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Methane Production from Glucose *in vitro* by Mixed Rumen Bacteria

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1. Methane was produced *in vitro* by incubating cell suspensions of rumen bacteria with glucose, under nitrogen. The amount of methane produced varied considerably and was lowered by high glucose concentrations. Carbon dioxide, acetic acid, propionic acid, butyric acid and lactic acid were also produced. An oxidation–reduction balance of near unity could be calculated, although carbon recovery was low. Under the experimental conditions, rumen bacteria used most of the metabolic hydrogen produced during the oxidation of glucose to form lactic acid. 2. Lower methane production at high glucose concentrations was balanced by higher lactic acid production. Low pH values due to a high production rate of lactic acid might explain the inhibition of methane production. 3. No lactic acid, less methane, but considerably more propionic acid were formed when nitrogen was replaced by carbon dioxide in the incubation system.

In earlier experiments *in vitro* with mixed rumen bacteria (Demeyer, 1965) pyruvate was shown to be an important precursor of methane. Indeed, Briggs (1964) stated: 'Much of the rumen methane probably originates from pyruvate'. Pyruvate can be detected in rumen fluid (Van der Horst, 1961) although its concentration is low; Bastie (1957) reported that only 6% of the total rumen acidity (determined as m-equiv.) was due to non-volatile acids. However, pyruvate concentration considerably increases after the addition of glucose to rumen fluid (Van der Horst, 1961) indicating the importance of pyruvate as a key intermediate in carbohydrate digestion in the rumen. In support of this conclusion, Baldwin, Wood & Emery (1963) found labelling patterns in acetate, after incubation of [1-^{14}C]-, [2-^{14}C]- and [6-^{14}C]-glucose with mixed rumen organisms, consistent with the almost exclusive operation of the Embden–Meyerhof pathway. Thus it can be assumed that hexose degradation by rumen organisms results in the production of pyruvate or a related C_3 intermediate that can be further metabolized to methane. Although glucose metabolism by rumen organisms *in vitro* has been extensively investigated since the pioneer work of Phillipson & McAnally (1942) and Elsden (1945), most of the literature is concerned with the production of lactic acid or volatile fatty acids or both; little attention has been given to the gaseous end products. Further, many reports of experiments dealing with the gaseous end products of glucose fermentation mention carbon dioxide as the only gas evolved (Lewis, 1951; Doetsch, Robinson, Brown & Shaw, 1953; Winkenwerder, 1961). [To our knowledge, methane production from glucose is only mentioned in a short communication (McNeill & Brown, 1954).] The absence of methane in these experiments could be due to damage to the methane bacteria during the preparation of mixed rumen bacteria for experiments. Also, in some cases, methane production could have been overlooked as, to our knowledge, no attempt has ever been made to calculate carbon and hydrogen recovery in the end products of glucose metabolism by mixed rumen bacteria *in vitro*.

In the present work, the production by mixed rumen bacteria *in vitro* of methane from glucose is described and a fermentation balance is presented, showing balanced carbon and hydrogen recoveries.

MATERIALS AND METHODS

Animals. Rumen contents were obtained from three fistulated wethers (sheep nos. 5, 6 and 7) kept indoors and fed on hay (*ad lib.*) and concentrates (40–45% of carbohydrates other than crude fibre; 500g./day). The animals were fed at 8·30a.m. and 5p.m. Samples were withdrawn with the apparatus described by Hungate (1950) at least 6hr. after feeding.

Cell suspensions. The rumen sample (400ml.) was immediately transported to the laboratory and filtered through muslin. The liquid was centrifuged for 3min. at 3000*g* in an Ecco-record-extra IVB apparatus (Ecco, Berlin, Germany). The supernatant liquid was centrifuged at 5000*g* for 30min. The pellet was then suspended in 67mM-phosphate buffer, pH 6·9, and recentrifuged. The final pellet was suspended in the same buffer (30–60ml.), containing $NaHCO_3$ (2%, w/v) when the suspension was to be used in

experiments with CO_2. N_2 (or CO_2) was bubbled through for 5min.

Incubation. All incubations were carried out under N_2 or CO_2 (commercial tank gas, purity 99·95%) in glass vessels provided with a silicone rubber septum to allow gas sampling with a Hamilton gas-tight syringe. During incubation, vessels were shaken in a water bath at 39°. Filling with gas was achieved by the evacuation technique (Umbreit, Burris & Stauffer, 1959).

Reagents. Sodium pyruvate and glucose were A.R. (E. Merck A.-G., Darmstadt, Germany) and were checked for purity by the iodometric method mentioned below. Standards for gas chromatography and lactate analysis were acetic acid, propionic acid and butyric acid (A.R.) (Fluka A.-G., Buchs SG, Switzerland), methane (N 35; Air Liquide, Liège, Belgium) and lithium lactate (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.). [1-^{14}C]-, [2-^{14}C]- and [3-^{14}C]-Pyruvate and [1-^{14}C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks.

Gas analysis. Gas–solid adsorption chromatography was applied, with a gas partitioner model 25 V (Fisher Scientific Co., Pittsburgh, Pa., U.S.A.). A silica-gel column (length 53cm., diameter 6mm.) was used, in series with a 2m. molecular-sieve column. The detector was a thermal conductivity cell (7mA), the carrier gas H_2 (30ml./min.) and the recorder a Kipp BDI apparatus (1mv, 72cm./hr.) (Kipp, Delft, The Netherlands).

Separation of CO_2 and CH_4 was achieved at room temperature, the peak heights were measured and the volume of gas in μl. was read from a standard curve. To calculate the total amount produced in μmoles, the volumes of the incubation vessels were measured and a conversion factor 1μmole$\equiv$ 22·4μl. was used. Correction for temperature was not made, the error thus introduced was counteracted by the effect of a pressure increase during incubation. Gas sampling was done after the incubation mixture had been acidified by injecting 1ml. of 10N-H_2SO_4.

Estimation of volatile fatty acids. A portion of the incubation mixture was made alkaline to phenolphthalein and evaporated to dryness. Ether (A.R) (10ml.) was added and the residue acidified under the ether layer with 1ml. of 6N-H_2SO_4. After 15min., separation was carried out as described by James & Martin (1952). A recording titration apparatus (Radiometer, Copenhagen, Denmark) was used as detector (Aunstrup & Djurtoft, 1960). Recoveries obtained with A.R. reagents were determined for each experiment and used to calculate correction factors. Mean values were: acetic acid, 86·5%; propionic acid, 97·0%; butyric acid, 100·0%.

Estimation of lactic acid. The micro-diffusion method described by Conway (1957) was used with 0·5 or 1ml. of the incubation mixture. The diffusion time, however, was lowered to 3hr. and the maximum concentration of the standard series raised to 25μmoles/ml.

Estimation of pyruvate and glucose. After deproteinization of a portion of the incubation mixture with equal volumes of 10% (w/v) $ZnSO_4,7H_2O$ and 0·5N-NaOH, dilution to a known volume and filtration, pyruvate and glucose were determined on a sample of the filtrate as described by Haag & Dalphin (1943). As only very small amounts of pyruvate were produced from glucose, pyruvate did not interfere with the glucose determination.

Radiochemical analysis. (*a*) Gases. After incubation, the mixture was acidified by the injection of 1ml. of 10N-H_2SO_4 and the gases were carried by O_2 (commercial tank gas) through a combustion train; CO_2 was first absorbed by 25ml. of 0·5N-NaOH (CO_2-free), then methane was burned over CuO containing 1% (w/v) of Fe_2O_3 (Murdock, Brooks & Zahn, 1948) at 900° in a quartz U-tube. The resulting CO_2 was absorbed as before in another bottle. CO_2 in both absorption bottles was then precipitated as $BaCO_3$ by 12% (w/v) $BaCl_2$, and the amount determined by titration of the residual alkali to the phenolphthalein end-point. The precipitate was centrifuged, washed with ethanol and ether, dried at 100° and assayed for radioactivity.

(*b*) Bacteria. To measure ^{14}C incorporation into bacteria these were isolated by centrifuging for 20min. at 20000*g* (Christ Junior centrifuge; Martin Christ, Osterode–Harz, Germany), washed with ethanol and ether, dried at 100°, weighed and a portion was burned to CO_2 by the method of Van Slyke & Folch (Neish, 1952). CO_2 was absorbed in NaOH, precipitated by $BaCl_2$ and determined by titration of residual alkali. The carbon content was 20–50μmoles of C/mg. of precipitate.

(*c*) Residual substrate and volatile fatty acids. Residual pyruvate was precipitated as the 2,4-dinitrophenylhydrazone (Bamann & Myrbäck, 1941), which was filtered and dried before combustion. Volatile fatty acids were isolated by column chromatography (Demeyer & Henderickx, 1964) before extraction and combustion. In Expt. 1 (Table 1) the supernatant, obtained after removal of the bacteria by centrifuging, was acidified with 6N-H_2SO_4 and fractionated by continuous ether extraction in a liquid–liquid extractor. Carrier glucose was added to the aqueous layer remaining after extraction, and both the ether extract (treated with 0.1 N-NaOH) and this aqueous layer were then evaporated to dryness. The residue from the ethereal extract was burned to CO_2 and assayed as $BaCO_3$ ($\equiv$ total compounds extractable by ether). The residue from the aqueous layer was also burned to CO_2 and assayed as $BaCO_3$ ($\equiv$ residual substrate). All compounds were finally converted into $BaCO_3$ by the method of Van Slyke & Folch (Neish, 1952) and assayed as such with a Tracerlab gas-flow counter. Corrections for background and self-absorption were made.

RESULTS

Preliminary experiments established that methane was formed from glucose by mixed rumen bacteria. Some indication of the predominant, though not exclusive, operation of the Embden–Meyerhof pathway was obtained. Thus, after incubation of rumen bacteria with [1-^{14}C]glucose, only 11·4% of the radioactivity recovered was present in methane and carbon dioxide, indicating a limited contribution of the hexose monophosphate pathway (Wood, 1961) (Table 1). However, incubations with [1-^{14}C]-, [2-^{14}C]- and [3-^{14}C]-pyruvate showed that labelled methane was formed only from carboxyl-labelled pyruvate. These results suggest that a small amount of methane is derived from intermediates other than pyruvate. It is probable, however, that the Embden–Meyerhof pathway, involving pyruvate

Table 1. *Distribution of radioactivity in the end products of labelled glucose and pyruvate metabolism*

In all experiments 15 ml. of cell suspension was incubated with 15 ml. of 67 mM-phosphate buffer, pH 6·9, containing 375 μmoles of glucose or 750 μmoles of pyruvate. Incubation was carried out under N_2 for 4 hr. (Expt. 2) or 15 hr. (Expts. 1, 3 and 4). One cell suspension was used in Expts. 1, 3 and 4 (total nitrogen 1·35 mg./ml.) and another in Expt. 2 (total nitrogen 1·10 mg./ml.). Analyses were carried out as described in the Materials and Methods section. N.D., Not determined.

				% of total ^{14}C in individual compounds				
Expt. no.	Substrate added	10^{-3} × Radioactivity added (counts/min.)	10^{-3} × Total radioactivity recovered (counts/min.)	Residual substrate	Bacteria	Volatile fatty acids or total compounds extractable by ether	CO_2	CH_4
1	[1-^{14}C]Glucose	1105	1205	3·3	17·0	68·3	8·6	2·8
2	[1-^{14}C]Pyruvate	1087	972	N.D.	0·2	16·1	68·6	15·3
3	[2-^{14}C]Pyruvate	1065	876	6·1	3·9	88·7	1·0	0·3
4	[3-^{14}C]Pyruvate	1061	1005	2·2	2·8	93·9	0·9	0·2

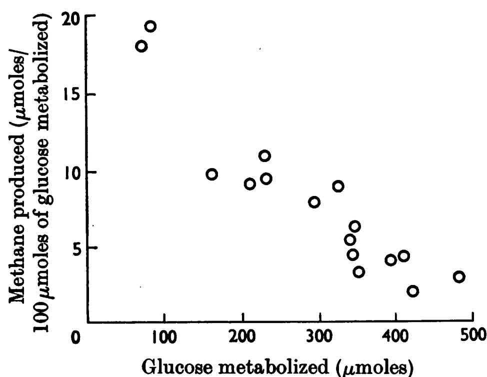

Fig. 1. Methane production from glucose by cell suspensions of rumen bacteria of varying activity in glucose fermentation. The incubations were carried out under N_2 for 3 hr. with 5 ml. of cell suspension and 5 ml. of 67 mM-potassium phosphate buffer, pH 6·9, containing 500 μmoles of glucose. Results were obtained from the mean values of at least two incubations, after correction for blank incubations.

as the key intermediate, is the major pathway involved (Baldwin, 1965). This assumption is further supported by the complete inhibition of methane production from glucose by 0·2 M-sodium fluoride.

Incubation of glucose with rumen bacteria under nitrogen. Nitrogen was chosen as incubation gas for the first experiments because, unlike carbon dioxide, it does not interfere with rumen metabolism. It soon appeared, however, that the amount of methane produced/unit of glucose metabolized varied considerably. Low values were always obtained with active suspensions that metabolized large amounts of glucose, but higher values were noted with less active suspensions metabolizing less glucose (Fig. 1). This effect is probably due to variations in the total numbers and proportions of different organisms in the rumen microbial population of the three sheep. Indeed, 'activity' was correlated with the total nitrogen content of the suspensions; low activity was associated with a low content (up to 0·5 mg./ml.) and high activity with a high content (up to 1·5 mg./ml.). Variability in methane production due to variability in damage by oxygen to the methane bacteria, although probably involved, cannot explain the obvious relation between methane production and overall glucose breakdown. With pyruvate and formate as substrates, a similar relation was indeed not found (D. I. Demeyer & H. K. Henderickx, unpublished work). The same effect could be produced by addition of increasing amounts of glucose to the same cell suspension; the more glucose that was added the more was metabolized and the less methane was produced/unit of glucose metabolized. The production of reduced compounds, other than methane, being either faster than methane production or inhibitory to methane production or both could explain this effect. To determine the nature of all reduced end products of glucose metabolism, large-scale incubations were carried out. Carbon dioxide, methane, acetic acid, propionic acid, butyric acid and lactic acid were the end products found. Classical fermentation balances (Wood, 1961) (Table 2) showed a low carbon recovery, probably owing to the use of glucose carbon in the synthesis of cellular material; e.g. Table 1 shows that 17% of the radioactivity added as [1-^{14}C]glucose is recovered in bacteria. An oxidation–reduction balance of near unity was obtained, however, indicating that carbon was assimilated into the cells at an oxidation level close to that of carbohydrate. It is clear that the major part of the metabolic hydrogen produced during the oxidation of glucose is consumed in lactic acid production. Incubation of rumen bacteria with increased amounts of glucose nearly always produced increased amounts

Table 2. *Glucose fermentation balance*

Incubation was carried out under N_2 for 15hr. as described in Table 1, with 1·5m-moles of glucose. (The cell suspension used had a total nitrogen content of 0·92mg./ml.) Fermentation balances were calculated as described by Wood (1961). Carbon recovery: 100 × 429·5/600 = 71·5%; oxidation–reduction balance: 63·0/57·9 = 1·09; C_1 recovery: 100 × C_1 observed/C_1 calculated = 78·4%.

	Amount (μmoles)			Products formed (μmoles/100 μmoles of glucose metabolized)	Carbon in products formed (μg.atoms of C)	Oxidation–reduction value	Products formed × oxidation–reduction value		C_1 calculated
	Blank incubation	Glucose incubation	Corrected value				Oxidized	Reduced	
Glucose metabolized			1194	100	600	0			
Products formed									
Acetic acid	311	902	591	49·5	99·0	0			49·5
Propionic acid	32	110	78	6·5	19·5	−1		6·5	
Butyric acid	30	116	86	7·2	28·8	−2		14·4	14·4
Lactic acid	0	924	924	77·4	232·2	0			
Carbon dioxide	178	554	376	31·5	31·5	+2	63·0		
Methane	57	278	221	18·5	18·5	−2		37·0	
					429·5		63·0	57·9	63·9

of lactic acid and less methane/unit of glucose metabolized. This is obvious from the results presented in Table 3. For the sake of clarity, blank values and oxidation–reduction balances are not indicated in Table 3; they are of the same magnitude as the values in Table 2. As lactic acid can be produced from glucose by a single organism (Baldwin *et al.* 1963), whereas at least two organisms are involved in methane production from glucose, lactic acid production might proceed faster than methane production in rumen symbiosis. The results shown in Table 4 indeed indicate such a difference in rate of production. Lactic acid itself could also be further metabolized to methane; at lower substrate concentrations (125 μmoles) more lactic acid was found after 0·5hr. than after 3hr. of incubation.

Probably, with high substrate concentrations, the significant fall in pH due to excessive lactic acid production partially prevents further metabolism of lactate or pyruvate or both to methane. Methane production from pyruvate was lower at pH values below 6·5 (Fig. 2) and such values were reached when glucose (500 μmoles) was incubated with rumen bacteria (values found were e.g. 5·3, 5·5 and 6·1).

Incubation of glucose with rumen bacteria under carbon dioxide. The concentration of lactic acid that accumulates during the incubation of glucose under nitrogen *in vitro* is greater than that usually present in the rumen. High concentrations oflactic acid are only found when excessive amounts of readily fermented carbohydrate are consumed by the animal (Annison & Lewis, 1959). The important difference between the conditions obtaining *in vivo* and in our experiments *in vitro* is that carbon dioxide is present in the former and absent in the latter. It is possible that the absence of carbon dioxide promotes the use of metabolic hydrogen for lactic acid production rather than for the production of other reduced compounds. For this reason, parallel incubations of rumen bacteria with glucose under carbon dioxide and nitrogen were carried out, and the end products compared. The presence of carbon dioxide strongly stimulated production of propionic acid, and less methane and butyric acid and no lactic acid were found (Table 5). It can also be seen that the total acid production is slightly greater than the amount (1·25 moles/mole of glucose) mentioned by Winkenwerder (1961). In similar experiments, with formate as substrate, the use of carbon dioxide stimulated methane production (Demeyer, 1965).

DISCUSSION

In the experiments of Doetsch *et al.* (1953) and Winkenwerder (1961), cell suspensions were pre-

Table 3. *End products of fermentation of varying amounts of glucose*

Incubations were carried out for 15 hr. as described in Table 1. All values were corrected for blank incubations.

Expt. no.	Total nitrogen (mg./ml.)	Substrate added (μmoles)	Products formed (μmoles/100μmoles of glucose metabolized)					
			Methane	Carbon dioxide	Acetic acid	Propionic acid	Butyric acid	Lactic acid
1	1·44	1560	16·8	33·9	35·0	4·6	5·6	57·3
		453	31·0	58·4	58·0	28·2	12·0	1·0
2	1·12	1490	25·8	31·6	70·7	10·1	6·9	87·1
		436	48·6	65·5	99·5	22·4	8·3	34·0
3	1·13	1540	25·3	34·0	69·5	3·3	5·2	109·0
		440	27·2	58·3	82·9	11·2	10·0	76·0

Table 4. *Rate of production of lactic acid and methane from glucose by rumen bacteria*

Incubations were carried out as described in Fig. 1 in two identical vessels, of which one was analysed after 3 hr. and one after 0·5 hr. All values were corrected for blank incubations.

Expt. no.	500 μmoles of glucose added				125 μmoles of glucose added			
	Lactic acid produced (μmoles) after		Methane produced (μmoles) after		Lactic acid produced (μmoles) after		Methane produced (μmoles) after	
	0·5 hr.	3 hr.	0·5 hr.	3 hr.	0·5 hr.	3 hr.	0·5 hr.	3 hr.
1	224	204	5·4	23·1	116	79	3·7	13·7
2	164	180	3·4	18·5	113	84	2·6	14·2
3	164	236	4·2	19·2	75	56	2·6	14·1
4	74	96	2·0	14·2	70	54	2·0	9·1

pared by methods differing only slightly from ours, but lower substrate concentrations and shorter incubation periods were used. Further, the direct Warburg method was used, so that methane was not determined as such, but all gases other than carbon dioxide were determined together. Carbon dioxide was absorbed continuously during incubation. Similar experiments with the same method, incubation time and substrate concentration were also carried out in our Laboratory and gases other than carbon dioxide were indeed detected, but in widely divergent amounts (D. I. Demeyer & H. K. Henderickx, unpublished work). This is not surprising, as Fig. 1 shows that the activity of cell suspensions in promoting methane production from glucose varies considerably and is often very low. Our results show, however, that hydrogen diverted from methane production is used in the production of lactic acid. Also, lactic acid is produced at a faster rate than methane. This explains the increased accumulation of lactic acid when increased glucose concentrations are used. Low pH values resulting from this accumulation were found to be inhibitory to methane production from pyruvate (Fig. 2). Possibly the composition of a rumen flora is such that lactic acid production from glucose is strongly promoted, resulting, *in vitro*, in a considerable pH fall so that methane production is suppressed. Such a rumen flora could be present in animals fed on a carbohydrate-rich diet. Indeed, according to Annison & Lewis (1959) more lactic acid-producing bacteria are present under these conditions. This promotes the growth of lactic acid-utilizing bacteria (Baldwin, Wood & Emery, 1962) but may suppress methane-producing bacteria. Similar changes in the rumen flora, due to differences in diet or in the preparation of cell suspensions, could account for the discrepancy between our results and those of earlier workers. It is noteworthy that in the earlier work beet cuttings ('rübenschnitzel'; Winkenwerder, 1961) and a grain mixture (Doetsch *et al.* 1953) were included in the diet of the experimental animals.

Replacement of nitrogen by carbon dioxide during incubations drastically changed the amounts of end products formed. Lactic acid was no longer found, and less methane and considerably more propionic acid were produced. A stimulation of propionic acid production by carbon dioxide is not surprising since Baldwin *et al.* (1963) established that propionic acid production from glucose occurs, mainly following the dicarboxylic acid pathway.

These authors speculated that this route was not very dependent on exogenous carbon dioxide as it involves only a transcarboxylation between methylmalonyl-CoA and pyruvate (Baldwin *et al.* 1962). However, the C_1 fragment produced by the succinate decarboxylation system of *Veillonella gazogenes* is readily released as carbon dioxide (Delwiche, Phares & Carson, 1956). This indicates the imorptance of carbon dioxide fixation in propionic acid production by this organism. The presence of carbon dioxide is probably necessary for the production of propionic acid by similar organisms in rumen symbiosis. The larger amount of metabolic hydrogen used in propionate production explains the lowered production of methane and the absence of lactic acid. However, further metabolism of lactic acid itself to propionic acid might also be promoted by carbon dioxide, though this is not so likely, as the dicarboxylic acid pathway is of minor importance in this process (Baldwin *et al.* 1962). It is surprising that carbon dioxide stimulates propionic acid production and lowers methane production. This might indicate that the role of gaseous carbon dioxide in rumen methane production is not as important as is generally accepted (Blaxter, 1962). Tentative evidence for methane production, not involving gaseous carbon dioxide as an intermediate, by mixed rumen bacteria has been reported (Fina, Sincher & DeCou, 1960; Williams, Hoernicke, Waldo, Flatt & Allison, 1963; Demeyer, 1965).

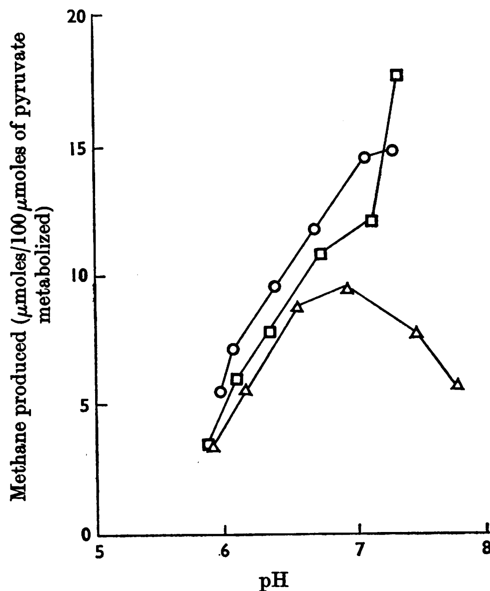

Fig. 2. Methane production from pyruvate by cell suspensions of rumen bacteria at different pH values. The incubations were carried out under N_2 for 3hr. with 9ml. of cell suspension made in 0·1M-potassium phosphate buffer of different pH values and 1ml. of water containing 500 μ-moles of pyruvate. The pH was measured after incubation. Three identical experiments with three different cell suspensions are shown (○, □ and △).

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Table 5. *Influence of carbon dioxide on the end products of glucose metabolism*

Incubations were carried out under N_2 or CO_2 as indicated, during 15hr. with 1·5m-moles of glucose. Further experimental details are given in Table 1. All values were corrected for blank incubations.

Expt. no.	Incubation gas	Products formed (μmoles/100 μmoles of glucose metabolized)						
		Methane	Carbon dioxide	Acetic acid	Propionic acid	Butyric acid	Lactic acid	Total acid
1	N_2	26·9	27·0	49·1	3·9	5·8	75·6	134·4
	CO_2	14·7		56·2	76·7	4·3	0·0	137·2
2	N_2	23·6	32·9	57·8	4·2	0·0	98·4	160·4
	CO_2	16·5		91·5	78·7	0·0	0·0	170·2
3	N_2	18·5	31·5	49·5	6·5	7·2	77·4	140·6
	CO_2	13·8		75·7	69·3	4·7	0·0	149·7

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