WRIGHT, D. E. (1960). *J. gen. Microbiol.* **22**, 713-725

The Metabolism of Carbon Dioxide by *Streptococcus bovis*

By D. E. WRIGHT

*Plant Chemistry Division, Department of Scientific and Industrial Research,
Palmerston North, New Zealand*

SUMMARY: Carbon dioxide stimulates the growth of *Streptococcus bovis* and the production of the extracellular enzyme dextransucrase. The growth of cultures in the presence of $^{14}\text{CO}_2$ resulted in the incorporation of carbon-14 into aspartic acid, with lesser amounts in threonine, glutamic acid, adenine, guanine, uridylic acid and cytidylic acid. Organisms grown with labelled aspartic acid did not utilize it to any extent; the principal source of this amino acid was from CO_2 -fixation reactions. The stimulation of growth by CO_2 is considered to be due to biosynthetic reactions involving CO_2 which lead to a sufficient supply of the intermediates required in nucleic acid and protein synthesis.

Several studies have been made of the growth of *Streptococcus bovis* and its ability to form dextran when grown in the presence of sucrose. Prescott & Stutt (1955) found that the growth of this organism with arginine as the major nitrogen source was stimulated by carbon dioxide. Since casein hydrolysate was able to replace carbon dioxide, they concluded that carbon dioxide was involved in the formation of amino acids. Another interesting effect of carbon dioxide in the metabolism of *S. bovis* is the stimulation of dextran formation by carbon dioxide. This effect has been studied by several workers but no satisfactory explanation for the role of CO_2 has been given. Dain, Neal & Seeley (1956) showed that most strains of *S. bovis* formed large mucoid colonies containing a dextran-like polysaccharide when grown with sucrose as the carbon source and in the presence of carbon dioxide. Bailey & Oxford (1958) examined the production of dextran from sucrose by several strains of *S. bovis* isolated from sheep and cattle rumen. While strain differences with regard to growth factors were found (Oxford, 1958), a requirement for carbon dioxide for both growth and appreciable dextran formation by most strains was noted. Another interesting observation by Bailey & Oxford (1958) was the confirmation of the results of Dain *et al.* (1956) that the presence of Tween 80 in the growth medium promoted some dextran synthesis in the absence of carbon dioxide and under anaerobic conditions. The role of carbon dioxide in stimulating growth has not been determined, but several suggestions have been made about the dextran-promoting properties of the gas. Bailey & Oxford (1958) suggested that carbon dioxide may either exert a physical effect or influence the metabolism of sucrose in such a way as to promote dextran formation. Bailey (1959) suggested that carbon dioxide might be involved in the production of dextransucrase or in its secretion by the bacteria into the culture medium. The present paper reports the results of a study of the role of carbon dioxide in the growth of *S. bovis*.

METHODS

Organism. The rumen strain *Streptococcus bovis* strain 1 (Bailey & Oxford, 1958), was used since it has an absolute requirement for carbon dioxide for growth and dextran formation.

Media. The following media were used for growing *Streptococcus bovis*:

Medium A. Sucrose 5 g.; sodium acetate 3 g.; Casamino Acids (Difco certified) 1.1 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.5 g.; sodium thioglycollate 0.03 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 g.; cystine 0.015 g.; tryptophan 0.015 g.; Bacto-yeast extract 0.5 g.; distilled water to 100 ml.; pH 7.0.

Medium B. The composition of medium B was the same as medium A except that the sodium acetate was 0.5 g. and the yeast extract was replaced by a mixture containing: biotin, 0.001 g.; calcium pantothenate, 0.005 g.; adenine sulphate, 0.005 g.; guanine, 0.002 g.; uracil, 0.002 g.

Medium C. Tryptone (Difco) 1.0 g.; Bacto-yeast extract 0.2 g.; sucrose 2 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 1.2 g.; KH_2PO_4 0.5 g.; agar 1.1 g.; water to 100 ml.; pH 7.2.

The media were sterilized by autoclaving at 121° for 20 min. All salts and organic compounds were of Analar quality when possible. When medium was required to be free from CO_2 , the procedure described by Bailey & Oxford (1958) was followed. Flasks were inoculated with a standard inoculum from an 18 hr. culture grown in air at 39° in glucose nutrient broth.

Growth of Streptococcus bovis in the presence of $^{14}\text{CO}_2$

The apparatus shown in Fig. 1 was used for introducing $^{14}\text{CO}_2$ into the growth flask *A*. After sterilizing separately the medium contained in *A*, and the tap *B*, the medium was inoculated, the apparatus assembled (using sterile grease) and then evacuated on a water pump. Tube *C*, containing a solution of $\text{NaH}^{14}\text{CO}_3$, was attached to *B* by pressure tubing and, with tap *D* closed, tap *B* was opened and 2N- H_2SO_4 slowly introduced from the dropping funnel *E*, releasing $^{14}\text{CO}_2$ from the labelled bicarbonate solution. When all the $^{14}\text{CO}_2$ had been released, a known volume of N_2 was flushed through *D* to carry over the labelled CO_2 into *A*. The growth flask was closed off at *B* and incubated at 39° for 1–2 days. After incubation, any residual $^{14}\text{CO}_2$ in the flask was removed by flushing CO_2 -free air through the flask; carbon dioxide was collected in a bubbler containing 3N-NaOH. At the end of each experiment the medium was decanted from the flask and centrifuged at 13,000 g for 30 min. to remove organisms. After measuring the pH value, the cell-free fluid was divided into three portions for analysis of organic acids, amino acids and dextran.

Analysis of organisms. The organisms were washed three times with water and freeze-dried. In the experiments with labelled carbon dioxide or labelled aspartic acid, the organisms were extracted successively with: 5% (w/v) trichloroacetic acid at 4°; ethanol (40–50°); ethanol + ether (40–50°); 5% trichloroacetic acid at 100° (Roberts, Abelson, Cowie, Bolton & Britten, 1957). The bacterial protein was hydrolysed with 6N-HCl at 110° in sealed tubes for 16 hr. After removing HCl by repeated evaporation *in vacuo*, the amino acids

were dissolved in water for paper chromatography or ion-exchange separation on Dowex 2 (Roberts *et al.* 1957).

Aspartic acid and glutamic acid were isolated from the Dowex 2 and separated by paper chromatography. After eluting the amino acids, the concentration of each was determined (Cocking & Yemm, 1954) and unlabelled carrier L-aspartic acid or L-glutamic acid added. The carbon-14 distribution within aspartic and glutamic acids was estimated by total combustion and reaction with ninhydrin. In general, the procedures and the apparatus were those described by Sakami (1955).

Fig. 1. Apparatus used for growing *Streptococcus bovis* in liquid media in the presence of ¹⁴CO₂. A, growth flask; B, tap; C, tube containing NaH¹⁴CO₃; D, 2-way tap; E, funnel containing 2N-H₂SO₄.

The nucleic acid fraction extracted with hot trichloroacetic acid was shaken with ether several times to remove the trichloroacetic acid and then hydrolysed in 0.5 N-HCl at 100° for 30 min. The hydrolysis products were separated on Whatman no. 1 paper with solvent mixture *iso*-propanol/conc. HCl/water (170/44/86) and the spots located by photographing under ultraviolet radiation.

Organic acids in the medium. The organic acids were isolated by acidifying a sample of the medium to pH 2–3 with 2N-H₂SO₄, adding to celite and eluting with 700 ml. ether (Colowick & Kaplan, 1957). The acids were neutralized with N/70 carbonate-free NaOH, with phenol red as indicator, and concentrated on a rotary evaporator to 1–2 ml. The aqueous solution of neutralized acids was acidified again and separated on celite (Swim & Krampitz, 1954).

Amino acids in the medium. Aspartic acid was isolated from the medium by loading a sample of the fluid on Dowex 50 and after washing the column,

eluting with 1.5 N-HCl (Roberts *et al.* 1957). The eluent was dried, loaded on Dowex 2 and eluted with increasingly acid solvents (Roberts *et al.* 1957). The fraction containing aspartic acid was then streaked on Whatman no. 3 paper and aspartic acid separated from glutamic acid by using solvent mixture phenol/ammonia sp.gr. (0.88)/water (80 g./0.3 ml./20 ml.). The position of the amino acids was located by radioautography and spraying with ninhydrin. Aspartic acid was eluted by water for analysis.

Dextran and dextransucrase in the medium. The dextran-dextransucrase complex was precipitated from the medium by adding two volumes of ethanol or ammonium sulphate solution (60 g./100 ml.) The precipitate was collected by centrifuging (10,000 g) for 20 min., redissolved in water or 0.1 M-acetate buffer (pH 5.8), reprecipitated and freeze-dried. The concentration of dextran was estimated either by comparing the opacity of dextran solutions in a nephelometer or by weighing the freeze-dried dextran. In some growth experiments the dextran was estimated by centrifuging off the bacteria and reading the optical density of the supernatant fluid in a nephelometer.

Dextransucrase activity was measured by the procedure described by Bailey (1959).

Bacterial growth was estimated by turbidity measurements or micro-Kjeldahl N analysis after centrifuging and washing the bacterial pellet three times with distilled water.

Radioactivity in the samples and chromatograms was counted with a thin end-window Geiger-Müller tube. The samples were counted in duplicate either evaporated to thin films in glass planchettes, or plated on filter-paper disks as $\text{Ba}^{14}\text{CO}_3$ in which case the counts were corrected for self-absorption to infinite thinness. The evaporated samples all contained less than 1 mg./cm². All counts were corrected for background radioactivity.

pH measurements were made with a glass electrode.

$\text{NaH}^{14}\text{CO}_3$ and L-(¹⁴C)-aspartic acid were obtained from the Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

The stimulation of growth of Streptococcus bovis and of dextran formation by carbon dioxide

Several experiments confirmed the results of Bailey & Oxford (1958). Growth and dextran production by *S. bovis* was greater under CO_2 than N_2 . The addition of Tween 80 (1 %, w/v) to the medium caused a slight initial inhibitory effect on growth but after incubation for 24 hr. the amounts of organism and dextran present in the tubes containing Tween 80 incubated under either CO_2 or N_2 were only slightly less than those in the tubes containing CO_2 but no Tween 80.

The growth of cultures in the presence of different amounts of bicarbonate and the analysis of the dextran-dextransucrase complex precipitated by ethanol from the culture fluid, showed that higher bicarbonate concentrations gave not only higher yields of polysaccharide (as found by Bailey & Oxford,

1958) but also increased the nitrogenous material 20-fold, suggesting that the HCO₃ content of the initial culture medium influenced not only the amount of cell protein but also the amount of extracellular protein.

The relation between growth of cells, pH of the medium and the excretion of dextransucrase

The previous results suggested that carbon dioxide is required by the organism at least in part for protein synthesis. A study was made of the relation between growth and the release of dextransucrase into the medium in order to determine the stage of growth during which the extracellular enzyme accumulated. A series of tubes containing 10 ml. medium A were inoculated and incubated under CO₂ at 39°; cultures were removed for analysis after different incubation times. After a lag phase of about 4 hr. (Fig. 2) growth was linear for the next 3.5 hr. during which time the pH value dropped from 6.2 to 5.3. The cell mass measured turbidimetrically was parallel to the increase in cell protein calculated from the nitrogen content of the organisms. Dextransucrase was present at pH 6.0 and reached a maximum concentration at pH 5.5 after which no increase was observed, although the organisms were still multiplying. This rise in dextransucrase concentration was accompanied by rapid dextran synthesis which slowed at pH 5.5 and gave little increase after pH 5.3. After incubation for 24 hr. the pH value was 4.8, the final 16.5 hr. of incubation showing a considerable increase in growth but little or no increase in dextran or dextransucrase concentration in the culture fluid.

Fig. 2. The relation between growth, pH, and dextransucrase excretion. ■—■, pH value; ○—○, dry-weight organism; ●—●, cell protein; □—□, dextransucrase activity; ▲—▲, dextran

The fixation of ¹⁴CO₂ by Streptococcus bovis during growth

The metabolism of CO₂ by *Streptococcus bovis* was studied by growing cultures in the presence of labelled CO₂. Two experiments were carried out, the

first with 0.45 mmole of $^{14}\text{CO}_2$ with an activity of 50 $\mu\text{c.}$, the second with 0.45 mmole of $^{14}\text{CO}_2$ with an activity of 240 $\mu\text{c.}$ In the first experiment organisms were grown on medium B for 24 hr.; in Expt. 2 on medium A for 40 hr. The higher concentration of isotope in the second experiment was necessary to obtain aspartic and glutamic acids of sufficiently high activity to determine the location of labelling by degradation. The results of these experiments are given in Tables 1-3.

Table 1. *The growth of Streptococcus bovis in $^{14}\text{CO}_2$*

Cultures were grown in 200 ml. medium at 39°. Expt. 1 was incubated for 24 hr. with medium B and 0.45 mmole of $^{14}\text{CO}_2$ (50 $\mu\text{c.}$); Expt. 2 for 40 hr. with medium A and 0.45 mmole of $^{14}\text{CO}_2$ (240 $\mu\text{c.}$).

		Experiment	
		1	2
Yield of bacteria (mg.)	...	60	200
		Counts/min./ mmole C	Counts/min./ mmole C
Dextran-dextranucrase	180 mg.	520	1800 mg. 206
Lactic acid	738 mg.	228	—
Acetic acid	710 mg.	40	—
Formic acid	11 mg.	60	—
		Counts/min. (% of counts in bacteria)	
Bacterial fractions			
Ethanol-ether soluble		600 (2.0)	1,060 (1.7)
Cold TCA soluble		1,380 (4.5)	2,600 (4.1)
Hot TCA soluble		7,920 (25.9)	16,180 (25.6)
Protein		20,700 (67.6)	43,200 (68.6)

Table 2. *Distribution of radioactivity in the nucleic acid and protein isolated from organisms in Expts. 1 and 2*

The results have been corrected for background counts.

	Experiment	
	1	2
	Counts/min.	
Nucleic acid hydrolysate		
Adenine	821	725
Guanine	683	720
Uridylic acid	617	75
Cytidylic acid	459	80
Percentage of total isotope in the protein		
Threonine	5	5
Glutamic acid	7	15
Aspartic acid	88	80

The yield of bacteria was far greater in Expt. 2 in which the medium contained yeast extract. The addition of yeast extract was observed invariably to give better growth and dextran synthesis than the mixture of growth factors. The distribution of carbon-14 amongst the bacterial fractions was similar in the two experiments. The protein fractions contained more than two-thirds of the radioactivity incorporated by the organisms. Hydrolysis

Table 8. *Distribution of carbon-14 in aspartic and glutamic acids isolated from the bacterial protein in Expt. 2*

	Specific activity (counts/min./ mmole C)
Aspartic acid	
α -COOH + β -COOH	727,500
Remainder of molecule	10,000
Glutamic acid	
α -COOH	480,000
Remainder of molecule	6,000

of the protein and two-dimensional paper chromatography of the dried hydrolysate followed by radioautography showed that all the isotope was located in the amino acids, threonine, glutamic acid and aspartic acid. Measurement of the radioactivity in the spots on the chromatograms showed that aspartic acid had by far the greatest activity, followed by glutamic acid and threonine (Table 2). Since the fixation of CO₂ by the Wood-Werkman reaction involves the formation of oxaloacetic acid labelled in the β -COOH leading to the formation of β -COOH-labelled aspartic acid and α -COOH labelled glutamic acid by the reactions of the citric acid cycle, samples of these two amino acids were isolated from the cell protein for degradation. Table 8 shows that virtually all the carbon-14 in the aspartic acid was contained in the carboxyl carbons, the combined specific activities being 727,500. The glutamic acid contained about 95 % of its radioactivity in the α -COOH.

Hydrolysis of the nucleic acid fractions extracted with hot trichloroacetic acid followed by paper chromatography and radioautography revealed four radioactive ultraviolet-absorbing spots which were identified as adenine, guanine, uridylic acid and cytidylic acid. Different results were obtained in the two experiments (Table 2). These four substances were all heavily labelled in Expt. 1 where adenine and guanine contained more carbon-14 than the pyrimidines. However, judging from the size and intensity of the ultraviolet absorbing spots on the chromatograms, both uridylic and cytidylic acids were present in much lower concentrations than the purines, indicating a higher specific activity in the pyrimidines. In Expt. 2, adenine and guanine were both highly labelled but the uridylic and cytidylic acids contained little carbon-14.

Ten times more dextran was found in Expt. 2 than in Expt. 1, the latter sample having the higher specificity activity. However, since the nitrogen content of the dextran-dextranase complex in Expt. 2, was very low (0.19 %) in comparison with that from the first experiment (2.12 %), it might be expected to have a lower activity since the protein carbon in this sample will be greatly diluted with polysaccharide carbon which is probably unlabelled.

The growth of Streptococcus bovis in the presence of labelled aspartic acid

Since the previous experiments had shown that the requirement for CO₂ seemed to be related to the formation of aspartic acid, the metabolism of this

amino acid was studied by growing a culture of *Streptococcus bovis* in 50 ml. medium A to which was added 3.2 μ -mole of L-(^{14}C)-aspartic acid containing 14 μc . of activity.

After incubation for 18 hr. under CO_2 the organisms were centrifuged from the medium. The yield of freeze-dried organism and dextran-dextranase were 64 and 660 mg., respectively; these were good yields considering the relatively short incubation period. Extraction of the organisms and estimation of the radioactivity in the fractions revealed a pattern quite different from that obtained in the $^{14}\text{CO}_2$ experiments. The ethanol+ether soluble fraction was the most heavily labelled fraction. Further fractionation of this sample into ethanol-soluble ether-soluble, and ethanol-soluble ether-insoluble material (Roberts *et al.* 1957) showed that the ether-soluble fraction contained most of the carbon-14. The protein contained about 25 % of the total radioactivity, threonine being the most heavily labelled amino acid; the aspartic acid had a very low activity and a negligible amount of carbon-14 was located in the glutamic acid. It was not possible to estimate the isotope content of the purines and pyrimidines because the counts/min. in these compounds were only just above the background count.

Samples of aspartic acid were obtained from the cell protein and from the culture medium and a comparison made between their specific activities (Table 4). The low specific activity of the cell-protein aspartic acid showed that little of this amino acid was derived from exogenous aspartic acid.

Table 4. *The metabolism of L-(^{14}C)-aspartic acid by Streptococcus bovis during growth*

Organisms were grown in 50 ml. medium A to which was added 14 μc . of L-(^{14}C)-aspartic acid (3.2 μ -mole). The flask was incubated under CO_2 at 39° for 18 hr. Fraction 1 b was estimated by difference.

Yield of organisms (mg.) ...	64	
Dextran (mg.) ...	660	
	Radioactivity of fractions	
	Counts/min.	% of total counts
Bacterial fractions		
1. Ethanol-ether soluble	3460	58.8
1 a. Ethanol soluble-ether soluble	3250	55.0
1 b. Ethanol soluble-ether insoluble	210	3.8
2. Cold TCA	197	3.3
3. Hot TCA	722	12.2
4. Protein	1200	25.7
	% of counts in protein	
Threonine	52	
Glutamic acid	6	
Aspartic acid	21	
	Specific activity	
	(counts/min./ μmole aspartic acid)	
Cell protein aspartic acid	12	
Culture fluid aspartic acid	1130	

The effect of Tween 80 on amino acid uptake by cultures of Streptococcus bovis

The synthesis of aspartic acid by CO₂-fixation reactions gives no clues to the ability of Tween 80 to replace CO₂ unless the detergent allows greater uptake of exogenous aspartic acid by the organisms. This possibility was examined by growing cultures in two flasks, each containing 20 ml. medium A to which was added 5 μ c. of labelled L-aspartic acid. In addition, the medium in flask 2 contained 1 % (w/v) Tween 80. The flasks were incubated for 2 days at 39°, culture 1 under CO₂ and culture 2 under N₂. The results are given in Table 5. The growth was similar in both flasks but considerably more labelled aspartic acid was taken up by the organisms grown in the presence of Tween 80. This increased uptake was reflected not only by the higher isotope content of the whole organisms but also by the cell-protein radioactivity.

Table 5. *The effect of Tween 80 on the uptake of L-(¹⁴C)-aspartic acid by cultures of Streptococcus bovis*

Cultures were grown in flasks each containing 20 ml. medium A to which was added 5 μ c. of L-(¹⁴C)-aspartic acid. Flask 1 was incubated under CO₂; flask 2 contained also 1 % (w/v) of Tween 80 and was incubated under N₂. The flasks were incubated at 39° for 2 days.

	Flask	
	1	2
Organism yield (mg.)...	14.7	18.0
	Counts/min.	
Organism:	1260	4175
Cell protein:	624	1400

The uptake of labelled aspartic acid by suspensions of Streptococcus bovis

Washed suspensions of *Streptococcus bovis* were used to investigate the uptake of labelled L-aspartic acid under different conditions. After incubating the organisms as described in Table 6 for 1 hr., they were centrifuged off, washed three times with water and samples dried in glass cups for counting. The counts for cell protein were found by extracting the organisms three times with 5 % (w/v) trichloroacetic acid at 100°, once with ethanol+ether at 50° and then plating a sample of the residual protein. The results show that the assimilation of aspartic acid by *S. bovis* required the presence of an energy source; only a slight uptake of carbon-14 was observed in the absence of sucrose. The presence of Tween 80 stimulated the uptake of labelled aspartic acid in both experiments by about 20 %, in presence or in absence of other amino acids. However, this increase in the total uptake in the organisms incubated in the presence of Tween 80 did not lead to an increase in the cell protein carbon-14. In fact, the radioactivity in the protein was slightly depressed by Tween 80. The addition of glutamic acid + lysine or a complete amino acid mixture (without aspartic acid) greatly decreased the assimilation of labelled aspartic acid; the complete mixture had the greater effect.

Table 6. *The uptake of labelled aspartic acid by washed suspensions of Streptococcus bovis*

Organisms grown on medium C were washed and suspended in a salt solution (Gale, 1947) in presence of compounds listed below. Each tube contained 0.2 μ c. of L-(14 C)-aspartic acid with a specific activity of 1 μ c./5 μ -mole aspartic acid. The salt solution had a final volume of 6.0 ml. and contained additions at the following concentrations: (a) sucrose, 100 mg., (b) Tween 80, 60 mg., (c) glutamic acid, 0.25 mg. + lysine, 0.25 mg., (d) a complete amino acid mixture without aspartic acid; 0.25 mg. of each acid. All tubes contained 0.1 mg. each of: guanine, uracil, adenine, xanthine.

Sucrose	Tween 80	Glutamic acid + lysine	Radioactivity in organisms (counts/min.)	
+	—	—	1913	
—	—	—	140	
+	+	—	2443	
+	—	+	1279	
		Amino acid mixture	Radioactivity in cells (counts/min.)	Radioactivity in cell protein (counts/min.)
+	—	—	3426	343
+	+	—	4235	320
+	—	+	1112	137
+	+	+	1305	113
—	—	—	150	12

DISCUSSION

There can be little doubt that while *Streptococcus bovis*, strain 1, is able to grow in a medium containing sucrose, phosphate, acetate, yeast extract and a casein hydrolysate, in the absence of added carbon dioxide or bicarbonate, its growth under these conditions is limited. This is in contrast with the strains studied by Prescott & Stutts (1955) which utilized casein hydrolysate in place of carbon dioxide. However, this is not surprising since considerable differences have been noted in the nutritional requirements of different strains of *S. bovis* (Oxford, 1958; Ford, Perry & Briggs, 1958). *S. bovis*, strain 1, seems to be a rather exacting organism, and it has not been possible to grow it in a completely defined medium of salts, sugar, vitamins and amino acids. Either yeast extract or Casamino Acids (certified) + pantothenate + biotin need to be added to the medium for growth to occur.

The marked incorporation of carbon-14 derived from $^{14}\text{CO}_2$ into aspartic acid suggests that in spite of an adequate amount of aspartic acid being present in the medium, the limiting factor in protein synthesis is the intracellular supply of this amino acid. Growing the organism in the presence of labelled aspartic acid has confirmed this view. When CO_2 is present in the growth flask, nearly all the cell aspartic acid appears to be synthesized by the fixation of CO_2 . The distribution of carbon-14 in aspartic acid indicates that the most probable route of fixation is into a 3-carbon compound forming oxaloacetic acid labelled in the β -COOH, followed by amination to aspartic acid. The formation of aspartic acid by CO_2 -fixation reactions possibly explains the

effect of biotin in stimulating dextran production which Oxford (1958) demonstrated. Biotin is known to participate in CO₂ fixation.

The presence of radioactivity in threonine is not surprising in view of the fact that aspartic acid is commonly regarded as being a metabolic precursor of threonine (Roberts *et al.* 1957). The presence of radioactivity from ¹⁴CO₂ in the α-COOH of glutamic acid can best be explained by assuming that the reactions of the citric acid cycle which lead to the synthesis of glutamic acid occur to some extent in this organism.

The effect of Tween 80 in partly replacing CO₂ is apparently due to the ability of *Streptococcus bovis* to take up more exogenous aspartic acid in the presence of the detergent. The site of action of Tween 80 is unknown; presumably it affects the permeability barrier of the cell membrane so as to enable a greater uptake of amino acid. In this connexion it is interesting to recall the high isotope content of the ethanol-soluble ether-soluble bacterial fraction in the labelled aspartic acid experiment. Since this fraction consists mainly of lipid it is surprising that its radioactivity is so high. It seems unlikely that the labelled aspartic acid is metabolized to acetate, which is presumably the precursor of the lipid, so it must be assumed that the isotope in this fraction is indeed aspartic acid bound to lipid. This suggests that the lipid exerts an effect on amino acid uptake and perhaps even protein synthesis. Several workers (Reith & Thomas, 1958; Westley, Wren & Mitchell, 1957) have shown that amino acids can be bound to lipid material, and Hendler (1958) suggested that such complexes may be factors in protein synthesis.

Two possibilities suggest themselves for the inability of *Streptococcus bovis* to utilize exogenous aspartic acid at a rate sufficient to permit maximum protein synthesis. First, since the uptake of aspartic acid is enzymic, either insufficient 'permease' is present in the cells or competition may exist between aspartic acid and, for example, glutamic acid for the same enzyme system. Gale & Folkes (1953) found that the rate of incorporation of (¹⁴C)-glutamic acid by *Staphylococcus aureus* was markedly decreased by the presence of aspartic acid, a result similar to that found with *S. bovis*. The second possibility is that inhibition of the utilization of exogenously derived aspartic acid may occur in the presence of other amino acids. Ravel, Felsing & Shive (1955) showed that glutamic acid inhibited the utilization of aspartic acid in reactions concerned with pyrimidine synthesis and also threonine formation in *Leuconostoc dextranicum*, and inhibited lysine, threonine and pyrimidine synthesis in *Lactobacillus arabinosus*. However, when bicarbonate or oxaloacetate was present, glutamic acid had no effect, suggesting that biosynthesized aspartic acid could be metabolized without interference from inhibitors.

Undue emphasis may have been placed in this discussion on the need for aspartic acid as a protein precursor, but the fact that most of the isotope from ¹⁴CO₂ is located in this amino acid does focus attention on it. It is possible that the role of the purines and pyrimidines derived partly from CO₂ is equally important, since the nucleic acids are regarded as having a controlling influence in protein formation. It might well be that when growth is occurring rapidly, as in the presence of CO₂, the metabolism of the organisms is able to

supply all the derivatives involved in nucleic acid synthesis, some of which are utilized to form the proteins involved in dextransucrase formation. When growth is not maximal, then the steps involved in dextransucrase synthesis may be poor competitors for the pool of compounds required in nucleic acid and protein formation. It was not possible to demonstrate satisfactorily the presence of labelled aspartic acid in the polysaccharide-protein complex because of the great excess of carbohydrate material in the complex with the consequent difficulty of separating protein from polysaccharide. Attempts at separation, followed by hydrolysis, paper-chromatography and radioautography did not give satisfactory results. However, the presence of carbon-14 in the complex makes it seem likely that carbon from $^{14}\text{CO}_2$ was incorporated into the enzyme.

Bailey & Oxford (1958) were not able to determine whether the stimulation of dextran formation by CO_2 was due to CO_2 gas in solution or to bicarbonate ion. Ochoa (1951) has suggested that most metabolic carboxylations and decarboxylations involve CO_2 in solution and not bicarbonate ions; thus it seems possible that a similar situation exists with *Streptococcus bovis*, with the dissolved gas being the effective agent. The observation by Bailey & Oxford (1958) that *S. bovis* strain 1 would not grow in a medium containing HCO_3^- through which CO_2 -free and O_2 -free H_2 was bubbled supports this view.

I wish to thank Miss J. Michael for her able technical assistance.

REFERENCES

- BAILEY, R. W. (1959). Transglucosidase activity of rumen strains of *Streptococcus bovis*. 2. Isolation and properties of dextransucrase. *Biochem. J.* **72**, 42.
- BAILEY, R. W. & OXFORD, A. E. (1958). A quantitative study of the production of dextran from sucrose by rumen strains of *Streptococcus bovis*. *J. gen. Microbiol.* **19**, 130.
- COCKING, E. C. & YEMM, E. W. (1954). Estimation of amino acids by ninhydrin. *Biochem. J.* **58**, xii.
- COLOWICK, S. P. & KAPLAN, N. O. (1957). *Methods in Enzymology*. New York: Academic Press Inc.
- DAIN, J. A., NEAL, A. L. & SEELY, H. W. (1956). The effect of CO_2 on polysaccharide production by *Streptococcus bovis*. *J. Bact.* **72**, 209.
- FORD, J. E., PERRY, K. D. & BRIGGS, C. A. E. (1958). Nutrition of lactic acid bacteria isolated from the rumen. *J. gen. Microbiol.* **18**, 273.
- GALE, E. F. (1947). The assimilation of amino acids by bacteria. 1. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. *J. gen. Microbiol.* **1**, 53.
- GALE, E. F. & FOLKES, J. P. (1953). The assimilation of amino acids by bacteria. 18. The incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus*. *Biochem. J.* **55**, 721.
- HENDLER, R. W. (1958). Possible involvement of lipids in protein synthesis. *Science*, **128**, 143.
- OCHOA, S. (1951). Biosynthesis of dicarboxylic and tricarboxylic acids by carbon dioxide fixation. *Symp. Soc. exp. Biol.* **5**, 29.
- OXFORD, A. E. (1958). The nutritional requirements of rumen strains of *Streptococcus bovis* considered in relation to dextran synthesis from sucrose. *J. gen. Microbiol.* **19**, 617.

- PRESTCOTT, J. M. & STUTTS, A. L. (1955). Effects of carbon dioxide on the growth and amino acid metabolism of *Streptococcus bovis*. *J. Bact.* **70**, 288.
- RAVEL, J. M., FELSING, B. & SHIVE, W. (1955). Comparative inhibitory effects of glutamic and cysteic acids on aspartic acid utilization. *Arch. Biochem. Biophys.* **54**, 541.
- REITH, W. S. & THOMAS, M. J. (1958). Isolation from human blood of a new substance associated with fat transport activity. *Nature, Lond.* **182**, 604.
- ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T. & BRITTEN, R. J. (1957). Studies of biosynthesis in *Escherichia coli*. *Publ. Carneg. Instn*, no. 607.
- SAKAMI, W. (1955). *Handbook of Isotope Tracer Methods*. Cleveland, Ohio, U.S.A.: Western Reserve University School of Medicine.
- SWIM, H. E. & KRAMPITZ, L. O. (1954). Acetic acid oxidation by *Escherichia coli*. Evidence for the occurrence of a tricarboxylic acid cycle. *J. Bact.* **67**, 419.
- WESTLEY, J., WREN, J. J. & MITCHELL, H. K. (1957). Phospholipides containing amino acids other than serine. I. Detection. *J. biol. Chem.* **229**, 131.

(Received 18 November 1959)