amino-2-deoxy-D-glucose 6-phosphate ketol isomerase (deaminating), EC 5.3.1.10 (Roseman, 1959; Strange & Dark, 1960). Addition of *N*-acetyl-D-glucosamine to the growth medium of *Bacillus subtilis* NCTC 1379 causes repression of the first enzyme (termed synthetase) and induction of the second enzyme (termed deaminase) (Clarke & Pasternak, 1961, 1962). D-Glucosamine, *N*-formyl-D-glucosamine and *N*-propionyl-D-glucosamine have been found to initiate the same effects, though to a lesser extent. The relation between the repression of synthetase and the induction of deaminase was not constant for the amino sugars tested. This indicates that repression of synthetase and induction of deaminase are independent processes. Derivatives of D-glucosamine with larger *N*-acyl side-chains or with a different configuration in the pyranose ring were inactive.

The addition of D-glucose to *B. subtilis* growing in the presence of *N*-acetyl-D-glucosamine causes the induced synthesis of deaminase to be decreased (Clarke & Pasternak, 1962). At the same time the repression of synthetase was partly relieved. Several other sugars had a similar effect as D-glucose in reversing the changes in enzyme synthesis caused by *N*-acetyl-D-glucosamine. Those that were active also inhibited the incorporation of *N*-acetyl-D-[1-^{14}C]glucosamine into growing cultures of *B. subtilis*. A slower uptake of *N*-acetyl-D-glucosamine from growth media containing D-glucose may therefore account for a part of the glucose effect (cf. Magasanik, 1961).

* Medical Research Council Training Scholar.

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