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Studies on the Lipid Content and Phosphate Requirement of Glucose- and Acetate-Grown *Escherichia coli*

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1. The phosphate requirement, i.e. the concentration of inorganic orthophosphate that just ceases to be limiting for growth, of *Escherichia coli* N.C.T.C. 5928 was determined for growth in ammonium–salts media containing glucose or acetate as the carbon and energy source, and compared with that of six other strains of *E. coli*. 2. The phosphate requirement for *E. coli* N.C.T.C. 5928 growing on acetate was about ten times that for growth on glucose, but this difference was not observed with any of the other strains. 3. After about 40 generations' growth on acetate with phosphate limitation in a chemostat, the phosphate requirement of the cells gradually decreased until it was equivalent to that of the glucose-grown organism; a single passage through glucose batch culture sufficed to restore the original high phosphate requirement, indicating a permeability phenomenon. 4. The lipid content of *E. coli* N.C.T.C. 5928 grown on glucose or acetate was measured isotopically by fractionation of cells grown on inorganic [^{32}P]orthophosphate and gravimetrically after extraction from the cells by three different methods; change of carbon source from glucose to acetate did not affect the lipid content, which remained constant at 8–9% of the bacterial dry weight.

The lipid content of *Escherichia coli* N.C.T.C. 5928 was reported to undergo substantial increase, and the carbohydrate content to decrease, if the carbon source in an ammonium–salts medium is changed from glucose to acetate (Dagley & Johnson, 1953). Additionally, the inorganic orthophosphate requirements of this organism were shown by Johnson (1953) to vary according to the carbon substrate. When *E. coli* N.C.T.C. 5928 was grown under conditions of phosphate limitation the phosphate requirement for growth on acetate was about ten times that for equivalent growth on glucose or succinate. As the lipids of *E. coli* are principally phospholipids (Kanfer & Kennedy, 1963), it seemed possible that the reported increases in lipid content and of phosphate requirement when growth occurs on acetate might be directly linked. Interest in the nature of storage materials in *E. coli*, and in the mechanisms controlling their deposition, led us to reinvestigate this phenomenon, since the system appeared to offer opportunities for a detailed study of the processes regulating the deposition of lipid and carbohydrate in the organism.

Preliminary accounts have been presented (Damoglou & Dawes, 1967*a*,*b*).

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EXPERIMENTAL

Materials

Organisms. *E. coli* N.C.T.C. 5928 was used for most of the work, except for some comparative experiments on phosphate requirements when *E. coli* N.C.I.B. strains 8114, 8270, 8545, 8571, 9342 and 9466 were used. The stock organisms were maintained on nutrient–agar slants.

Growth methods. *E. coli* was grown aerobically at 37° in one of three ways. When only small volumes of culture were required the cells were grown in 12·5ml. of medium in conical flasks (50ml.) on a Gallenkamp reciprocating shaker (120 oscillations/min.; stroke 4cm.). To follow the growth cycle a conical flask (50ml.) with a 6in. × $\frac{1}{2}$in. test tube fused on the side was used and the bacterial density was measured turbidimetrically. For larger culture volumes cells were grown in a 1l. of medium in a conical flask (2l.), or in 2l. of medium in a 3l. conical flask in an incubator and flushed with sterile air (2l./min.) through a sintered-glass sparger.

The organism was also grown in a chemostat of the 0·5l. 'Porton' type (Herbert, Phipps & Tempest, 1965). The pH was maintained at 7·1 ± 0·1 and the temperature at 37 ± 0·05°. Sterile air was passed through the culture at 0·7l./min., and a Mackereth oxygen electrode indicated that at the two dilution rates used (0·15 and 0·40hr.$^{-1}$) the culture was respectively 100% and 80% saturated with oxygen.

Growth on acetate as the sole carbon source was very slow when the organism was transferred directly from a slant to synthetic media; cells in glucose media were therefore trained to grow on acetate by gradually decreasing the

glucose concentration in the presence of increasing concentrations of acetate, until the glucose was completely withdrawn. This technique was used for both batch and chemostat growth.

Media. All cells were grown on defined media. Glass-distilled water was used throughout. Citrate was not utilized by *E. coli* in an ammonium–salts medium (Johnson, 1953), and was used as a buffer for experiments in which the phosphorus source was limiting for growth.

Media were made up in two or more parts and mixed aseptically after being autoclaved; they are designated by the carbon source and buffer used. The carbon source and Mg^{2+} comprised one part [to give final concentrations (g./l.): glucose, 8, or sodium acetate trihydrate, 7; $MgSO_4,7H_2O$, 0·4]. The buffer–nitrogen source used was either phosphate [to give final concentrations (g./l.): KH_2PO_4, 5·4; $(NH_4)_2SO_4$, 1·2; pH adjusted to 7·1 with 5N-NaOH] or citrate–phosphate A [to give final concentrations (g./l.): trisodium citrate dihydrate, 10; $(NH_4)_2SO_4$, 1·2; K_2SO_4, 3·0; KH_2PO_4, 0·1; pH adjusted to 7·1 with 2N-H_2SO_4], or citrate–phosphate B, which differed from citrate–phosphate A only in final concentration of KH_2PO_4 (1·0g./l.). Citrate–phosphate A provides a slight excess of phosphate over minimum growth requirements on glucose, and citrate–phosphate B does likewise for acetate-grown cells.

For certain experiments slightly different media were used. In those designed to show that citrate was not metabolized, the media were as follows. Citrate-buffered media: these contained glucose to give a range of final concentrations of 3–30mM, with citrate–phosphate solution A and $MgSO_4,7H_2O$ (0·4g./l.). Phosphate-buffered media: these contained glucose to give a range of final concentrations of 3–30mM, with phosphate solution containing (g./l.): KH_2PO_4, 10·8; $(NH_4)_2SO_4$, 1·2; $MgSO_4,7H_2O$, 0·4.

In experiments to determine the phosphate requirement for *E. coli* the media used were as follows: carbon source as previously described; citrate buffer–nitrogen source [final concentration (g./l.): trisodium citrate dihydrate, 10·0; $(NH_4)_2SO_4$, 1·2; K_2SO_4, 3·0; the pH was adjusted to 7·1 with 5N-NaOH]; phosphate was added to give a final concentration either in the range 10–100mg. of KH_2PO_4/l. or in the range 0·1–1·0g. of KH_2PO_4/l.

The phosphate and glucose solutions were sterilized by filtration through a Millipore filter (Millipore Filter Corp., Bedford, Mass., U.S.A.), pore size 0·25μ, to prevent possible concentration changes that might result from autoclaving.

Chemostat media. In all chemostat experiments inorganic orthophosphate was the factor limiting growth. As described in the Results section, a final KH_2PO_4 concentration of 25mg./l. was used for growth on glucose, but 250mg./l. was necessary when acetate was the carbon source. Media were prepared from three separately sterilized solutions: (1) buffer–nitrogen source: in a calibrated 20l. aspirator (g./18l.): trisodium citrate dihydrate, 400; K_2SO_4, 47; $(NH_4)_2SO_4$, 53·2; $MgSO_4,7H_2O$, 16; pH adjusted to 7·1 with 2N-H_2SO_4; (2) carbon source: glucose, 144g. in approx. 1l., or for growth on acetate sodium acetate trihydrate, 108·9g. in approx 1l.; (3) phosphate solution: for glucose as carbon source, 0·5g. of KH_2PO_4 in approx. 500ml.; for acetate, 5g. of KH_2PO_4 in 500ml.; pH adjusted to 7·1 with 5N-NaOH. Solutions (2) and (3) as appropriate were added to solution (1) aseptically, and the volume was made up to 20l. with sterile water.

During the transition from glucose to acetate as the carbon source the glucose concentration was decreased in 10mM steps as the acetate was introduced in 10mM stages. Only when the glucose had been completely withdrawn was it necessary to increase the phosphate concentration to 250mg./l. (see the Results section).

Buffers. The phosphate buffer solution used was 0·067M-KH_2PO_4 adjusted to pH7·1 with 5N-NaOH; the citrate buffer was 0·067M-trisodium citrate dihydrate adjusted to pH7·1 with 2N-H_2SO_4.

Chemicals. A.R.-grade chemicals (from Hopkin and Williams Ltd., Chadwell Heath, Essex, or British Drug Houses Ltd., Poole, Dorset) were used whenever possible; other chemicals were of the best available purity from the same suppliers, or as noted in the text.

Radiochemical. Carrier-free [^{32}P]orthophosphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Analytical methods

Bacterial densities. Bacterial densities were determined turbidimetrically in a Unicam SP. 600 spectrophotometer at 570mμ; extinctions were converted into bacterial densities from curves relating bacterial dry weight (after drying for 18hr. at 105°) to extinction. Growth in the modified conical flasks was estimated with the test-tube attachment of the Unicam SP. 600.

Growth measurements. Total growth of the organism as a function of the glucose concentration in media buffered with citrate (containing phosphate in slight excess of the growth requirements) or phosphate and the relationship between the total growth and the phosphate concentration in media containing glucose or acetate as the carbon source were determined.

Cells were harvested from media of composition identical with that being used in the experiment, washed twice with 0·85% NaCl soln. at room temperature under sterile conditions and made up to the original volume. Portions (0·1ml.) were used to inoculate 12·5ml. of medium in the modified conical flasks, which were placed in a Gallenkamp reciprocating shaker bath. The growth of the cultures was followed, and as each reached the stationary phase the flask was removed and the culture quantitatively transferred, with the appropriate buffer solution, to a volumetric flask and made up to the mark. The volumetric flask was of such volume that the resulting cell suspension had E_{570} between 0·3 and 0·7.

Total lipid. Lipid was estimated isotopically and gravimetrically.

(*a*) Isotopic method. Cells grown in a medium containing [^{32}P]orthophosphate were fractionated by the method of Roberts, Abelson, Cowie, Bolton & Britten (1957). The cellular lipid is contained in the ethanol–diethyl ether-soluble fraction in this procedure. The fractions were each digested with 2ml. of 10N-H_2SO_4 containing KH_2PO_4 (9g./l.) as carrier. They were cleared with 2 drops of 100-volume H_2O_2 and quantitatively transferred to 10ml. volumetric flasks with phosphate buffer (0·067M-KH_2PO_4 adjusted to pH7·1 with 5N-NaOH). Portions (5ml.) of each of these fractions were counted in liquid Geiger tubes (M6; 20th Century Electronics, Croydon, Surrey) for 1ksec. with an Ekco automatic scaler type N530G. The Geiger tubes had been calibrated against a halogen-quenched tube (M6H), which was kept as a reference standard. Corrections

were applied for background, dead time and the half-life of ^{32}P.

(*b*) Gravimetric method. The lipid was extracted from cells in three ways. (i) Washed cells (60–70mg. dry wt.) were extracted with 40ml. of aq.75% (v/v) ethanol at 40° for 30min., and the cells were centrifuged and then re-extracted with 40ml. of aq.75% (v/v) ethanol–diethyl ether (1:1, v/v) at 40° for 30min. This is a modification of the procedure of Roberts *et al.* (1957). (ii) Freeze-dried cells were extracted overnight in a Soxhlet apparatus with ethanol–diethyl ether (1:1, v/v) (Dagley & Johnson, 1953). (iii) Freeze-dried cells, after being ground in a Potter homogenizer fitted with a Teflon plunger, were extracted with methanol at 45° for 15min. The extraction was repeated twice with methanol and then with ethanol–diethyl ether (3:1, v/v) at room temperature (Knivett & Cullen, 1965).

The extracted lipid material was in each case evaporated almost to dryness in a rotary evaporator at 30° and finally to complete dryness at room temperature under a high vacuum. The lipid was then dissolved in chloroform, leaving a residue that was mostly water-soluble. Most of the chloroform was evaporated with a gentle stream of air and the remainder was completely removed in a vacuum desiccator. The residue, lipid freed from protein and carbohydrate, was then weighed.

Carbohydrate. Carbohydrate was measured by the anthrone method of Trevelyan & Harrison (1952) as modified by Binnie, Dawes & Holms (1960).

RESULTS

Phosphate requirements of E. coli

For the present purpose the 'phosphate requirement' is defined as the concentration of inorganic orthophosphate that just ceases to be limiting for growth when all other nutrients are present in excess. As *E. coli* does not utilize citrate in an ammonium–salts medium citrate was used as the growth-medium buffer in experiments designed to determine the phosphate requirement of the organism. With a concentration of citrate adequate to buffer the acid produced from glucose, the relationship between total growth and glucose concentration was identical for phosphate- and citrate-buffered media (Fig. 1).

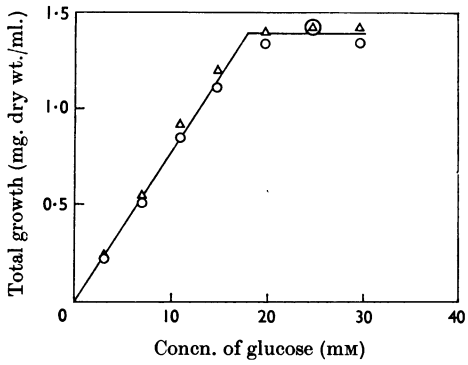

Fig. 1. Relationship between total growth of *E. coli* N.C.T.C. 5928 and glucose concentration in an ammonium–salts medium buffered with phosphate (△) or with citrate (○). The citrate-buffered medium contained sufficient phosphate to meet growth requirements.

Batch-culture experiments. Experiments to determine the phosphate requirements of *E. coli* N.C.T.C. 5928 for growth on glucose or acetate as the carbon and energy source revealed that growth was limited by phosphate concentrations of up to 0·456mM for growth on glucose, and of up to 5·73mM for growth on acetate (Fig. 2). These observations are in accord with those of Johnson (1953).

It seemed unlikely that this tenfold increase in requirement for phosphate could have been accounted for by incorporation into cellular material, even when one allows for the reported higher lipid content of this strain during growth on acetate (Dagley & Johnson, 1953). A comparison was therefore made with six strains of *E. coli* selected at random from the N.C.I.B. collection. The comparison of phosphate requirements and total growth of these strains with those of *E. coli* N.C.T.C. 5928 shows (Table 1) that the abnormally high phosphate requirement of *E. coli* N.C.T.C. 5928 with acetate as the carbon source is peculiar to this strain. Studies with [^{32}P]orthophosphate revealed that this large amount of phosphorus is not retained by the acetate-grown cells, e.g. the phosphorus incorporation was found to be 16·3 and 16·8mg./g. dry wt. for glucose- and acetate-grown cells respectively. We therefore undertook a study of growth of *E. coli* N.C.T.C. 5928 on glucose and on acetate under conditions of phosphate limitation in the chemostat.

Chemostat experiments. To secure comparable cell densities it was necessary to use concentrations of 25 and 250mg. of potassium dihydrogen phosphate/l. for glucose and acetate respectively and, as noted above, growth on acetate was achieved by the gradual decrease in 10mM stages of the glucose concentration coupled with the corresponding increase in acetate concentration. It was necessary to increase the concentration of potassium dihydrogen phosphate to 250mg./l. only after complete withdrawal of glucose.

After a period of growth on acetate in the chemostat corresponding to about 40 generations, the cell density began to increase, and this increase could be controlled by decreasing the potassium dihydrogen phosphate concentration. Eventually the concentration of the phosphate was decreased to 25mg./l. and the organism then displayed the 'normal' phosphate requirement characteristic of glucose-grown cells. If some of these cells were inoculated into glucose batch culture one passage through this

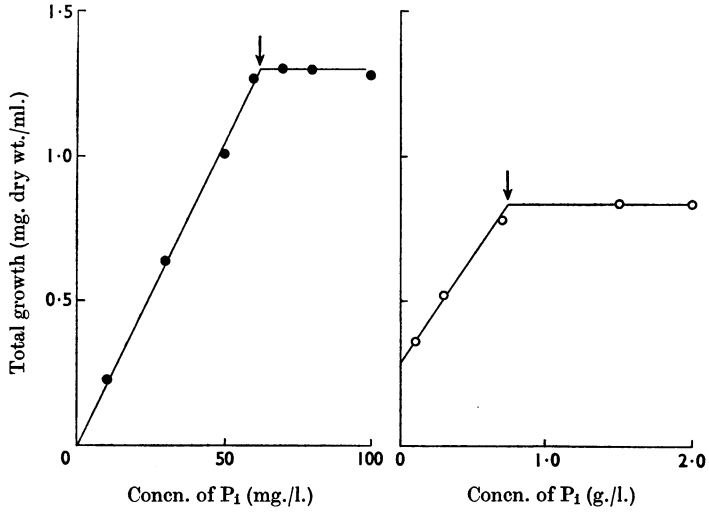

Fig. 2. Relationship between total growth of *E. coli* N.C.T.C. 5928 and KH_2PO_4 concentration in ammonium–salts medium with glucose (●) and acetate (○) as the carbon and energy source. The different scales for the abscissae should be noted. The arrows indicate the values taken as the phosphate requirement, which are 0·456 mM for glucose and 5·73 mM for acetate.

Table 1. *A comparison of the phosphate requirement and total growth of various strains of* E. coli *grown on glucose or acetate*

'Phosphate requirement' is defined as the concentration of phosphate that just ceases to be limiting for growth when all other nutrients are in excess.

Carbon source for growth	Glucose			Acetate		
Strain of *E. coli*	Phosphate requirement (mM)	Total growth (mg./l.)	Phosphorus content (mg./mg. dry wt. of bacteria)	Phosphate requirement (mM)	Total growth (mg./l.)	Phosphorus content (mg./mg. dry wt. of bacteria)
N.C.T.C. 5928	0·456	1300	10·9	5·73	840	212
N.C.I.B. 8114	0·617	1200	16·0	0·213	425	15·6
N.C.I.B. 8270	0·411	725	17·6	0·294	550	16·6
N.C.I.B. 8545	0·602	1500	12·5	0·199	385	16·0
N.C.I.B. 8571	0·404	730	17·2	0·096	300	9·9
N.C.I.B. 9342	0·588	1040	17·5	0·411	500	23·7
N.C.I.B. 9466	0·529	1150	14·3	0·096	270	11·0

medium (about ten generations) sufficed to restore the original phosphate requirement for growth on acetate; we conclude that a permeability phenomenon is involved.

Lipid content of glucose- and acetate-grown cells

Lipid determinations were initially performed on cells grown in media containing [^{32}P]orthophosphate. They were fractionated by the procedure of Roberts *et al.* (1957), and the relative proportions of ^{32}P in the different fractions were determined. The cells were grown on glucose or acetate with citrate as buffer and a range of potassium dihydrogen phosphate concentrations (30–1000 mg./l. for the glucose-grown cells and 0·3–3·0 g./l. for the acetate-grown cells). The results (Table 2) provide no evidence that acetate-grown cells contained more lipid than glucose-grown cells, a finding in conflict with that of Dagley & Johnson (1953). Further determinations were carried out by the other extraction procedures described in the Experimental section

Table 2. *Fractionation of* E. coli *N.C.T.C.* 5928 *labelled with* [^{32}P]*orthophosphate*

The fractionation procedure of Roberts *et al.* (1963) was used and the values are expressed as percentages of the total radioactivity incorporated by the cells when they are grown in the presence of various concentrations of [^{32}P]orthophosphate with glucose or acetate as the carbon source.

	Radioactivity (% of total radioactivity incorporated)							
Carbon source	Glucose				Acetate			
Concn. of KH_2PO_4 in medium (mg./l.)	1000	75	50	30	3000	1000	700	300
Metabolic intermediates	9·0	6·8	9·1	9·5	21·7	19·3	21·8	22·8
Lipids	15·2	15·5	17·8	18·7	11·5	11·8	12·1	14·5
Ethanol-soluble protein	2·5	2·6	2·3	3·4	1·9	1·7	1·4	1·3
Nucleic acids	71·0	73·3	69·1	65·0	60·8	62·9	61·9	56·6
Residual protein	2·2	2·2	1·8	3·4	4·1	4·0	2·8	4·8
Total counts/ksec. (corresponding to 100%)	20845	34304	27986	38808	24237	27396	28787	25371

Table 3. *Comparison of the lipid content of glucose- and acetate-grown* E. coli *N.C.T.C.* 5928 *with three different methods of extraction*

The numbers of determinations are given in parentheses.

Method of extraction	Lipid content (% of dry wt. of bacteria)	
Carbon source ...	Glucose	Acetate
(1) Modified from Roberts *et al.* (1957)	8·6±0·7 (2)	8·3±1·3 (2)
(2) Dagley & Johnson (1953)	9·3±0·4 (2)	9·0±0·5 (2)
(3) Knivett & Cullen (1965)	8·5±0·2 (2)	7·6±0·2 (2)

and these results (Table 3) also indicate that the purified lipid content of *E. coli* N.C.T.C. 5928 was stable irrespective of the carbon source used for growth.

In an attempt to discover whether our culture of *E. coli* N.C.T.C. 5928 might have changed in some characteristics during the intervening years between the work of Dagley & Johnson (1953) and the present study, another culture of the same strain, which had had an independent history, was obtained from Dr J. Sykes. This strain displayed characteristics indistinguishable from those of our own culture, and its lipid content was identical. It seems unlikely therefore that the different results could be attributed to a change of characteristics peculiar to our organism.

The lipid content of cells grown with phosphate limitation in the chemostat was measured by the method of Knivett & Cullen (1965). The results (Table 4) reveal that the lipid content changed very little with alteration of the dilution rate, and there was no significant difference between growth on glucose and growth on acetate as sole carbon source.

DISCUSSION

The present work confirmed the high inorganic orthophosphate requirement of *E. coli* N.C.T.C. 5928 noted by Johnson (1953) when he grew the organism on acetate in batch culture. However, our finding that six other strains of *E. coli*, selected at random from the N.C.I.B. catalogue, did not display this phenomenon, and that they all had similar phosphate requirements whether grown on glucose or on acetate, suggested anomalous behaviour of *E. coli* N.C.T.C. 5928. In support of this belief, experiments with [^{32}P]orthophosphate revealed that the increased requirement for inorganic orthophosphate when growth occurs on acetate was not reflected by an increase in the phosphorus bound by the cell. It seemed probable therefore that growth on acetate produced some permeability barrier to inorganic orthophosphate necessitating a higher external concentration to secure the required internal concentration for growth. This interpretation is supported by the observation that, after about 40 generations' growth on acetate in the chemostat, the organism's phosphate requirement gradually decreased until it was identical with that for cells grown on glucose. However, one passage in batch culture through glucose medium was sufficient to restore the high phosphate requirement during growth on acetate.

We could not confirm the report of Dagley & Johnson (1953) that changing the carbon source from glucose to acetate resulted in a higher lipid content, despite the use of various environmental conditions and different methods of assay. This stability of the lipid content of *E. coli* has been noted

Table 4. *Carbohydrate and lipid contents of* E. coli *N.C.T.C. 5928 grown with phosphate limitation in a chemostat, or in batch culture providing a slight excess of phosphate*

Batch cultures were analysed at the onset of the stationary phase in citrate–phosphate medium A (glucose) and B (acetate). The numbers of determinations are giveni n parentheses.

Carbon source	Glucose				Acetate	
Conditions	Aerobic			Anaerobic	Aerobic	
Dilution rate ($hr.^{-1}$)	0·15	0·40	Batch culture	0·15	0·15	Batch culture
Carbohydrate content (% of dry wt.)	3·7±0·5 (2)	7·4±0·4 (4)	15·0	3·6±0·6 (2)	4·7±0·5 (2)	4·0
Lipid content (% of dry wt.)	8·8±0·7 (2)	8·4±0·7 (2)	8·6	7·1±0·5 (2)	8·8±0·4 (2)	8·4

by other workers (Dr J. H. Law, personal communication). The explanation for the discrepancy between our results and those of Dagley & Johnson (1953) is not apparent, but a change in the characteristics of the strain does not seem likely.

At the outset of this work we had conjectured that the reported increased lipid, i.e. phospholipid Kanfer & Kennedy, 1963), content of acetate-grown *E. coli* N.C.T.C. 5928 might bear some relationship to the higher phosphate requirement characteristic of growth on this carbon source. Our findings have ruled out this hypothesis, and the observed constancy of the lipid content under various conditions casts doubt on the possible role of this material as an endogenous substrate. Dawes & Ribbons (1965) reported that the lipid of *E. coli* grown anaerobically on [U-^{14}C]glucose was not degraded during starvation in phosphate buffer, as measured by the radioactivity of the lipid fraction obtained by the procedure of Roberts *et al.* (1957).

In those organisms for which lipid has been shown to serve as a reserve material, e.g. *Rhodotorula graminis* and *Rhodotorula glutinis* (Mulder, Dienema, van Veen & Zevenhuizen, 1962), and *Mycobacterium phlei* (Stephenson & Whetham, 1922), it accumulated during growth in amounts far in excess of those required for structural purposes, a feature never observed in our studies with *E. coli*.

There is no evidence to suggest that other reserve materials, such as poly-β-hydroxybutyrate or polyphosphate, play a significant role as reserve materials in *E. coli*. Poly-β-hydroxybutyrate has never been detected in the organism, and although polyphosphate is present in small amounts (Kornberg, 1957; Hughes & Muhammed, 1962) the conditions of phosphate limitation used in the chemostat would prevent polyphosphate deposition. Indeed, a rough calculation of the molecular weight of the lipid extracted from *E. coli*, based on one atom of phosphorus/molecule, gives a value of 720, which is in the region expected for phosphatidylethanolamine with two C_{17} fatty acids.

The carbohydrate content of *E. coli* in relation to the environment of the cells has been the topic of considerable study (Dagley & Dawes, 1949; Holme & Palmstierna, 1956*a*,*b*,*c*). Palmstierna (1955) showed that *E. coli* contained a glycogen-like polysaccharide, which was broken down during starvation (Holme & Palmstierna, 1956*c*), and Dawes & Ribbons (1965) demonstrated that this carbohydrate served as a primary endogenous substrate, having a sparing action on the net degradation of nitrogenous materials. The glycogen content of *E. coli* has been shown to be under direct environmental control, being high for nitrogen limitation and low for carbon limitation in the chemostat (Holme, 1957). Phosphate limitation in batch culture (Holme & Palmstierna, 1956*b*) resulted in low carbohydrate content, and we have shown this to be true also of phosphate-limited cells from the chemostat.

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