The Metabolism of Glucuronic Acid by Xylose-fermenting Coliform Bacteria

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Although the ability of coliform bacteria to ferment D-xylose and L-arabinose is well known, there is relatively little information concerning their ability to ferment the related homomorphous uronic acids, D-glucuronic and D-galacturonic acids.

Kay (1926) found that twenty-one members of the coli-typhosus group were able to utilize glucuronic acid as a carbon source. Some cultures studied further were found to ferment glucuronic acid to form qualitatively the same end products as from glucose. However, Kay pointed out that the lactic and succinic acids found may have arisen from the peptone in the medium. Quick & Kahn (1929) extended this work and in surveying a large number of colon typhoid-dysentery bacteria, found that most strains were capable of fermenting glucuronic acid to form some unspecified acidic products.

It has frequently been observed (cf. Hirst, 1949) that in natural products, such as xylans, both glucuronic acid and xylose occur together, as part of the same molecule, and this has led to the suggestion that xylose may arise by the decarboxylation of glucuronic acid. Indeed, in 1902, Salkowski & Neuberg claimed to have isolated xylose as a product of the decarboxylation of glucuronic acid by bacteria in a putrefying mince. It has, however, been demonstrated (Franken, 1932) that glucuronic and galacturonic acids can be decarboxylated to the homologous pentoses by heating with sulphuric acid. In a recent examination of the hypothesis, Cohen (1949) studied the ability of the K-12 strain of *Escherichia coli* to ferment certain uronic acids and their related pentoses, and showed that cells grown on the one type of substrate would not immediately ferment the other type of substrate. From this, the conclusion was drawn that uronic acids were not metabolized by an initial decarboxylation.

In a previous communication (Heald, 1952) it was shown that the products of the fermentation of xylose and glucuronic acid by certain strains of coliform bacteria were not at all dissimilar, but differed from the fermentation products of glucose. This suggested that glucuronic acid and xylose might be metabolized by the same pathway, which might differ in some respects from the pathway for the anaerobic metabolism of glucose. As a result of experiments carried out to test this hypothesis, it has been found that cultures grown on xylose or glucose were able to adapt to the utilization of glucuronic and galacturonic acids in resting suspension, under anaerobic conditions, and in the absence of fixed exogenous nitrogen. Further it was found that the rate at which adaptation occurred was markedly stimulated by the presence of a readily metabolized second substrate, by a factor (or factors) which could be washed from the cells, or by adenosinetriphosphate (ATP).

MATERIALS AND METHODS

Cultures. The principal cultures used have already been described (Heald, 1952). After the general results described below had been established with five cultures, the remaining work was carried out with four strains of culture 14. These were obtained by plating the culture on to a basal medium agar containing 0·5% xylose and selecting four colonies. These strains were indistinguishable by their fermentation reactions, but were used as separate strains in order to confirm each set of data obtained. At the end of the experiments a comparison of the cultural and general morphological characteristics of these strains, with similar characteristics of the original freeze-dried stock cultures, failed to show that any change had taken place. *Escherichia coli* N.C.T.C. no. 86 and *Aerobacter aerogenes* N.C.T.C. no. 8197 were obtained from the National Collection of Type Cultures.

Culture medium. The medium originally described (Heald, 1952) was modified in order to obtain reproducible growth. The rumen-liquor supplement was replaced by 0·06% Bacto-Tryptone (Difco Laboratories, Detroit, Michigan) and the concentrations of $NaHCO_3$ and acetate were reduced by half. All the cultures grew well on this medium, with 0·4% of the required carbohydrate, but did not grow at all on the medium alone, in 24 hr. at 40°.

Preparation of cells for experiments. Except where stated below, the cells were obtained by inoculating 0·1 ml. of a 24 hr. culture into the liquid basal medium containing 0·4% of the required carbohydrate and incubating at 40° for 16–20 hr. The suspension was centrifuged and the cells were washed twice in bicarbonate saline buffer (Krebs & Henseleit, 1932) saturated with 95% N_2–5% CO_2 at pH 7·2 (Umbreit, Burris & Stauffer, 1945). The suspensions were diluted with buffer to roughly the same density, and suitable quantities were taken for manometric experiments.

Manometry. Acid production was measured in bicarbonate-saline, in Warburg manometers, at 40° under an atmosphere of 95% N_2–5% CO_2. In experiments requiring two substrates, vessels with two side arms were employed.

Anaerobiosis was obtained by placing a small stick of scraped yellow phosphorus in the centre cup.

It was shown that when gas evolution ceased, all the substrates had been utilized (Table 1), and consequently the rate of gas production was taken as a measure of substrate utilization.

Rates are calculated as $q_{CO_2}^{N_2}$, (μl. CO_2/0·1 mg. N/hr.).

Table 1. *Quantities of xylose and glucuronate remaining in solution after the initial rapid gas evolution had ceased in fermentation by washed suspensions of coliform bacteria*

Substrate	Amount originally taken (μg.)	Amount found (μg.)	Amount used (μg.)	Amount used (%)
Xylose	688·5	32·5	656·0	95·5
	687·5	29·5	658·0	95·0
Glucuronate	605·0	49·5	555·5	92·0
	612·0	49·5	562·5	91·8

Analytical methods. Carbohydrates were estimated by the method of Hagedorn & Jensen (1923) in solutions from the manometers after first inhibiting further bacterial action by tipping in acid from a second side arm at the end of the experiment. The solution was then brought to pH 7·0 with NaOH and the bacteria were removed by precipitation with $ZnSO_4$ and NaOH (Neish, 1946).

Nitrogen was estimated in samples of the cell suspensions and in other liquids by the method of Chibnall, Rees & Williams (1943).

Substrates. D-Glucose (British Drug Houses Ltd., 'Analar' Dextrose); D-galactose, D-xylose (T. Kerfoot Ltd.); L-arabinose, D-galacturonic acid (L. Light and Co. Ltd.). D-Glucurone was given by Dr G. A. Levvy, a gift to him from Corn Products Co., Ltd. It was converted to the Na salt of the acid by electrometric titration with NaOH to a stable pH of 8·0. At this pH the acid is most probably in the pyranose form (Challinor, Haworth & Hirst, 1931; Smith, 1939) and is therefore comparable to D-galacturonic acid, D-galactose, D-glucose, D-xylose, and L-arabinose. α- and β-glucuronic acid-1-phosphates were given by Mr C. A. Marsh. The sample of β-acid contained 30% of the phosphate, the remainder being most probably mainly potassium phosphate and a small amount of β-glucose-1-phosphate. Glucose and glucuronic acid were absent. ATP was used as the sodium salt, prepared from a sample of the dibarium salt given by Mrs K. Heald.

For use as substrates for growth, the required carbohydrates were sterilized in 25% aqueous solution, either by autoclaving at 15 lb. pressure for 15 min. or, in the case of the sugar acids, by Seitz filtration. In the manometric experiments, the sugars were used as 0·01 M solutions in bicarbonate saline.

RESULTS

The result of shaking cells grown on xylose with glucuronic and galacturonic acids, glucose, galactose xylose, arabinose and α- and β-glucuronic acid-1-phosphates is shown in Fig. 1. Glucose and xylose were fermented immediately. Galactose and α- and β-glucuronic acid-1-phosphates were not fermented

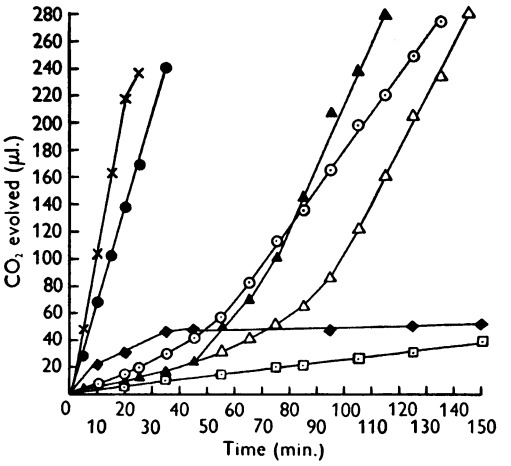

Fig. 1. Acid production from various substrates by cells previously grown on xylose. Flasks contained 153 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95% N_2–5% CO_2. Temperature 40°. ×—×, xylose; ●—●, glucose; △—△, galacturonic acid; ▲—▲, glucuronic acid; ⊙—⊙, arabinose; ◆—◆, α-glucuronic acid-1-phosphate; ⊡—⊡, galactose, β-glucuronic acid-1-phosphate, and no substrate.

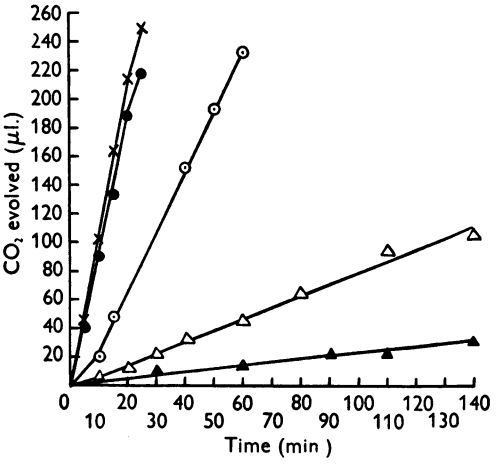

Fig. 2. Acid production from various substrates by cells previously grown on glucuronate. Flasks contained 60 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95% N–5% CO_2. Temperature 40°. ×—×, glucuronate; ●—●, galacturonate; ⊙—⊙, glucose; △—△, xylose; ▲—▲, arabinose, galactose, α- and β-glucuronic acid-1-phosphates, and no substrate.

to any extent while glucuronic and galacturonic acids and arabinose were used after a more or less short lag period. This was interpreted to mean that the cells formed adaptive systems for the fermentation of these substrates while in resting suspension.

In Fig. 2 is shown the results of shaking cells grown on glucuronic acid with the above substrates. Glucuronate and galacturonate were fermented immediately and at the same rate. Glucose was also fermented immediately, but at a somewhat slower rate, while xylose, arabinose, galactose and α- and β-glucuronic acid-1-phosphates were fermented either very slowly or not at all. The results of these

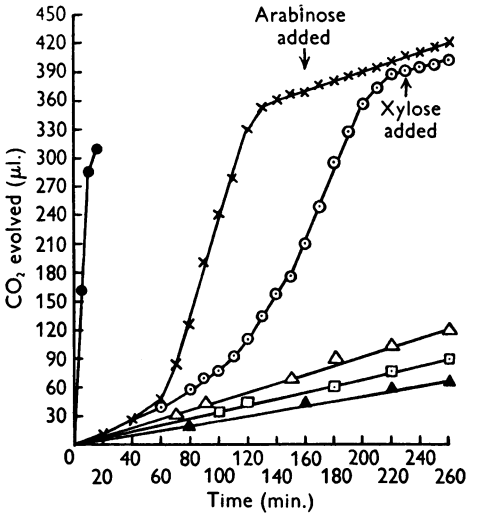

Fig. 3. The utilization of xylose and arabinose, by cells grown on glucose and adapted to glucuronic and galacturonic acids. Vessels with two side arms used. Each vessel contained 300 μg. cell N in 2·25 ml. bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate or 0·5 ml. buffer. Gas 95% N_2–5% CO_2. ●—●, glucose; ×—×, galacturonic acid; ⊙—⊙, glucuronic acid; △—△, xylose; ⊡—⊡, arabinose; ▲—▲, no substrate.

experiments suggested that the uronic acids were not metabolized by a primary step involving decarboxylation to the homologous pentoses. However, since the preparation of the cells involved growth, these results may have been caused by the selection of certain cells during growth. Because of this, it was not possible to state definitely that the system for xylose fermentation was replaced by the system for glucuronate fermentation when cells were grown on glucuronate, or that the failure of cells grown on glucuronate to ferment xylose, showed that xylose was not an intermediate in glucuronate metabolism. It was possible, however, to test both these points under conditions in which growth could not be a major factor in the results. The first point was investigated as follows. It had been found that cells which had been grown on glucose were able to form adaptive systems to ferment both glucuronic and galacturonic acids, but did not ferment xylose, arabinose or galactose (Fig. 3). However, when the pentoses were added to cells which had developed adaptive systems to the corresponding uronic acids, no further increase in gas production was observed (Fig. 3), and from this it was concluded that the fermentation of the uronic acids did not proceed directly through the pentose. In order to investigate the second point, cells grown on xylose were adapted to glucuronate and then allowed to metabolize xylose (Fig. 4). From this experiment it was clear that the formation of a system for the metabolism of glucuronate did not in itself affect the utilization of xylose.

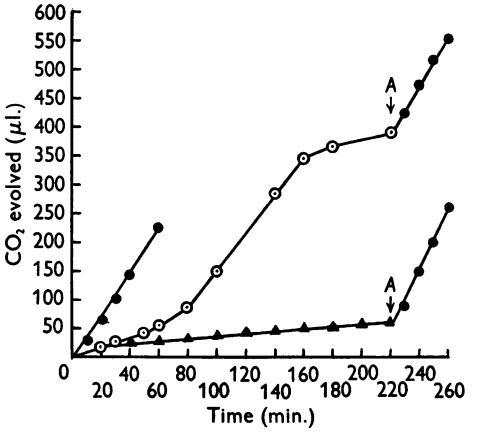

Fig. 4. The utilization of xylose by cells previously grown on xylose and adapted to glucuronate metabolism. Vessels contained 120 μg. cell N in 2·25 ml. bicarbonate saline. Two side arms contained the substrates 0·5 ml. of 0·01 M in each. Gas 95% N_2–5% CO_2. Temperature 40°. Xylose tipped into glucuronate and into no substrate control, at *A*. ●—●, xylose; ⊙—⊙, glucuronate; ▲—▲, no substrate.

The rate of formation, in resting cells, of the adaptive system for the fermentation of glucuronate

In many experiments it was found that there was considerable variation in the period before which cells, grown on xylose, were able to ferment glucuronate. This period was increased by using cells which had been well washed (Fig. 5). The original activity could be restored by adding to the well washed cells some of the first washings (Fig. 6), thereby showing that the decrease in activity shown in Fig. 5 was not owing to the cells suffering damage. Also the effect was not due to the presence of amino-acids since the addition of a mixture of amino-acids (casein hydrolysate + 3% tryptophan) in a quantity equal to the amount of nitrogen determined in the cell washings (8–15 μg./200 μg. cell nitrogen) had no effect on the rate of formation of the system for glucuronate.

The period could be reduced considerably by 'priming' the cells with small quantities of either glucose or xylose (Table 2), but it was also found

that such an effect was obtained only if glucuronate was present during the period of active metabolism of the added 'priming' substrate (Fig. 7). This was interpreted to mean that an energy source was necessary to enable glucuronate to be metabolized. Addition of ATP (100 μg./manometer) produced a

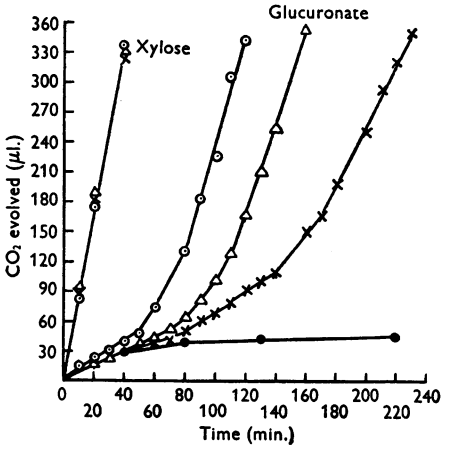

Fig. 5. The effect of extensive washing of cells previously grown on xylose, on the rate of adaptation to glucuronate metabolism. Vessels contained 135 μg. cell N in 2·3 ml. bicarbonate saline. Side arms contained 0·5 ml. 0·01 M substrate. Gas 95% N_2–5% CO_2. ⊙—⊙, not washed; △—△, twice washed; ×—×, washed six times; ●—●, controls, no substrate.

similar shortening of the period (Table 3). This lag period could be abolished almost completely by growing cells overnight, first on glucuronate and then on either xylose or glucose. Such an experiment is shown in Fig. 8, and it is seen that the rate of fermentation of glucuronate ($q_{CO_2}^{N_2} = 400$) was equal to that of xylose ($q_{CO_2}^{N_2} = 404$) and was also similar to the rate for cells grown on glucuronate ($q_{CO_2}^{N_2} = 450$). When these cells were once again cultured on glucuronate the system for xylose was lost, and the metabolic pattern followed that shown in Fig. 2.

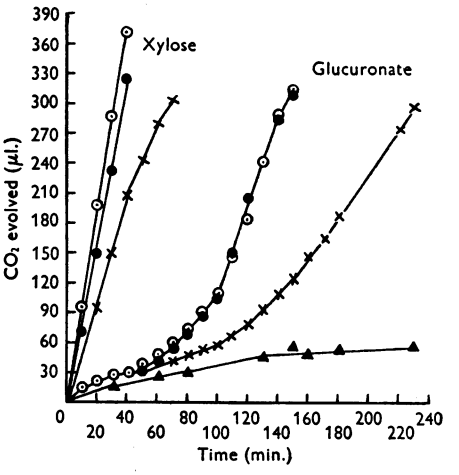

Fig. 6. The effect on the rate of adaptation to glucuronate metabolism of adding the first washings to well washed cells. Each vessel contained 100 μg. cell N in 2·25 ml. of bicarbonate saline. The side arms contained 0·5 ml. 0·01 M substrate. The washings, included in one of the duplicate sets of flasks, were added with the cell suspension. Gas 95% N_2–5% CO_2. ⊙—⊙, unwashed cells; ●—●, cells washed six times, +first washings; ×—×, cells washed six times; ▲—▲, no substrate.

With cells grown on glucuronate, however, the addition of glucose (0·1 ml. 0·01 M solution), or glucuronate (0·1 ml. 0·01 M solution) or ATP

Table 2. *Effect of adding a small quantity of xylose on the production of the adaptive system for glucuronate metabolism in cells grown on xylose*

(Each manometer contained a total volume of 0·6 ml. in the side arm and 200 μg. of cell nitrogen in the flask. The total volume of liquid was 2·85 ml. bicarbonate saline. The values represent μl. of gas evolved.)

	Carbohydrate in side arm of manometer flask			
Time (min.)	D-Xylose (0·5 ml. 0·01 M)	D-Xylose (0·1 ml. 0·01 M) +sodium D-glucuronate (0·5 ml. 0·01 M)	Sodium D-glucuronate (0·5 ml. 0·01 M)	D-Xylose (0·1 ml. 0·01 M)
0	0	0	0	0
10	140	75	16	65
20	274	101	17	71
30	351	113	22	69
40	356	127	25	68
50	—	148	30	69
60	—	180	38	71
70	—	225	51	72
80	—	291	60	74
90	—	352	73	76
100	—	396	85	78

(100 μg.), did not promote the fermentation of xylose. In each instance, after the added carbohydrate had been fermented, the rate of gas production from xylose resembled that shown in Fig. 2.

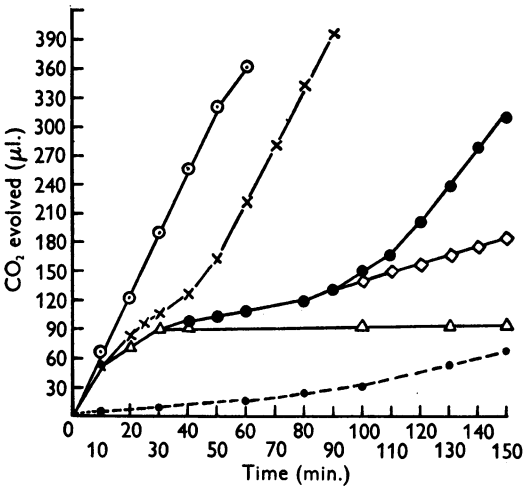

Fig. 7. The effect of the metabolism of xylose on the rate of formation of the adaptive system for glucuronic acid fermentation in cells grown on xylose. Vessels with two side arms used. Vessels contained 115 μg. cell N in 2·3 ml. bicarbonate saline in the centre compartment. Side arms contained 0·1 ml. 0·01 M-xylose or 0·1 ml. buffer; and 0·5 ml. 0·01 M-xylose, or 0·5 ml. 0·01 M-glucuronate, as required. Gas 95% N_2–5% CO_2. ⊙—⊙, xylose (0·5 ml. 0·01 M); ×—×, xylose (0·1 ml. 0·01 M), and sodium glucuronate (0·5 ml. 0·01 M) added at start; ●—●, xylose (0·1 ml. 0·01 M) added at start, glucuronate (0·5 ml. 0·01 M) added after 20 min.; ◇—◇, xylose (0·1 ml. 0·01 M) added at start, glucuronate (0·5 ml. 0·01 M) added after 30 min.; △—△, xylose (0·1 ml. 0·01 M); •- - -•, glucuronate (0·5 ml. 0·01 M) added at start, no xylose present.

Experiments with other cultures

Cells of *Esch. coli* type 86, previously grown on xylose, developed an adaptive system for the fermentation of glucuronate under the conditions

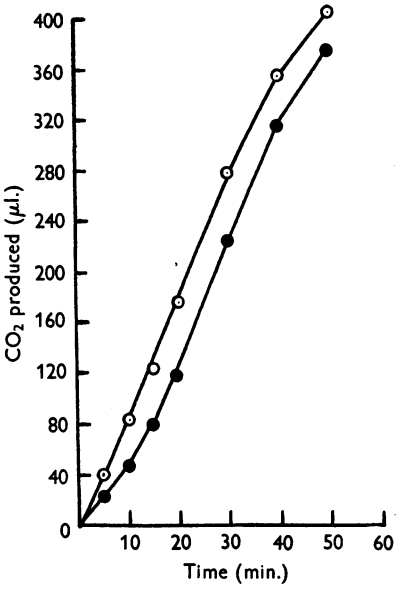

Fig. 8. The utilization of glucuronic acid and xylose by cells grown overnight firstly on glucuronic acid and then on xylose. Flasks contained 150 μg. cell N in 2·25 ml. of bicarbonate saline. Side arms contained 0·5 ml. of 0·01 M substrate. Gas 95% N_2–5% CO_2. Temperature 40°. ⊙—⊙, xylose; ●—●, glucuronate. $q^{N_2}_{CO_2}$ (xylose) = 404. $q^{N_2}_{CO_2}$ (glucuronic acid) = 400.

described above. The rate of formation of this system was increased by the presence of xylose, as in Fig. 7. When grown on glucuronate, the cells were unable to ferment xylose. When cultured again on

Table 3. *Effect of ATP on the utilization of glucuronate by washed suspensions of cells grown on xylose*

(Each manometer flask contained the substrate in the side arm and in the main compartment 120 μg. cell nitrogen in 2·3 ml. bicarbonate saline. Values in μl. gas evolved.)

	Contents of side arm			
Time (min.)	D-Xylose (0·5 ml. 0·01 M) +0·1 ml. buffer	D-Glucuronate (0·5 ml. 0·01 M) +0·1 ml. buffer	D-Glucuronate (0·5 ml. 0·01 M) +100 μg. ATP in 0·1 ml.	0·5 ml. Buffer +100 μg. ATP in 0·1 ml.
0	0	0	0	0
5	31	12	15	6
10	72	12	19	16
15	105	20	23	22
20	138	20	28	19
30	224	18	34	28
40	281	28	55	39
50	308	33	94	40
60	343	45	167	45
70	345	56	262	53
80	—	74	392	56
90	—	90	485	60

xylose, these cells did not ferment glucuronate immediately, but only after a lag period, as in Fig. 1. *Aerobacter aerogenes* 8197, when grown on xylose, did not ferment glucuronate and the addition of a trace of xylose, as a 'primer', was without effect. The culture would, however, grow in a medium containing glucuronate as the sole carbon source.

DISCUSSION

It was mentioned in the introduction that one hypothesis for the mechanism of the formation of pentoses involved an initial decarboxylation of the homomorphous uronic acid. The results presented above show that, as far as the coliform organisms tested are concerned, there is not such a simple relation between the metabolism of uronic acids and of the homologous pentoses. While such studies with intact cells involving simultaneous adaptation are necessarily subject to the limitations discussed fully by Stanier (1950), the experiments shown in Figs. 2 and 3 demonstrate that although the sugar acids can be metabolized the initial step does not involve a primary decarboxylation to the pentose. The lag in fermentation of the uronic acids by cells grown either on glucose or xylose is considered to be due to the formation of an adaptive system. This term is used in the sense discussed by Reiner & Spiegelman (1947), Spiegelman (1950) and Stanier (1951) and is taken to mean that certain essential units of the enzyme system(s) involved must be already present in the cells, and must increase during the metabolism of the substrates, in this instance the uronic acids.

This system, which is considered, at least in the initial stage, to be the same for both glucuronic and galacturonic acids, was formed in washed, resting cells, in the absence of fixed exogenous nitrogen, under anaerobic conditions, and was developed very rapidly. This appears to be the first instance of the demonstration of such a carbohydrase system in resting bacteria.

The fact that cells grown either on glucose or xylose were able to ferment glucuronate, implies that a small quantity of the necessary enzyme system is always present in the cells, and suggests that the metabolism of uronic acids may be of fundamental importance to these organisms. Xylose was fermented only to a small extent, if at all, by cells grown on glucuronate or glucose and since this low rate was not increased by supplying sources of energy in the form of glucuronate, glucose and ATP, it is considered that energy alone is not sufficient for the development of the xylose-fermenting system, which increases only during growth. The results in Fig. 4 show that the development of an adaptive system for the metabolism of glucuronic acid in resting cells grown on xylose does not involve a loss or decrease of the system for the metabolism of xylose. This does not entirely agree with the results of Spiegelman & Dunn (1947) who found that the formation of galactozymase in a strain of *Saccharomyces cerevisiae*, previously grown on glucose, caused a fall in glucozymase activity. However, Wainwright & Pollock (1949) found that the adaptive production of tetrathionase in resting cells of *Esch. coli* did not cause a reduction in the activity of the nitratase system already present.

The marked acceleration of formation of the system for glucuronate metabolism, brought about by xylose, glucose and ATP is similar to that reported by Spiegelman, Reiner & Cohnberg (1947) who showed that, in yeast grown on glucose, formation of galactozymase and maltozymase was facilitated by addition of small amounts of glucose. These results imply that only a source of energy was necessary, but since the presence of some factor which could be washed from the cells also enhanced the rate of adaptation, it does not appear that an energy source alone is sufficient. This conclusion remains uncertain, however, in the absence of further knowledge of the factor.

It is of interest to note that in Fig. 6 the rate of utilization of xylose by well washed cells is less than the rate for unwashed cells, or for supplemented washed cells. This result was not always observed (cf. Fig. 5) in this type of experiment, but it had been previously found that the rate of utilization of xylose by cells grown on xylose might vary considerably from the usual $q_{CO_2}^{N_2}$ (xylose) of 400–450. In one instance a value of $q_{CO_2}^{N_2}$ (xylose) of 250 was obtained. Perhaps the factor removed by washing is also involved in the metabolism of xylose.

So far, comparisons have been made with only two stock strains of coliform organisms and of these only *Esch. coli* N.C.T.C. no. 86 behaved in a similar manner to the strains investigated above. *Aerobacter aerogenes* N.C.T.C. 819 did not ferment glucuronic acid after growth on xylose. These results are too few to permit of any definite conclusions, but it would appear that all coliform organisms do not behave in the same way in metabolizing glucuronic acid.

SUMMARY

1. Washed suspensions of strains of *Escherichia coli* previously grown on xylose are able to form adaptive systems for the fermentation of glucuronic and galacturonic acids and L-arabinose. Glucose was fermented immediately, but galactose and α- and β-glucuronic acid-1-phosphates were not fermented.

2. Cells grown on glucose could ferment glucose, and formed adaptive systems for glucuronate and galacturonate, but did not ferment galactose,

xylose, arabinose or α-glucuronic acid-1-phosphate.

3. Cells grown on glucuronate could ferment glucose, glucuronate and galacturonate but not galactose, xylose, arabinose or α- and β-glucuronic acid-1-phosphate.

4. In the case of cells grown on xylose the adaptation period was greatly shortened by adding glucose, xylose or adenosinetriphosphate, and also by the presence of a 'factor' which could be washed from the cells.

5. Cells grown on glucuronate could not be made to ferment xylose by the addition of glucuronate, glucose or adenosinetriphosphate.

6. The possible significance of these results is discussed.

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Tracer Studies with the B_{12} Vitamins

1. NEUTRON IRRADIATION OF VITAMIN B_{12}

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The presence of cobalt and phosphorus in the vitamin B_{12} molecule invites attempts to label the vitamin with radioactive isotopes to facilitate metabolism studies. Soon after our isolation of crystalline vitamin B_{12} we tried direct chemical exchange with radioactive cobalt compounds and radioactive phosphate (Fantes, Page, Parker & Lester Smith, 1949). Not surprisingly, in the light of later knowledge about the constitution of vitamin B_{12}, no significant radioactivity was introduced into the molecule during these experiments. These observations have recently been confirmed and extended by Baldwin, Lowry & Harrington (1951), and also by Boos, Rosenblum & Woodbury (1951), who found no evidence of exchange with ^{60}Co after 1 or 3 months contact.

About the same time we considered direct irradiation of vitamin B_{12} in the atomic pile, bearing in mind the observation of Ball, Solomon & Cooper (1949) that the Szilard-Chalmers reaction does not go to completion, and their claim to have obtained ^{35}S-labelled cystine in small yield by direct neutron bombardment of cystine. We were advised that the experiment was not worth attempting with valuable material, but later Dr A. G. Maddock suggested that irradiation with thermal neutrons might be expected to induce some radioactivity in the vitamin B_{12} molecule without causing excessive decomposition of the material if it were properly prepared.

RESULTS

Neutron irradiation: first experiment

Anhydrous crystalline vitamin B_{12}, sealed *in vacuo* in a silica tube, was exposed to a low neutron flux in British Experimental Pile (BEPO) for 4 weeks. After irradiation the material was unchanged in appearance and measurement of the colour of its