NATIONAL LIBRARY
OTTAWA

CANADA

BIBLIOTHÈQUE NATIONALE
OTTAWA

8073

NAME OF AUTHOR.....B.I.J.A.N...E.M.M.A.N.U.E.L.....

TITLE OF THESIS...E.N.Z.Y.M.A.T.I.C...A.S.P.E.C.T.S...O.F..
...G.L.U.T.A.M.I.C...A.C.I.D...S.Y.N.T.H.E.S.I.S..
....I.N....R.U.M.E.N...M.I.C.R.O.O.R.G.A.N.I.S.M.S

UNIVERSITY.....O.F...A.L.B.E.R.T.A.....

DEGREE FOR WHICH THESIS WAS PRESENTED.....P.h..D.....

YEAR THIS DEGREE GRANTED.....S.P.R.I.N.G...1.9.7.1.....

Permission is hereby granted to THE NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(Signed).....

PERMANENT ADDRESS:

...Box...3.5.8.....
...Univ....of...Alberta
.....

DATED....April...14....19 71

NL-91 (10-68)

THE UNIVERSITY OF ALBERTA

ENZYMATIC ASPECTS OF GLUTAMIC ACID SYNTHESIS IN RUMEN MICROORGANISMS

by

© BIJAN EMMANUEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ANIMAL SCIENCE

EDMONTON, ALBERTA

SPRING, 1971

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Enzymatic aspects of glutamic acid synthesis in rumen microorganisms" submitted by Bijan Emmanuel, B.Sc.(Agr.), M.Sc.(Agr.), in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....
Supervisor

.....

.....

.....

.....
External Examiner

Date April 6, 1971

ABSTRACT

Cell-free extracts of mixed rumen microorganisms were incubated *in vitro* in order to determine the existence and nature of metabolic conversions leading from succinate to glutamate. When succinate was incubated as the substrate in the presence of reduced ferredoxin, a product was formed, which reacted with 2,4-dinitrophenylhydrazine to give the hydrazone of succinic semialdehyde as determined by paper chromatography. When succinic semialdehyde was used in the presence of reduced ferredoxin and ^{14}C-bicarbonate, the fixation of label was catalyzed by the extracts. The labelled product was isolated as an organic acid and identified as 2-hydroxyglutarate by means of paper chromatography. In the presence of ammonia and NAD, 2-hydroxyglutarate was aminated. The amino acid formed, was identified as glutamate by paper chromatography, and the quantity was estimated colorimetrically, using ninhydrin. The conversion of 2-hydroxyglutarate to glutamate may entail 2-ketoglutarate as an intermediate as the activity was inhibited by hydrazine hydrochloride and 2-ketoglutarate was readily reduced in the presence of NADH and was reductively aminated by the extracts.

From the information obtained, a new pathway for the synthesis of glutamate in rumen microbes was proposed. This pathway entails the reduction of succinate to succinic semialdehyde, followed by reductive carboxylation of succinic semialdehyde to yield 2-hydroxyglutarate, which is then aminated to glutamate. This pathway would agree with the labelling pattern reported in the literature in glutamate produced in the presence of ^{14}C-bicarbonate by mixed rumen microorganisms.

The proposed pathway of glutamate synthesis entails implications opposite to those of the route involving the intermediates of the oxidative tricarboxylic pathway. The energetic and electron transfer aspects of this pathway in relation to the metabolism of rumen microbes are discussed.

The presence of ferredoxin and the activity of pyruvate synthase, which catalyzes the reductive carboxylation of acetyl coenzyme A to pyruvate, were also shown in extracts of mixed rumen microorganisms.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. L.W. McElroy, Head of the Department of Animal Science, for placing the facilities of the Deparment at his disposal. My deepest gratitude is due to Dr. L.P. Milligan, Associate Professor of Animal Biochemistry, for his guidance and consultation during the course of my Ph.D. studies. I am also grateful for his constructive instructions in the preparation of the manuscript.

The author wishes to thank Mr. T.W. Fenton and Mr. B.V. Turner for their useful technical advice. I am greatly indebted to Mr. J.A. Francis, for taking care of the animal.

My sincere thanks and appreciation are given to Miss Margaret Sandene, for showing such understanding and patience during the winter of 1968-1969.

The financial grant from the National Research Council of Canada is hereby acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A high concentration of ammonia is present in the rumen under various dietary regimes (Lewis, 1957) and many rumen microorganisms can use ammonia as their sole source of nitrogen (Bryant and Robinson, 1962). Glutamate likely occupies a key role in the metabolism of nitrogenous compounds, particularly ammonia, in rumen organisms. The synthesis of glutamate from 2-ketoglutarate is catalyzed by NAD-and NADP-linked glutamic dehydrogenases (EC 1.4.1.3 and EC 1.4.1.4) (Joyner and Baldwin, 1966; Palmquist and Baldwin, 1966) and its further amination is catalyzed by glutamine synthetase (EC 6.3.1.2) (Chalupa et al., 1970). These conversions provide sites for ammonia assimilation in the rumen microflora. Furthermore, glutamate is a precursor of a family of amino acids (Niederman and Wolin, 1967) and it is an amino donor in a number of transaminase reactions (Chalupa et al., 1968; Somerville, 1968). Experiments entailing incubation of whole rumen contents in the presence of $NaH^{14}CO_3$ (Milligan, 1970) have indicated the occurrence of three pathways for the biosynthesis of glutamic acid in the mixed rumen microbes, namely, a forward tricarboxylic acid (TCA), an atypical TCA (Stern et al., 1966; Gottschalk and Barker, 1966; Isle and O'Brien, 1967) and a reverse TCA pathway.

The object of this investigation was to establish the presence of the enzymes needed for the biosynthesis of glutamic acid by way of the reverse TCA pathway in mixed rumen microorganisms.

REVIEW OF LITERATURE

The synthesis of glutamate through the reaction sequence consisting of the carboxylation of phosphoenolpyruvate or pyruvate to yield oxaloacetate, followed by intermediates similar to that of the oxidative tricarboxylic acid (TCA) cycle will be referred to as the forward TCA pathway. The route of glutamate synthesis as reported by Burchall et al.(1964) and Somerville and Peel (1964, 1967) supports the occurrence of the forward TCA pathway in the rumen microorganisms.

The pathway in which citrate synthetase (EC 4.1.3.7) or aconitase (EC 4.2.1.3) functions with atypical stereospecificity will be referred to as the atypical TCA pathway. Stern et al.(1966); Gottschalk and Barker (1966) and Isle and O'Brien (1967) have reported the occurrence of the atypical citrate synthetase in several anaerobic bacteria.

The reductive biosynthesis of glutamate from oxaloacetate through the intermediates malate, fumarate, succinate and 2-ketoglutarate will be referred to as the reverse TCA pathway. Hopgood and Walker (1969) have reported the presence of the enzymes which catalyze the conversion of oxaloacetate to succinate through this route in the rumen isolate Ruminococcus flavefaciens. These conversions also occur in the succinate pathway of propionate formation in rumen microbes (Baldwin et al., 1962; Baldwin et al., 1963).

The forward, atypical and reverse TCA pathways in the presence of $H^{14}CO_3^-$ would result in label in the positions (C-1), (C-5) and (C-1, C-2 and C-5) of glutamate, respectively (Milligan, 1970).

Milligan (1970), who incubated whole rumen contents in the

presence of $H^{14}C\bar{O}_3$, measured a labelling pattern in microbial glutamic acid indicating the operation of the above three pathways. The report of Allison and Robinson (1970) on the labelling pattern of glutamate in the rumen isolate Bacteroides ruminicola also supports the occurrence of the reverse TCA pathway in this rumen microorganism.

An enzyme which catalyzes the reductive carboxylation of succinyl coenzyme A to 2-ketoglutarate has been designated as 2-ketoglutarate synthase (Buchanan and Evans, 1965).

Because of the similarity in the catalytic function of pyruvate synthase (Equation 1) and 2-ketoglutarate synthase (Equation 2), it is worthwhile to briefly review the characteristics of the former enzyme.

(1) Acetyl coenzyme A + $H\bar{C}O_3$ + ferredoxin (reduced) ⟶
pyruvate + coenzyme A + ferredoxin (oxidized)

(2) Succinyl coenzyme A + $H\bar{C}O_3$ + ferredoxin (reduced) ⟶
2-ketoglutarate + coenzyme A + ferredoxin (oxidized)

The exchange reaction between carbon dioxide and the carboxyl group of pyruvate, catalyzed by extracts of a number of bacteria (Sagers and Beck, 1956; Rabinowitz, 1960; Stern, 1965), suggested the possible participation of CO_2 in the formation of pyruvate from acetate or an acetate derivative. On the other hand, this exchange reaction may be catalyzed by a system involved in pyruvate degradation (Whiteley and McCormick,1963). Palmquist and Baldwin (1966) have found this exchange reaction to be catalyzed by extracts of ruminal bacteria.

Pyruvate synthase activity has been detected in photosynthetic

bacteria (Buchanan et al., 1964; Evans and Buchanan, 1965; Evans, 1968) and anaerobic bacteria (Mortlock and Wolfe, 1959; Bachofen et al., 1964; Andrew and Morris, 1965).

More than twenty years ago Ajl and Werkman (1948) proposed the reductive carboxylation of succinate. Their hypothesis was based on the observations that 2-ketoglutarate would replace the CO_2 requirement of E. coli. In addition, extracts catalyzed an exchange reaction of 2-ketoglutarate with CO_2. Furthermore, they suggested the participation of a phosphorylated intermediate in the reductive carboxylation reaction.

Shigesada et al. (1966) have observed completely different labelling patterns in glutamic acid after synthesis under light-anaerobic or dark-aerobic conditions by photosynthetic bacteria. The substrates used were $KH^{14}CO_3$ in the presence of acetate or succinate, or ^{14}C-succinate plus $KHCO_3$. They suggested a mechanism for the biosynthesis of glutamate under light-anaerobic conditions similar to the reductive synthesis of pyruvate.

Buchanan and Evans (1965) obtained the first experimental evidence indicating the presence of 2-ketoglutarate synthase in photosynthetic bacteria. In their cell-free extracts, succinyl coenzyme A was reductively carboxylated to 2-ketoglutarate. Ferredoxin was required as the electron donor. This result is consistent with the experiments of Knight (1962) and Evans (1965) who observed that glutamic acid was one of the major labelled products in Rodospirillum rubrum extracts after a short exposure to labelled propionate or succinate. In the following years

Buchanan et al.(1967) and Evans (1968) demonstrated the existence of 2-ketoglutarate synthase in other photosynthetic bacteria. In the carboxylation of acetyl coenzyme A and succinyl coenzyme A, the strong reducing potential of ferredoxin (Tagawa and Arnon, 1962) allows the reaction to proceed.

Recently, the ferredoxin-dependent reductive carboxylation of propionyl coenzyme A to 2-ketobutyrate in photosynthetic and anaerobic bacteria has been reported (Buchanan, 1969), and the enzyme catalyzing this reaction was called 2-ketobutyrate synthase.

It is relevant at this point to refer to a number of carboxylation reactions which have been reported to occur in the ruminal microbes. The branched-chain volatile fatty acids including isobutyrate, isovalerate and 2-methylbutyrate (Allison, Bryant and Doetsch, 1962; Allison and Bryant, 1963; Allison et al.,1966) appear to be carboxylated and then aminated by mixed rumen organisms and isolates to yield valine, leucine and isoleucine, respectively. In a similar fashion phenylacetic acid (Allison, 1965) and indolacetic acid (Allison and Robinson, 1967) are the precursors of phenylalanine and tryptophan. The biochemical mechanism for these carboxylation reactions has not been elucidated. However, the following sequential mechanism was suggested (Allison, 1969):

(1) $R\text{-}COOH + ATP \longrightarrow R\text{-}CO\text{-}OPO_3H_2$

(2) $R\text{-}CO\text{-}OPO_3H_2 + CoASH \longrightarrow R\text{-}CO\text{-}S\text{-}CoA + HPO_4^{-2}$

(3) $R\text{-}CO\text{-}S\text{-}CoA + TPP\text{-enzyme} \longrightarrow R\text{-}CO\text{-}TPP\text{-enzyme} + CoASH$

(4) $R\text{-}CO\text{-}TPP\text{-enzyme} + 2FdH \longrightarrow R\text{-}CHOH\text{-}TPP\text{-Enzyme} + 2Fd$

(5) $RCHOH\text{-}TPP\text{-enzyme} + CO_2 \longrightarrow R\text{-}CO\text{-}COOH + TPP\text{-enzyme}$

This proposed mechanism was based on the information obtained with Peptostreptococcus elsdenii and upon studies with pyruvate synthase in other microorganisms.

Robinson and Allison (1969) and Allison and Robinson (1970) were the first workers to report the possibility of reductive carboxylation of succinyl coenzyme A in the rumen isolate Bacteroides ruminicola. Coenzyme A, 1,4-^{14}C-succinate, ATP and cell-free extracts were included in their reaction mixture. The information obtained suggests the possible carboxylation of succinate in the above fashion in this bacteria. However, further experimental evidence is required to establish a definite mechanism for the succinyl coenzyme A carboxylation, since, in the absence of ATP and coenzyme A, the reaction proceeded fairly readily.

In reviewing the literature, the data on the reductive carboxylation of succinyl coenzyme A in photosynthetic bacteria as well as the labelling pattern of glutamate formed by mixed and isolated rumen bacteria suggested the possible occurrence of 2-keto-glutarate synthase in the rumen microorganisms.

The present experiments were designed to establish the presence of the enzymes required for the biosynthesis of glutamic acid by the route of the reverse TCA pathway in cell-free extracts of mixed rumen microorganisms.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

Materials and methods

A. Materials

I. Animal and diet

One rumen-fistulated cow was used in these studies. This cow was fed 2.5 kg of concentrate (15.3% protein, Table 1) and 0.9 kg of good quality alfalfa-bromegrass hay at 8:00 a.m. and 4:00 p.m. daily. The animal had free access to drinking water and to a mixture of one part by weight of sodium-tripolyphosphate and four parts of cobaltized-iodized salt.

Table 1

Composition (%) of the concentrate mixture*

Ingredients	(%)
Barley	61.5
Oats	20.5
Soybean meal	15.5
Dicalcium phosphate	2.0
Cobalt-iodized salt	0.5
Total	100.0

* Contained 4,400 I.U. vitamin A and 1,050 I.U. vitamin D per kg of concentrate.

II. Chemicals

Succinyl phosphate was prepared by the method of Hildebrand and Spector (1969). Succinyl coenzyme A and acetyl coenzyme A were made from their respective anhydrides and coenzyme A using the procedure of Sly and Stadtman (1963). The hydroxamate method of Stadtman and

Barker (1950) was employed to identify the chemical formation of succinyl phosphate and the enzymatic synthesis of succinyl coenzyme A, or acetyl coenzyme A from their respective acids, ATP and coenzyme A.

Succinic semialdehyde was prepared using the method described by Jakoby (1962). The product was identified by infrared spectrometry, nuclear magnetic resonance and reactivity with 2,4-dinitrophenylhydrazine. Attempts to further concentrate the preparation were unsuccessful. The results indicated the formation of a polymer, which is in accordance with the report of Bessman et al.(1953) who concluded that succinic semialdehyde is unstable and polymerizes readily.

Ferredoxin was obtained from mixed rumen microorganisms as described in Appendix II. A zinc-methyl viologen system was used to reduce ferredoxin (Eisenstein and Wang, 1969). The chemicals obtained commercially are listed alphabetically in Table 2.

Table 2

Commercial source of compounds used

Compound	Company
Acetyl phosphate	Sigma Chemical Co., St. Louis, U.S.A.
Adenosine-5'-triphosphate	" " " " " "
Coenzyme A	P-L Biochemical Inc., Milwaukee, U.S.A.
β-dihydronicotinamide adenine dinucleotide (β-NADH)	Sigma Chemcial Co., St. Louis, U.S.A.
Dihydronicotinamide adenine dinucleotide phosphate (NADPH)	" " " " " "
2-hydroxyglutaric acid	" " " " " "
2-ketoglutaric acid	" " " " " "
2-mercaptoethanol	Matheson Coleman and Bell, Cincinnati, U.S.A.
Methyl viologen	Mann Research Laboratories, N.Y., U.S.A.
β-nicotinamide adenine dinucleotide (β-NAD)	Sigma Chemical Co., St. Louis, U.S.A.
Sodium bicarbonate ($NaH^{14}CO_3$)	Amersham/Searle Co., Arlington Heights, U.S.A.
Thiamine pyrophosphate	Sigma Chemical Co., St. Louis, U.S.A.

B. Methods

I. Preparation of cell-free extracts (CFE)

Rumen ingesta was obtained from a fistulated cow and placed in a warmed vacuum flask immediately after sampling. Portions of this rumen ingesta were then diluted twofold with 0.01 M potassium

phosphate buffer, pH 7.5 at 37°C and subjected to low-speed treatment in an electric blendor for 2 min to release the microorganisms from food particles. Subsequently, the diluted rumen content (RC) was strained through four layers of cheesecloth. The strained RC was allowed to settle for 15 min and the supernatant was discarded. The dense lower layer was centrifuged at 48,000 x g for 30 min at 0°C in a refrigerated centrifuge. All the following steps were carried out at 0°C. The centrifuged microbial paste was diluted with one-half volume of 0.5M potassium phosphate buffer, pH 7.5 containing 0.05 M 2-mercaptoethanol and homogenized in a Vitris "23" homogenizer. Eight grams of this preparation were placed in a glass tube (20 x 60 mm) and were frozen in dry ice. Using a modified Hughes apparatus (Mandell and Roberts, 1966) the frozen preparation was subjected to 24,000 psi in a hydraulic press. The exudate from the press was centrifuged at 48,000 x g for 30 min to remove unbroken cells and cellular debris. Two ml of the crude extract was then passed through a column of Sephadex G-25 (10 x 100 mm) equilibrated with the 0.05 M buffer to remove endogenous substrates. The Sephadex-treated extract was diluted with one-fourth volume (v/v) of 0.5 M 2-mercaptoethanol to protect labile sulfhydryl groups and used for enzyme assays.

II. Incubation procedure

All the substrates used were first neutralized and then added to centrifuge test tubes (15 x 100 mm) equipped with rubber serum stoppers. Immediately after adding the enzyme, the test tubes were alternately evacuated and refilled with hydrogen gas for 30 sec using

hypodermic needles inserted through the stopper. The incubation was carried out under a hydrogen atmosphere in a shaking bath at 37°C for 45 min. The reaction was stopped with 0.1 ml of 12N HCl. The samples then were centrifuged. Unfixed $H^{14}C\bar{O}_3$ was removed by flushing the acidified samples with CO_2 for 10 min. Capryl alcohol was used as anti-foaming reagent.

III. Radioactive counting

The radioactivity was measured by dissolving 0.3 ml of the reaction mixture in 20 ml of the scintillation liquid described by Harlan (1966) and counting the vials in a Nuclear-Chicago Mark I apparatus. Correction was made for quenching by applying the channels-ratio procedure (Bruno and Christian, 1961). The usual efficiency of counting was about 60%. To avoid loss of labelled $H\bar{C}O_3$ from stock solution, 2 mmoles of unlabelled $NaHCO_3$ was added to 1 ml of solution containing 20 umoles $NaH^{14}CO_3$. This preparation was adjusted to pH 9 and stored at -15°C in a test tube equipped with a rubber stopper. Radioactivity on paper chromatograms was counted using a Nuclear-Chicago Actigraph III.

IV. Protein measurement

The protein concentration in CFE was estimated from the absorbancies at 280 nm and 260 nm as reported by Layne (1957). The formula "mg protein/ml = $1.55 \times A_{280} - 0.76 \times A_{260}$" was employed.

V. Total amino acid estimation

Excess ammonia was removed from the deproteinized reaction mixture by boiling to one-half the original volume after adding 0.2 ml of 10N KOH to the solution. Then 0.2 ml of the above solution

was neutralized and placed in a test tube. Subsequently, the procedure of Matheson et al.(1961) was applied. Two ml of the ninhydrin-hydrindantin reagent was added. The contents were mixed gently and the test tubes were covered with aluminum foil and heated in a vigorously boiling water bath for 30 min, following which the test tubes were placed in ice water for 2 min. The contents of each tube were then made to a volume of 10 ml by addition of 50% (v/v) ethanol. The absorbancy was measured at 570 nm using 10 mm path cuvettes in the Bausch and Lomb Spectronic 20. A standard curve was prepared with glutamic acid.

VI. Isolation of end products

Fifteen ml of the $H^{14}CO_3^-$-fixation reaction mixture were incubated when it was intended to isolate the products of reaction. When it was desired to detect the formation of amino acids, 1 mmole of ammonium chloride and 50 umoles of NAD or NADH (depending on the enzyme assay used) were added to a separate test tube containing the above volume. At the end of incubation time, 1 ml of 12N HCl was added. The sample was then centrifuged at 48,000 x g for 20 min and the supernatant was evaporated to dryness under vacuum at 50°C. The residue was dissolved in 1 ml of distilled water. The aqueous solutions were used in the following procedures of isolation:

(a) To isolate organic acids, the 1 ml sample was treated with 10 ml of ether (containing 1 ml of 12N HCl per 50 ml ether) while shaking vigorously. This process was repeated 6 to 8 times until almost all the radioactivity was removed from the aqueous solution. The ether was removed by evaporation and the residue was dissolved

in a minimum volume of water and applied to a column (10 x 100 mm) of Dowex-1 resin (acetate form). The column was prepared with 2.5N acetic acid and eluted with 6N formic acid.

(b) To isolate amino acids, a column (10 x 60 mm) of Dowex 50W-X8(H^+form 200-400 mesh) was prepared and washed with 2N HCl (60-100 ml). Subsequently, deionized water was added until the effluent was neutral. The 1 ml of aqueous sample, containing the product of the reaction carried out in the presence of ammonia, was applied to this hydrogen column and the adsorbed amino acids were eluted with 1N ammonium hydroxide.

The acidic (a) or basic (b) compounds in the respective effluents were pooled at the peak radioactive region and evaporated to dryness under vacuum at 50°C. The residues were then dissolved in a minimum volume of distilled water and used for paper chromatography.

VII. Paper chromatography

The chromatograms were run in a descending manner, using Whatman No. 1 chromatographic paper in a presaturated steel chromatographic cabinet. The organic acids were identified by applying two-dimensional chromatography. The two solvent systems used were n-butanol-formic acid-water (4:1.5:1 v/v/v) in the first direction and n-butanol-pyridine-water (1:1:1 v/v/v) in the second. The amino acids were separated in one-dimensional chromatography, using the latter solvent system. The organic acids were located by spraying the paper with aroyl-glycine reagent, as described by Nordmann and Nordmann (1960), and heating at 125°C for 5 min. The amino acids were detected by spraying with 0.5% ninhydrin in butanol and heating.

Known organic and amino acids were used as standard solutions.

VIII. Enzyme assays

a. Dehydrogenases

The activities of 2-hydroxyglutarate and 2-ketoglutarate dehydrogenases studied were estimated by measuring the change in the absorbancy due to NADH at 340 nm, using a Gilford Recording Spectrophotometer (Model 2000). The reactions were carried out at room temperature in a total volume of 0.3 ml, using microcuvettes. The quantity of NADH oxidized was calculated using the following formula:

$$\text{NADH oxidized (umoles)} = \frac{\text{optical density change}}{\text{molar extinction coefficient}} \times \frac{\text{assay volume}}{100}$$

The molar extinction coefficient applied for NADH was $6.22 \times 10^3 cm^{-1}$.

a-1. 2-hydroxyglutarate dehydrogenase (EC 1.1.99.2)

This enzyme catalyzes the following reversible reaction:

$$COOH\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}COOH + NADH \longrightarrow COOH\text{-}CH_2\text{-}CH_2\text{-}CHOH\text{-}COOH + NAD$$

In the presence of NADH, 2-ketoglutarate is reduced to yield 2-hydroxyglutarate. The reaction mixture was composed of potassium phosphate buffer (pH 7.5), 50 umoles; CFE, 5 mg protein; NADH, 0.04 umoles; 2-ketoglutarate, 1 umole. The enzyme assay is essentially the same as described by Beppu and Arima (1960).

a-2. 2-ketoglutarate dehydrogenase (EC 1.2.4.2)

The enzyme catalyzes the following reaction:

$$\text{2-ketoglutarate} + NAD + CoASH \longrightarrow \text{succinyl coenzyme A} + NADH + CO_2$$

This enzyme was assayed using the method of Kaufman (1955). The

reaction mixture was composed of the same concentration of substrates in a total volume of 0.3 ml.

b. Reduction of succinic acid

The reaction mixture contained CFE, 4 mg; potassium phosphate buffer (pH 7.5), 2.5 mmoles; potassium succinate, 0.5 mmoles; ferredoxin, 800 ug; methyl viologen, 4 ug; NADPH, 2.5 umoles; and zinc powder in a total volume of 5 ml. The reaction was carried out under a hydrogen atmosphere at 37°C for 30 min. Succinic semialdehyde formation was detected, using the method of Bessman et al.(1953). The solvent system used for chromatography was isoamyl alcohol equilibrated with 0.1 M acetate buffer, pH 5.0.

c. Exchange reaction

The method of Palmquist and Baldwin (1966) was employed to measure catalysis of exchange between carbon dioxide and the carboxyl group of pyruvic, 2-hydroxyglutaric, or 2-ketoglutaric acid. The reaction mixture contained Sephadex-treated CFE, 2 mg; K_2HPO_4 (pH 8), 1.6 mmoles; $MgCl_2$, 4 umoles; 2-mercaptoethanol, 4 umoles; GSH, 2 umoles; CoASH, 0.068 umoles; Na-pyruvate, Na-2-hydroxyglutarate, or Na-2-ketoglutarate, 20 umoles; $NaHC^{14}O_3$, 20 umoles (5 uCi) in a total volume of 0.6 ml. The reaction was carried out under a hydrogen atmosphere at 37°C for 45 min.

Results

I. The reduction of succinic acid to succinic semialdehyde

The reaction mixture used was as described (Methods - VIII). The reaction was stopped using 1 ml of 15% (w/v) trichloroacetic acid, and the protein was precipitated. To detect the succinic

semialdehyde formation, 3 ml of a solution of 5N HCl, containing 15 mg of 2,4-dinitrophenylhydrazine, was added. The hydrazone derivative was subjected to treatment as described by Bessman et al. (1953) and applied to a descending paper chromatogram, using the acid isoamyl alcohol solvent. The R_f value obtained was the same (0.96) as that of a standard solution of succinic semialdehyde hydrazone. In the absence of enzyme, succinate, reducing reagents, or ferredoxin, there was no formation of a product that would react with 2,4-dinitrophenylhydrazine. Ferredoxin was removed by adding DEAE-cellulose to the Sephadex-treated CFE. This preparation was allowed to settle for 15 min and was centrifuged at 48,000 x g for 20 min at 0°C. The supernatant was used for ferredoxin-free enzyme assays. The omission of NADPH from the reaction mixture had no effect on the reaction.

II. Bicarbonate fixation in the presence of succinic semialdehyde

A system was devised in which CFE catalyzed the fixation of $HC\bar{O}_3$ in the presence of succinic semialdehyde. The absolute dependence of $HC\bar{O}_3$ fixation on the reducing system, succinic semialdehyde and the soluble enzyme is shown in Table 3. The table also demonstrates that the reaction does not require added thiamine pyrophosphate, ATP, or $MgCl_2$.

The effect of pH on $HC\bar{O}_3$ fixation is illustrated in Figure 1. The fixation was optimum at pH 7.5 and then dropped more sharply on the basic side than the acidic side of this optimum.

The dependence of $HC\bar{O}_3$ fixation on time, substrate and enzyme concentration is demonstrated in Figures 2, 3 and 4, respectively.

The $HC\bar{O}_3$ fixation in various treatments is given in Table 4.

The experiments were done at different times and conditions. Therefore, the following comparisons are not between, but within experiments.

In Experiment I there was as much fixation in a N_2 atmosphere as in a H_2 atmosphere.

In Experiment II the omission of the reducing reagents resulted in no fixation. Furthermore, NADH or NADPH could not replace the reducing system.

In Experiment III the removal of endogenous ferredoxin, coupled with omission of ferredoxin from the complete reaction mixture, caused a marked decrease in the $HC\bar{O}_3$ fixation. There was restoration of activity when ferredoxin was added to a system that had had ferredoxin removed.

The product formed in this reaction was first extracted with ether, and then applied to a Dowex-resin column as described (Methods - VI). The radioactivity eluted as an organic acid. Subsequently, the product was applied to a paper chromatogram (two-dimensional) and a pink spot was noted when the paper was sprayed with aroyl-glycine reagent. The pink color was seen with a standard solution of 2-hydroxyglutarate. This reagent develops a yellowish-brownish color with 2-ketoglutarate. The profile of the reaction product on the paper chromatogram is shown in Figure 5. This chromatographic behaviour was the same as that of standard 2-hydroxyglutarate. The R_f value in the first direction was 0.7 and in the second direction was 0.5.

To further confirm the results, a standard solution of 2-hydroxyglutarate was added to the treated sample just prior to

applying it on the paper chromatogram. Only one pink spot was detected and this was coincident with the radioactivity on the chromatogram strip.

In addition, the following experiment was designed to detect the formation of glutamate from succinic semialdehyde. Fifteen ml of a reaction mixture, as described in Table 4, was incubated. After 15 min incubation 2 mmoles of NH_4Cl, 50 umoles of NADH and 100 umoles of NAD were added and the incubation was carried out for another 30 min. The product formed was treated and applied to a paper chromatogram as described (Methods - VI and VII). The R_f value (0.29) obtained corresponded well with standard glutamic acid.

Table 3

Requirements for $H\bar{C}O_3$ fixation in the presence of succinic semialdehyde by extracts of mixed rumen microorganisms*

Treatment	$H^{14}\bar{C}O_3$ fixed (dpm)
Complete	11,600
Minus ATP	11,000
Minus thiamine pyrophosphate	10,950
Minus magnesium chloride	14,500
Minus enzyme	300
Minus succinic semialdehyde	260
Minus reducing reagents	205

*The complete reaction mixture contained Sephadex-treated bacterial extract 1 mg, and the following in umoles: potassium

phosphate buffer (pH 7.5), 250; thiamine pyrophosphate, 2.5; ATP, 5; $MgCl_2$, 5; succinic semialdehyde, 50; and sodium bicarbonate, 5 (containing $NaH^{14}CO_3$, 2.5 x 10^6dpm). The reducing system was composed of 1 ug methyl viologen, 200 ug ferredoxin and zinc powder (enough to reduce the methyl viologen); final volume 1.5 ml. The reaction mixture was incubated under a hydrogen atmosphere at 37°C in a shaking bath for 45 min.

Table 4

$H\bar{C}O_3$ fixation in various treatments*

Experiment		$H^{14}\bar{C}O_3$ fixed (dpm)
I.	(a) Complete (N_2)	21,000
	(b) Complete	20,500
II.	(a) Complete	10,000
	(b) Minus reducing reagents	300
	(c) Replacement of the reducing reagents by NADH or NADPH	300
III.	(a) Complete	14,500
	(b) DEAE-cellulose treated, no added ferredoxin	5,350
	(c) DEAE-cellulose treated, ferredoxin added	11,000

*The complete reaction mixture was the same as given in Table 3, except for omitting ATP, thiamine pyrophosphate and $MgCl_2$. The experiments were carried out under a hydrogen atmosphere unless otherwise specified.

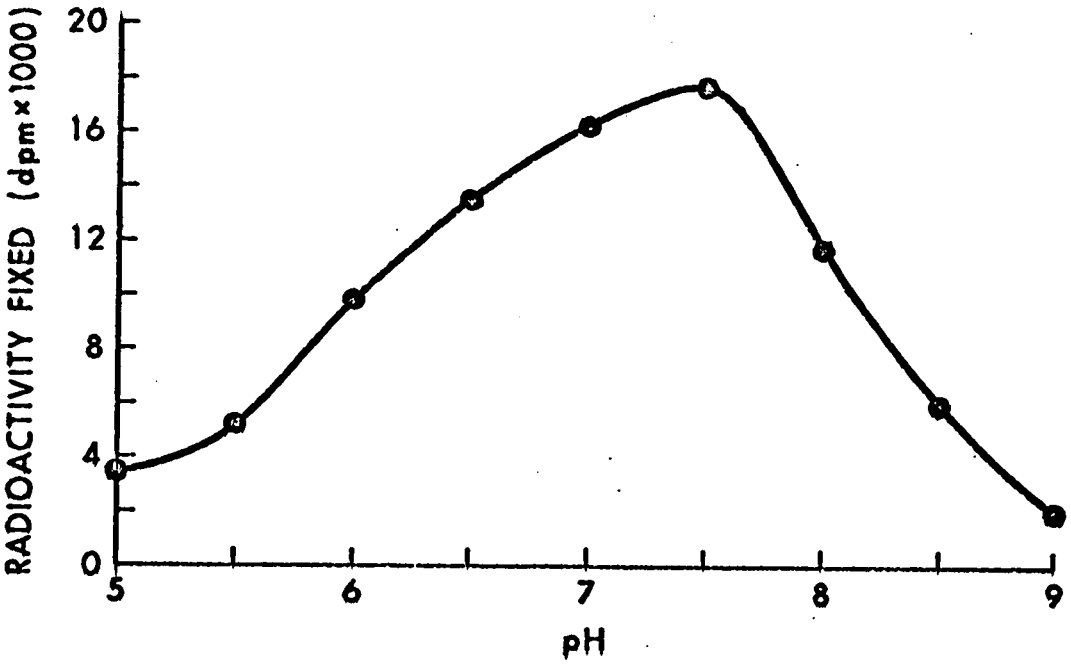


Figure 1. The effect of pH on $HC\bar{O}_3$ fixation in the presence of succinic semialdehyde. Experimental conditions were the same as given in Table 3, except for the following changes: ATP, thiamine pyrophosphate and magnesium chloride were omitted. The buffering system was composed of 83 umoles of each of the following buffer solutions: potassium phosphate (pH7.5), tris-buffer (pH7.0) and N-2-hydroxyethylpiperazine-N-2-ethane sulfonate (pH6.0). The buffer solutions were adjusted to various pH values by adding concentrate KOH or HCl to the original solution.

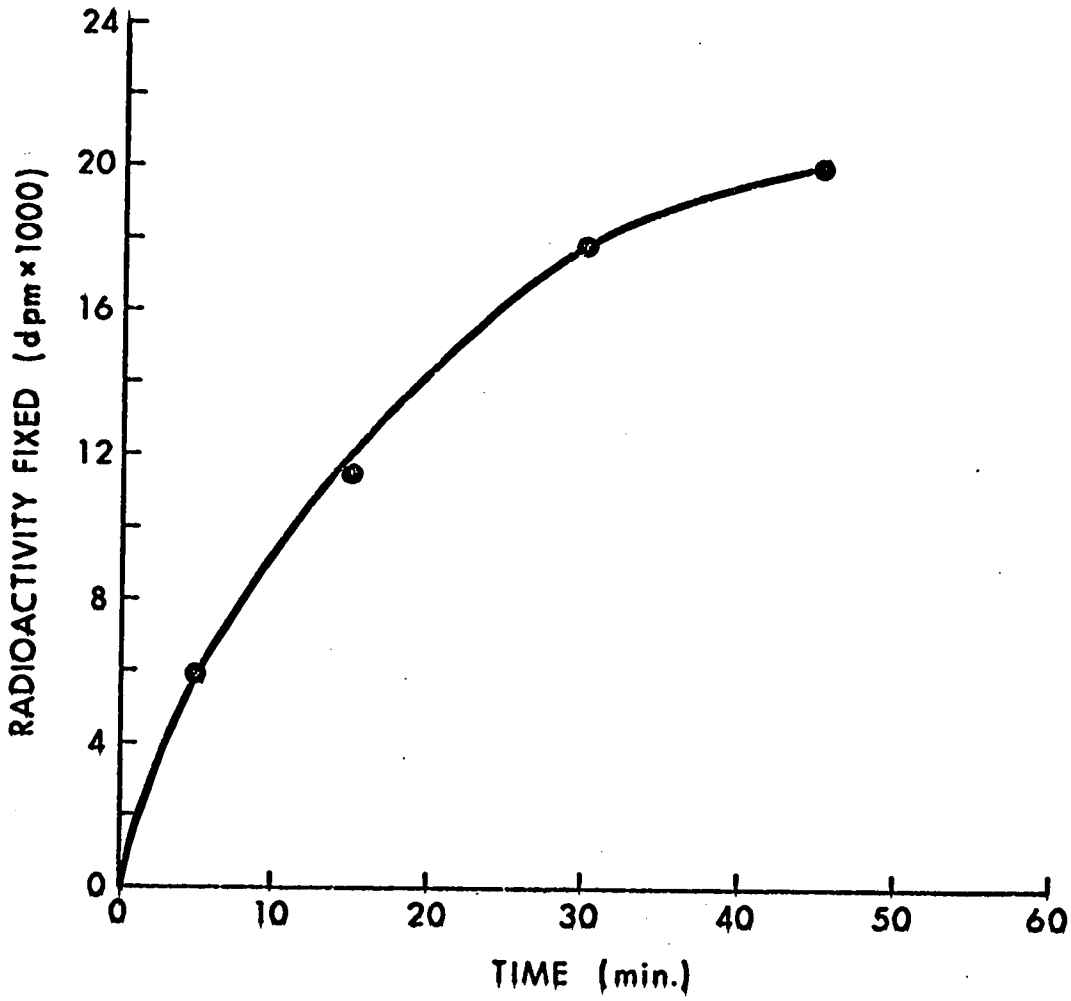


Figure 2. Time course of $HC\bar{O}_3$ fixation in the presence of succinic semialdehyde. Experimental conditions were the same as given in Table 3, except for varying the incubation time and omitting ATP, thiamine pyrophosphate and magnesium chloride.

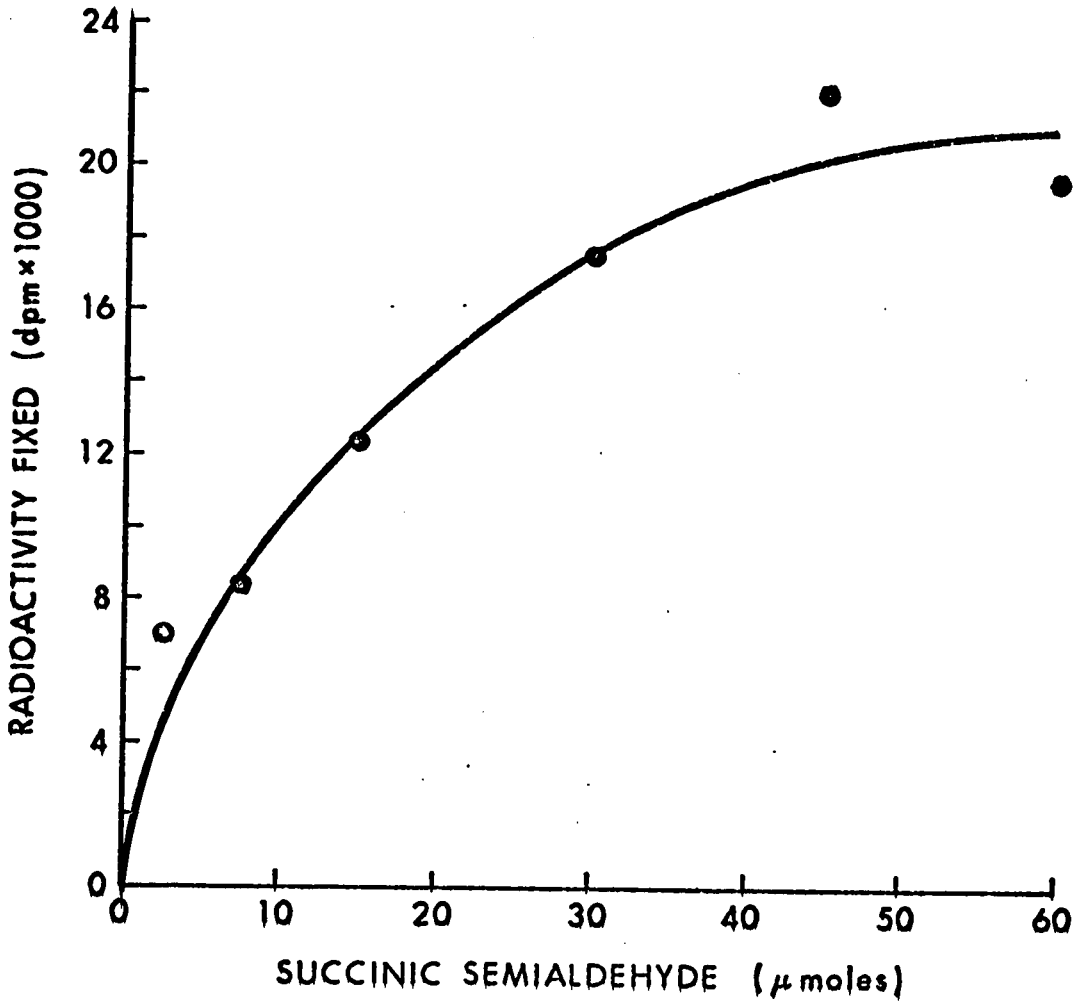


Figure 3. Dependence of $HC\tilde{O}_3$ fixation on succinic semialdehyde concentration. Experimental conditions were the same as given in Table 3, except for varying the substrate concentration and omitting ATP, thiamine pyrophosphate and magnesium chloride.

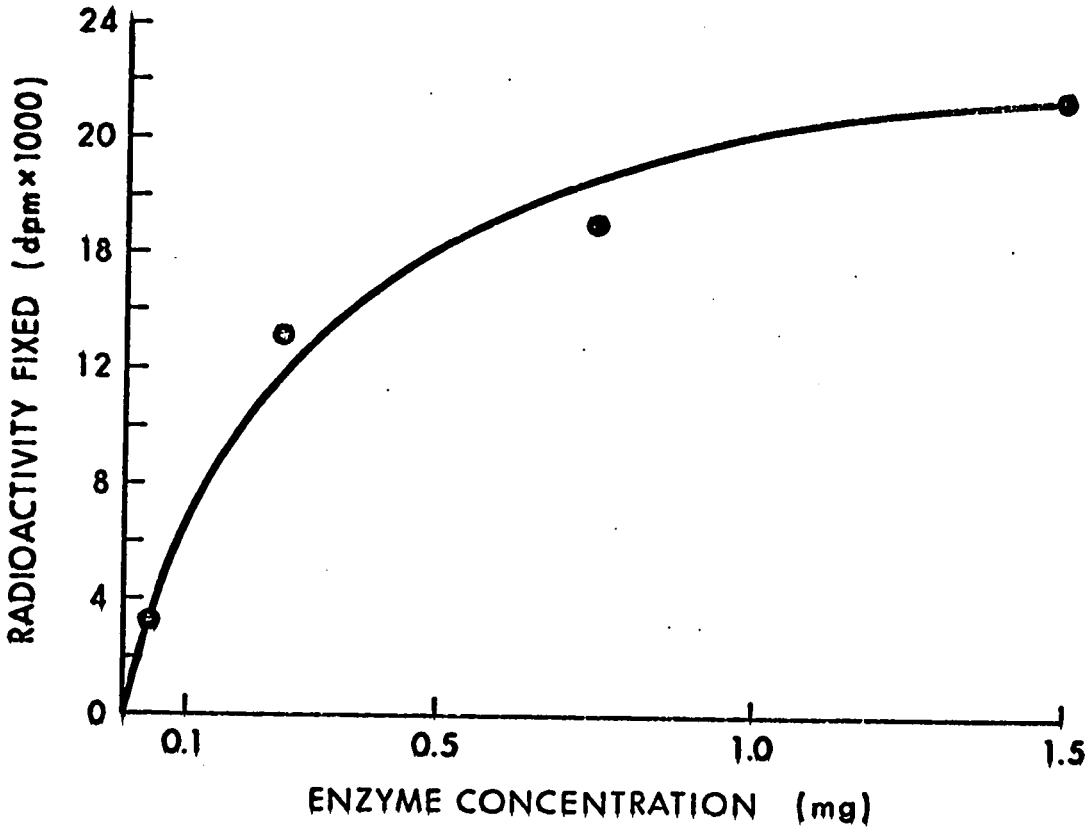


Figure 4. Dependence of $HC\bar{O}_3$ fixation on the concentration of enzyme in the presence of succinic semialdehyde. Experimental conditions were the same as given in Table 3, except for varying the enzyme concentration and omitting ATP, thiamine pyrophosphate and magnesium chloride.

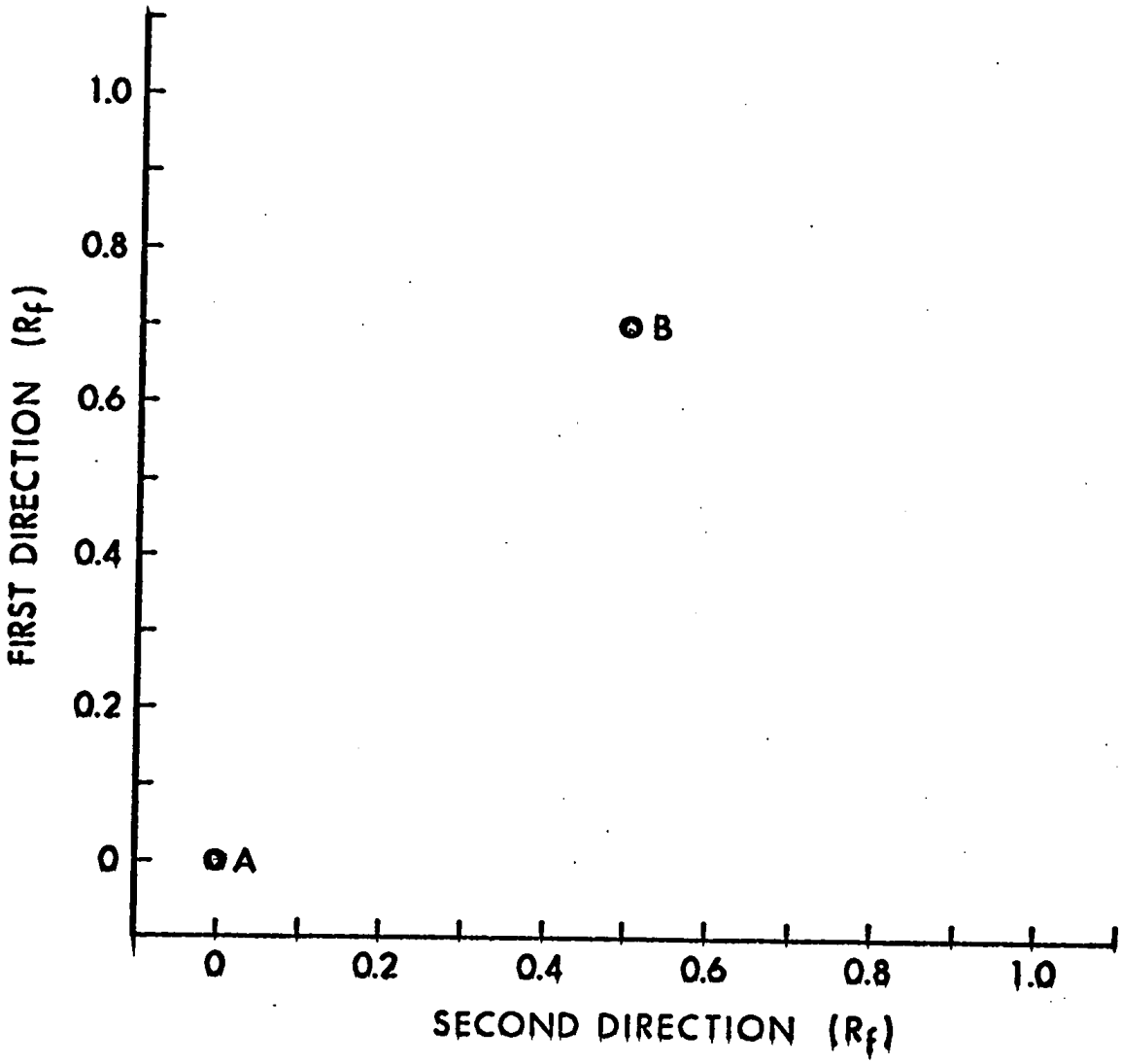


Figure 5. The profile of the reaction product on two-dimensional paper chromatogram. The solvents used were as described (Methods-VII). Points A and B represent the origin and the final location of the spot, respectively.

III. Amination of 2-hydroxyglutarate

The requirements for formation of ninhydrin-reactive products in the presence of 2-hydroxyglutarate are shown in Table 5. The reaction is dependent on the soluble enzyme, NH_4Cl, NAD and 2-hydroxyglutarate.

Figure 6 illustrates that the optimum synthesis occurs between pH 7.0 and pH 7.5.

The dependence of amino acid synthesis on time and on enzyme and substrate concentration is demonstrated in Figures 7 and 8, respectively.

To identify the amino acid formed, 15 ml of the reaction mixture was incubated as outlined in Table 5. The reaction was stopped and treated as described (Methods-VI and VII) until the 1 ml preparation was obtained. This sample was applied to a Dowex 50W-X8 column. Aliquots (1 ml) of the effluent were collected and evaporated to dryness under vacuum at 50°C. These samples were then dissolved in a minimum volume of water and tested for amino acids with 0.5% ninhydrin in butanol. Thus the concentration peak of amino acids was found. To a second column, 1 ml of a standard solution of 0.04 M glutamic acid was applied and was similarly treated. The concentration peak of glutamic acid corresponded quite well with the respective peak in the first column. Subsequently, the amino acid peak from the column was subjected to paper chromatography. The R_f value (0.29) obtained was identical to that of a standard solution of glutamic acid.

When 2-hydroxyglutarate plus NAD were replaced by 2-ketoglutarate

plus NADH, the total amino acid synthesis was increased 3.5 fold (0.92 umoles versus 0.264 umoles).

To establish whether the amination of 2-hydroxyglutaric acid to glutamate involves the participation of 2-ketoglutarate as an intermediate, the following experiment was done. To the 1 ml reaction mixture containing either 2-ketoglutarate plus NADH or 2-hydroxyglutarate plus NAD, 50 umoles of the carbonyl trapping reagent (hydrazine hydrochloride) were added. In both instances no synthesis of amino acid occurred.

In addition, in a reaction mixture containing the substrates 2-ketoglutarate and NADH as described (Methods - VIII) the activity of 2-hydroxyglutarate dehydrogenase was shown. The activity was 0.28 umoles NADH oxidized/min/mg protein. Correction was made for NADH oxidase.

Table 5

Requirement for amination of 2-hydroxyglutarate by CFE of mixed rumen microorganisms*

Treatment	Total Amino Acids (umoles)
Complete	0.320
Minus 2-hydroxyglutarate	0.048
Minus ammonium chloride	0.064
Minus NAD	0.080
Minus enzyme	0.024

*The complete reaction mixture contained in a final volume of 1 ml, 325 umoles potassium phosphate buffer, pH 7.5; Sephadex-treated extract, 1 mg; ammonium chloride, 0.1 mmoles; NAD, 5 umoles; and 2-hydroxyglutarate, 10 umoles. The incubation was carried out at 37°C for 45 min.

IV. Other enzyme assays

a. 2-ketoglutarate dehydrogenase

The attempts to demonstrate any activity of 2-ketoglutarate dehydrogenase in the CFE of mixed rumen microorganisms were unsuccessful.

b. Exchange reaction

An exchange reaction between $H^{14}C\bar{O}_3$ and pyruvate, 2-ketoglutarate and 2-hydroxyglutarate was shown. The $H^{14}C\bar{O}_3$ (dpm) fixed were 9,600, 4,500 and 2,000, respectively.

c. Acetyl coenzyme A and succinyl coenzyme A synthetase (EC 6.2.1.1 and EC 6.2.1.5)

The presence of these enzymes in the mixed rumen microorganisms was shown. The hydroxamate assay of Stadtman and Barker (1950) was employed. The two reaction mixtures used contained identical concentrations of enzyme and coenzyme A. Acetyl coenzyme A synthetase showed a higher activity than that of succinyl coenzyme A synthetase (3.2 umoles hydroxymate versus 1.5 umoles).

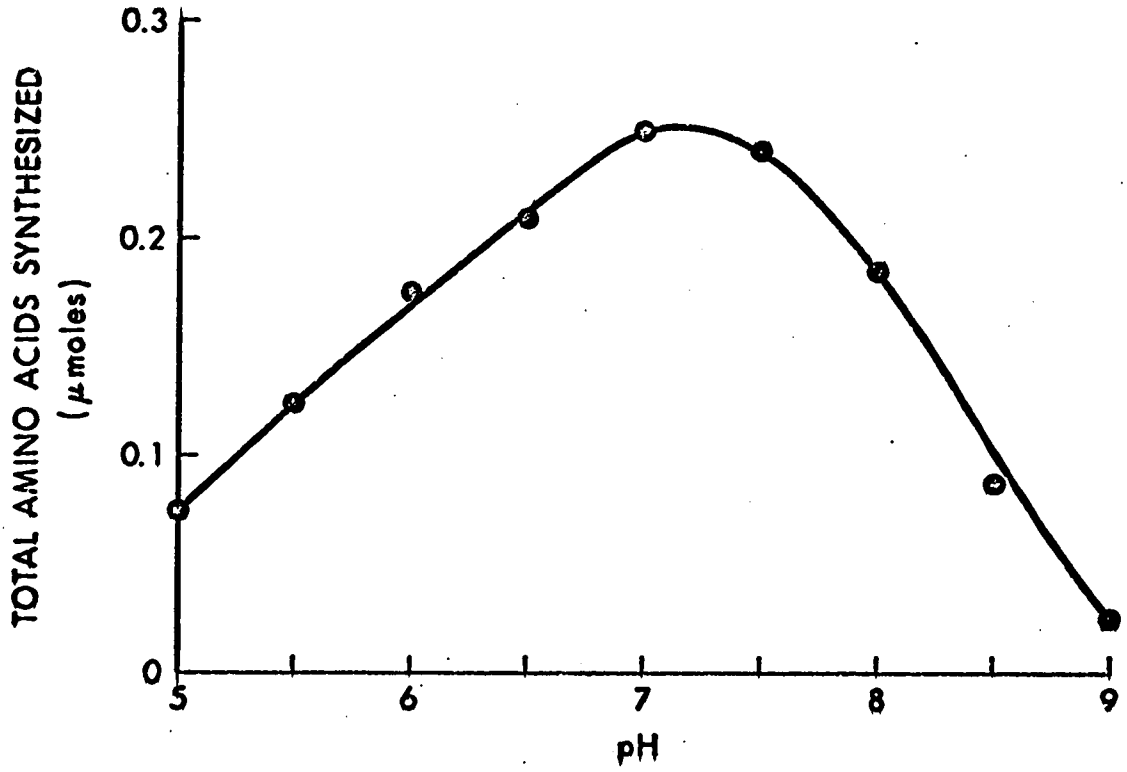


Figure 6. The effect of pH on the synthesis of total amino acids. Experimental conditions were the same as given in Table 5, except that the buffer was replaced with the buffering system as described in Figure 1.

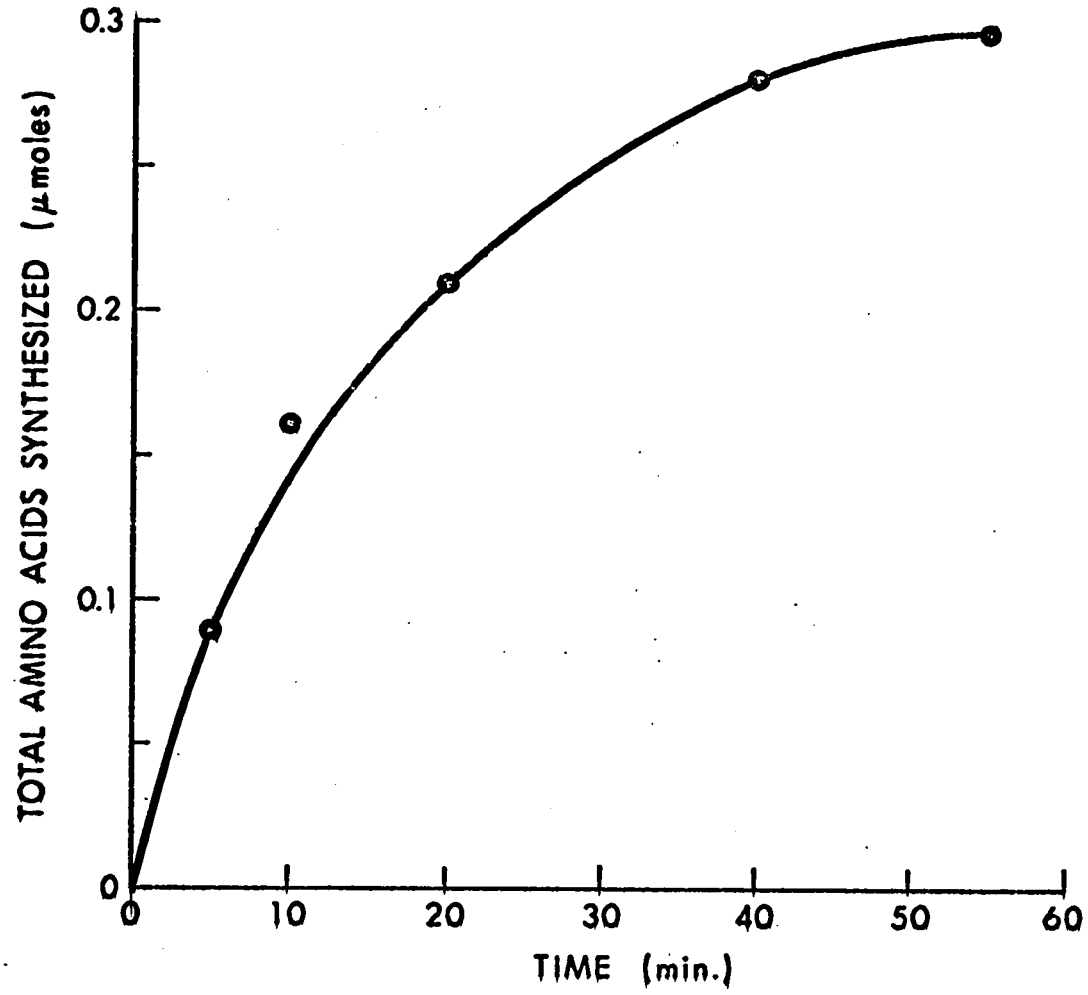


Figure 7. Time course of total amino acid synthesis. Experimental conditions were the same as given in Table 5, except for varying the time.

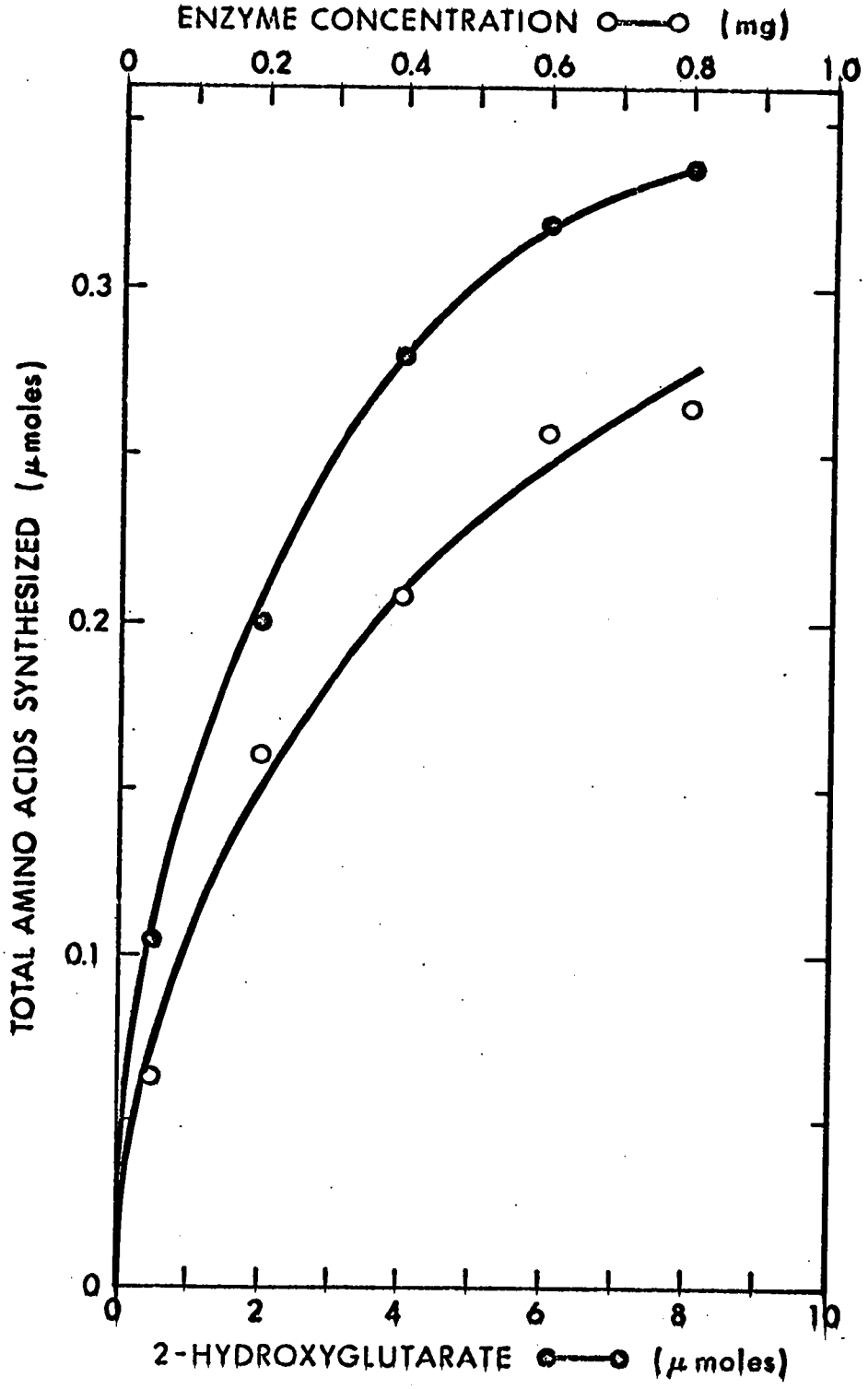


Figure 8. Dependence of total amino acid synthesis on substrate and enzyme concentration. Experimental conditions were the same as given in Table 5, except for varying the concentration of enzyme and substrate in the respective experiments.

DISCUSSION

Reports on the reductive carboxylation of succinyl coenzyme A in a number of photosynthetic bacteria (Buchanan and Evans, 1965; Buchanan et al.,1967; Evans, 1968) and the labelling pattern of glutamate produced by mixed rumen microorganisms incubated in the presence of $NaH^{14}CO_3$ (Milligan, 1970) suggested the possible existence of this carboxylation reaction in rumen microbes. The observations of Allison and Robinson (1970) on the labelling pattern of glutamic acid with growing cultures and with CFE of the rumen isolate Bacteroides ruminicola using ^{14}C-succinate as a substrate also support the occurrence of the reductive carboxylation in this rumen organism.

The experiments in the present studies were designed to investigate the existence of the enzymes which catalyze the biosynthesis of glutamate in the reverse TCA pathway in mixed rumen microorganisms. The experimental data obtained suggests the sequential reactions as described in Figure 9 for the reductive biosynthesis of glutamate.

Figure 9

Succinate route of glutamate synthesis by mixed rumen microorganisms

(1) Succinic acid + FdH ⟶ Succinic semialdehyde + Fd

(2) Succinic semialdehyde + FdH + HCO_3^- ⟶ 2-hydroxyglutarate + Fd

(3) 2-hydroxyglutarate + NH_4^+ ⟶ glutamate

Sum: succinic acid + 2 FdH + HCO_3^- + NH_4^+ ⟶ glutamate + 2 FdH

where Fd = oxidized ferredoxin and FdH = reduced ferredoxin.

The formation of succinate prior to Equation 1 in the reverse TCA pathway would entail the reductive conversion of oxaloacetate to succinate through malate and fumarate. Hopgood and Walker (1969) have found the enzymes, DPNH-malate dehydrogenase (EC 1.1.1.37), fumarate hydratase (EC 4.2.1.2) and fumarate reductase which are necessary for formation of succinate from oxaloacetate in CFE of Ruminococcus flavefaciens. These conversions also occur in the succinate pathway of propionate formation in rumen microbes (Baldwin et al.,1962; Baldwin et al., 1963). Oxaloacetate, in turn is likely synthesized from the carboxylation of phosphoenolpyruvate or pyruvate.

In the presence of $H^{14}C\bar{O}_3$ the intermediates of glutamate synthesis by the way of the reverse TCA pathway as proposed (Figure 9) would be anticipated to be labelled in positions shown in Figure 10.

Figure 10

Labelling patterns of the intermediates in the biosynthesis of glutamate by the reverse TCA pathway in the presence of $H^{14}C\bar{O}_3$

(1) $COOH\text{-}CO\text{-}CH_3 + H\overset{*}{C}\bar{O}_3 \xrightarrow{ATP} COOH\text{-}CO\text{-}CH_2\text{-}\overset{*}{C}OOH$

(2) $COOH\text{-}CO\text{-}CH_2\text{-}\overset{*}{C}OOH \xrightarrow{2H} COOH\text{-}CHOH\text{-}CH_2\text{-}\overset{*}{C}OOH$

(3) $COOH\text{-}CHOH\text{-}CH_2\text{-}\overset{*}{C}OOH \xrightarrow{-H_2O} \overset{*}{C}OOH\text{-}CH = CH\text{-}\overset{*}{C}OOH$

(4) $\overset{*}{C}OOH\text{-}CH = CH\text{-}\overset{*}{C}OOH \xrightarrow{2H} \overset{*}{C}OOH\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH$

(5) $\overset{*}{C}OOH\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH \xrightarrow[-H_2O]{2H} \overset{*}{C}HO\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH$

(6) $\overset{*}{C}HO\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH + H\overset{*}{C}\bar{O}_3 \xrightarrow{2H} \overset{*}{C}OOH\text{-}\overset{*}{C}HOH\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH$

(7) $\overset{*}{C}OOH\text{-}\overset{*}{C}HOH\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH \xrightarrow{NH_4^+} \overset{*}{C}OOH\text{-}\overset{*}{C}HNH_2\text{-}CH_2\text{-}CH_2\text{-}\overset{*}{C}OOH$

*^{14}C-labelled; (1) pyruvic acid (2) oxaloacetic acid (3) malic acid (4) fumaric acid (5) succinic acid (6) succinic semialdehyde, and (7) 2-hydroxyglutarate.

Thus the reverse TCA pathway would result in incorporation of $H^{14}C\bar{O}_3$ in the positions C-1, C-2 and C-5 of glutamate. This would agree with the labelling pattern of glutamate formed by mixed rumen microorganisms incubated in the presence of $NaH^{14}CO_3$ (Milligan, 1970). The theoretical distribution in positions C_2 and C_5 would be 25% of the total incorporated isotope because of the randomization at the level of fumarate and succinate, whereas the anticipated activity in C-1 would be 50%.

Conversion of succinate to succinic semialdehyde (Equation 1)

In this reaction succinic acid is reduced to yield succinic semialdehyde. The reaction is dependent on the soluble enzyme, succinate and reduced ferredoxin. The product was found to be reactive with 2,4-dinitrophenylhydrazine and the resultant 2, 4-dinitrophenylhydrazone behaved as standard succinic semialdehyde 2,4-dinitrophenylhydrazone on paper chromatography. The enzyme which catalyzes this reaction seems to be different from NADP-linked succinic semialdehyde dehydrogenase (EC 1.2.1.16) (Jakoby, 1962), since the omission of the reduced ferredoxin from the reaction mixture resulted in no further formation of succinic semialdehyde, whereas the removal of NADPH had no effect.

The conversion of succinic acid to succinic semialdehyde is energetically unfavorable in the absence of a very powerful reductant; however, the mass action of succinic acid present in the rumen (Bryant et al., 1958), or the high reducing potential of ferredoxin likely overcomes this energy barrier.

Reductive carboxylation of succinic semialdehyde (Equation 2)

In this reaction succinic semialdehyde is reductively carboxylated to yield 2-hydroxyglutarate. The product formed was isolated and identified as described (Methods - VI and VII).

The results show that the enzyme does not require ATP and therefore would not entail the expenditure of phosphate bonds. The optimal pH is within the physiological range. In addition to the identification of the product as 2-hydroxyglutarate, an exchange reaction between 2-hydroxyglutarate and bicarbonate was observed to be catalyzed by CFE, suggesting the possibility of CO_2 fixation during synthesis of 2-hydroxyglutarate. Ferredoxin in the reaction mixture was reduced using powdered zinc in the presence of methyl viologen. Apparently, the reaction requires a very powerful reductant, since NADH and NADPH could not replace the reducing system.

Because of the role of ferredoxin in Equations 1 and 2, it is worthwhile to broaden the discussion on this topic.

Ferredoxin is the most electronegative electron carrier protein known in cellular oxidation-reduction reactions (Tagawa and Arnon, 1962). Baldwin and Milligan (1964) have demonstrated the presence of ferredoxin in the rumen isolate Peptostreptococcus elsdenii. Also, large quantities of ferredoxin were obtained from mixed rumen microorganisms (Appendix II). Therefore, it is quite possible that many rumen bacteria may contain ferredoxin. Under natural rumen conditions, ferredoxin may be reduced by the H_2-hydrogenase system or by pyruvate oxidation. Palmquist and Baldwin (1966) have shown the existence of hydrogenase (EC 1.12.1.1) in rumen microflora. The reactions in Equations 1 and 2 did not proceed

in a hydrogen atmosphere when the zinc and methyl viologen were removed suggesting the lack of hydrogenase activity. Under these conditions the failure to obtain the conversions in the presence of hydrogen may be due to the binding of hydrogenase to the cell membranes as is the case in Vibrio succinogenes (Niederman and Wolin, 1969). Alternatively, the enzyme may be inactivated by the removal of cofactor(s) during the process of preparation of CFE. Pyruvate was not included because of the probability of confounding side reactions. Palmquist and Baldwin (1966) have shown exchange between CO_2 and the carboxyl group of pyruvate in extracts of rumen microbes. Similarly, in the present studies, this exchange reaction was observed.

Recently, Tagawa and Arnon (1968) have demonstrated that the redox potentials of spinach and clostridial ferredoxins are independent of pH in the physiological range. If the rumen microbial ferredoxins exhibit a similar property, the stable redox potential would be of advantage to the metabolic function of the rumen microbes as the pH values in the rumen reflect many factors and fluctuate to the extent of 1.2 pH units (Emmanuel et al., 1969).

The carboxylation of succinic semialdehyde would likely be thermodynamically unfavorable; however, ferredoxin with its highly negative electrode potential (E_o' = -0.39 to -0.42 volts) probably favors this conversion. The relationship between the free energy of the reaction and the redox potential expressed in terms of calories is as follows:

$$\Delta F^\circ = \frac{nF\Delta E'_o}{4.18}$$

where ΔF° is the standard free energy of the reaction, n is the number of electrons involved, F is the Faraday constant (96,500 coulombs) and $\Delta E'_o$ is the difference between the E'_o values of two half electrodes. It can be seen that the negative potential of ferredoxin would tend to give rise to negative ΔF° and thus favors the occurrence of overall reaction.

Amination of 2-hydroxyglutarate (Equation 3)

The biosynthesis of glutamate from 2-hydroxyglutarate was shown by identification of the product by paper chromatography and by measuring requirements for formation of glutamate. This amination may proceed in two steps. First 2-hydroxyglutarate in the presence of NAD is likely converted to 2-ketoglutarate. The latter is then reductively aminated to glutamate (Palmquist and Baldwin, 1966).

The information obtained in the present studies on the presence of 2-hydroxyglutarate dehydrogenase, the absolute dependence of amination of 2-hydroxyglutarate to glutamate on NAD and the pronounced negative effect of hydrazine hydrochloride (carbonyl trapping reagent) on this amination supports the proposed mechanism.

Reitz and Rodwell (1969) have found the enzyme 2-hydroxyglutarate dehydrogenase which catalyzes the conversion of 2-hydroxyglutarate to 2-ketoglutarate in Pseudomonas putida. The report of Johnson and Westlake (1969) on the pattern of degradation of glutamate in CFE of Peptococcus aerogenase also supports the suggested mechanism. They concluded that glutamate is oxidatively deaminated to

2-ketoglutarate, and the latter is then reduced to 2-hydroxyglutarate. Johnson (1971) has purified the respective enzymes in this bacteria. The proposed reactions in Figure 9 are the reverse process of glutamate degradation, and possibly the reduced NAD formed in the conversion of 2-hydroxyglutarate to 2-ketoglutarate is used in the reductive amination of the latter to glutamate. It is also possible that this amination may occur in a coordinated fashion without free intermediates. Studies with purified enzymes are needed to establish the actual mechanism involved.

Reactions 1 and 3 in Figure 9 are reported for the first time in rumen microorganisms. Reaction 2 is a new mechanism of reductive carboxylation which previously has not been reported in the literature.

The reductive carboxylation of succinic semialdehyde may not be the only mechanism for the biosynthesis of glutamate by a reverse TCA pathway. Carboxylation of other possible substrates would also result in a similar labelling pattern of glutamate.

The carboxylation of 4-aminobutyrate was considered. It was postulated that such conversion could entail the following reaction sequence:

(1) Succinic acid + 2H $\longrightarrow$ succinic semialdehyde

(2) Succinic semialdehyde + glutamate $\longrightarrow$ 4-aminobutyrate + 2-ketoglutarate

(3) 4-aminobutyrate + $HC\bar{O}_3$ $\longrightarrow$ glutamate

(4) 2-ketoglutarate + 2H + NH_4^+ $\longrightarrow$ glutamate

---

Sum: succinic acid + 4H + $HC\bar{O}_3$ + NH_4^+ $\longrightarrow$ glutamate

The reduction of succinic acid to succinic semialdehyde in Equation 1 was found in the present studies. Reaction 2 is catalyzed by glutamic-succinic semialdehyde transaminase (Jakoby, 1962). The conversion of 2-ketoglutarate to glutamate in Equation 4 is catalyzed by NAD and NADP-linked glutamic dehydrogenases (Palmquist and Baldwin, 1966). When 4-aminobutyrate was used as the substrate in the present system there was no fixation of $HC\bar{O}_3$, indicating the lack of the proposed carboxylation reaction.

One of the steps in the succinate pathway of propionate formation in rumen microorganisms is the transcarboxylation between methylmalonyl coenzyme A and pyruvate to yield propionyl coenzyme A and oxaloacetate (Baldwin et al., 1962). Similarly, an analogous transcarboxylation reaction between succinyl coenzyme A and pyruvate to yield 2-ketoglutarate and acetyl coenzyme A was considered. Such a transcarboxylation may itself require no high energy phosphate bond expenditure. The possibility of such transcarboxylation was investigated, employing a mixture of CFE, K_2HPO_4 buffer, succinyl coenzyme A and pyruvate. At the end of the incubation time, the reaction was stopped, using perchloric acid (70%,w/w), centrifuged and then neutralized with KOH. To detect the possible formation of 2-ketoglutarate, the glutamate dehydrogenase enzyme assay was applied (Bergmeyer and Bernt, 1963). The attempts to show this reaction were unsuccessful. Nevertheless, the possibility of the occurrence of this transcarboxylation reaction in the rumen microorganisms should not be ruled out. In reviewing the literature, subsequently, the report of Northrop and Wood (1969) on the presence of zinc and cobalt at the active site of methylmalonyl coenzyme A -

pyruvate carboxyltransferase (EC 2.1.3.1) was noted. If the postulated transcarboxylase exhibits similar properties and ions are loosely bound to the active site, the treatment with Sephadex may have resulted in the inactivation of the enzyme.

The activity of 2-ketoglutarate synthase, which catalyzes the reductive carboxylation of succinyl coenzyme A to yield 2-ketoglutarate has been observed in photosynthetic bacteria (Buchanan and Evans, 1965). At the outset, experiments were conducted in the present studies to show the presence of this enzyme in the rumen microorganisms using a similar procedure to that reported by these authors. In the pilot experiments, a commercial ferredoxin from spinach was reduced by illumination of chloroplasts in the presence of 2-mercaptoethanol as described by Whatley and Arnon (1963). Maximum precaution was taken to carry out the experiments under anaerobic conditions. In addition, ferredoxin prepared from mixed rumen microbes as described in Appendix II was used, and reduced by dithionite ($Na_2S_2O_4$) or powdered zinc in the presence of methyl viologen. Synthetically prepared succinyl phosphate and succinyl coenzyme A as well as succinate plus ATP and coenzyme A were used as substrates. The bivalent ions manganese and magnesium were included in the reaction mixture. In no instance was there $H^{14}C\bar{O}_3$ fixation that could be attributed to 2-ketoglutarate synthase.

Concurrent to the present investigations, Robinson and Allison (1969) and Allison and Robinson (1970) have reported on the reductive carboxylation of succinyl coenzyme A in the rumen isolate Bacteroides ruminicola. These authors suggested pyruvate may function as the

electron donor. However, their results are not concrete at present and further experimental evidence is needed to confirm the reaction in this bacteria. In their Sephadex-treated extract, the omission of coenzyme A and ATP should result in a very low activity, whereas the reaction still proceeded fairly readily. The authors were also unable to demonstrate any activity of succinyl coenzyme A synthetase (personal communication). Nevertheless, the possibility of the carboxylation of succinyl coenzyme A in the rumen microflora can not be excluded. The activity of succinyl coenzyme A sythetase, and also the exchange reaction between bicarbonate and 2-ketoglutarate in extracts of mixed rumen microorganisms were observed in the present studies, suggesting the possible formation of 2-ketoglutarate in the fashion as proposed by Allison and Robinson (1970). The exchange reaction may, however, be catalyzed by a system involved in the degradation of 2-ketoglutarate, if there is a "clastic" type of system for 2-ketoglutarate analogous to that for pyruvate degradation (Mortlock et al., 1959; Peel, 1960; Whiteley and McCormick, 1963). In this connection, NAD-linked 2-ketoglutarate dehydrogenase could not be detected in CFE of mixed rumen organisms by the method described earlier (Methods - VIII).

Alternatively, the biosynthesis of 2-ketoglutarate as proposed by Allison and Robinson (1970) may occur through a transcarboxylation between succinyl coenzyme A and pyruvate, since pyruvate was included in their reaction mixture in the presence of coenzyme A, ATP and ^{14}C-succinate. In general, the reductive carboxylation in Bacteroides rumincola may be distinct from other species of rumen microorganisms because of the lack of ferredoxin and hydrogenase in this organism

(Allison and Robinson, 1970).

In a reducing environment such as the rumen, the disposal of electrons and the conservation of energy and carbon are important factors for the optimal growth of rumen microorganisms. The forward and the reverse TCA pathways are compared on the basis of these factors in schematic Figure 11.

The conversion of fumarate to succinate in this Figure is considered to be a site of energy conservation. Barton et al. (1970) working with anaerobic sulfate reducing bacteria suggested that the formation of a high energy bond in this conversion was coupled to the oxidation of molecular hydrogen catalyzed by hydrogenase. This oxidative phosphorylation may also occur in ruminal microbes because of the presence of both hydrogenase (Palmquist and Baldwin, 1966) and fumarate reductase (Hopgood and Walker, 1969). Other sufficiently electro-negative reductants such as ferredoxin, or NADH could provide sufficient free energy in the conversion of fumarate to succinate to allow for formation of a high energy phosphate bond. Hopgood and Walker (1969) have found a NADH-linked fumarate reductase in a rumen isolate. White et al. (1962) suggested the possible formation of a high energy phosphate bond during the synthesis of succinate from fumarate which is catalyzed by cytochrome-linked fumaric reductase in the rumen isolate Bacteroides ruminicola.

Figure 11 shows that the overall conversion in the biosynthesis of glutamate from pyruvate by way of forward TCA pathway is associated with a yield of electrons and the release of CO_2. On the other hand, the reverse TCA pathway results in the fixation of CO_2 and utilization of electrons. Moreover, in the forward TCA pathway,

greater input of pyruvate is required per unit of glutamate formed. The extra pyruvate used could have yielded a high energy phosphate bond if it was instead degraded to acetyl coenzyme A or acetyl phosphate, and then acetate (Baldwin, 1965). Therefore, considering the extra input of pyruvate and the overall reactions in Figure 11, the synthesis of glutamic acid by the reverse TCA pathway via carboxylation of succinyl coenzyme A and through carboxylation of succinic semialdehyde would result in the conservation of one and two high energy phosphate bonds, respectively, over the forward TCA pathway.

The opposite characteristics of the forward and the reverse TCA pathways may be the main reason for their concurrent occurrence in the rumen organisms.

It is not possible yet to conclude whether the operation of more than one pathway for the synthesis of glutamic acid occurs simultaneously in one species of bacteria. Perhaps only the more favorable pathway under the circumstances functions. The report of Allison and Robinson (1970) on the probable absence of isocitric dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42) in Bacteroides ruminicola and the operation of only reductive carboxylation of succinate for the synthesis of glutamate indicates this. However, it is also possible that under different conditions in the rumen certain enzymes of the various pathways may be induced or supressed within any one organism.

The biosynthesis of a number of amino acids by reductive carboxylation of organic acids in rumen microorganisms (Allison, 1969) may follow a similar fashion as proposed in Figure 9.

Figure 11

The production and utilization of ATP, CO_2 and electrons in the biosynthesis of glutamate from pyruvate through different pathways by rumen microorganisms

---

A. Forward TCA pathway:

Pyruvate + CO_2 + ATP ⟶ oxaloacetate + ADP

Pyruvate + CoASH ⟶ acetyl-CoA + 2H + CO_2

Acetyl-CoA + oxaloacetate ⟶ citrate + CoASH

Citrate ⟶ 2-ketoglutarate + 2H + CO_2

2-ketoglutarate + 2H + NH_4^+ ⟶ glutamate

Sum: 2 pyruvate + ATP + NH_4^+ ⟶ glutamate + ADP + 2H + CO_2

B. Reverse TCA pathway (carboxylation of succinyl-CoA):

Pyruvate + CO_2 + ATP ⟶ oxaloacetate + ADP

Oxaloacetate + 2H ⟶ malate

Malate ⟶ fumarate

Fumarate + 2H + ADP ⟶ succinate + ATP

Succinate + CoASH + ATP ⟶ succinyl-CoA + ADP

Succinyl-CoA + 2H + CO_2 ⟶ 2-ketoglutarate + CoASH

2-ketoglutarate + 2H + NH_4^+ ⟶ glutamate

Sum: Pyruvate + ATP + $2CO_2$ + 8H + NH_4^+ ⟶ glutamate + ADP

cont'd.....

C. Reverse TCA pathway (carboxylation of succinic semialdehyde):

$$\text{Pyruvate} + CO_2 + \text{ATP} \longrightarrow \text{oxaloacetate} + \text{ADP}$$

$$\text{Oxaloacetate} + 2H \longrightarrow \text{malate}$$

$$\text{Malate} \longrightarrow \text{fumarate}$$

$$\text{Fumarate} + 2H + \text{ADP} \longrightarrow \text{succinate} + \text{ATP}$$

$$\text{Succinate} + 2H \longrightarrow \text{succinic semialdehyde}$$

$$\text{Succinic semialdehyde} + 2H + CO_2 \longrightarrow \text{2-hydroxyglutarate}$$

$$\text{2-hydroxyglutarate} \longrightarrow \text{2-ketoglutarate} + 2H$$

$$\text{2-ketoglutarate} + 2H + NH_4^+ \longrightarrow \text{glutamate}$$

Sum: $\text{Pyruvate} + 2CO_2 + 8H + NH_4^+ \longrightarrow \text{glutamate}$

BIBLIOGRAPHY

Ajl, S.J. and C.H. Werkman. 1948. Enzymic fixation of carbon dioxide in ∝-ketoglutaric acid. Proc. Nat. Acad. Sci. U.S.A., 34:491-498.

Allison, M.J. 1965. Phenylalanine biosynthesis from phenylacetic acid by anaerobic bacteria from the rumen. Biochem. Biophys. Res. Comm. 18:30-35.

Allison, M.J. 1969. Biosynthesis of amino acids by ruminal microorganisms. J. Anim. Sci. 29:797-807.

Allison, M.J. 1970. Nitrogen metabolism of ruminal-organisms. p. 456 to 473. In A.T. Phillipson (ed.) Physiology of digestion and metabolism in the ruminant. Oriel Press Ltd., Newcastle upon Tyne.

Allison, M.J., M.P. Bryant and R.N. Doetsch. 1962. Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for Ruminococci. I. Incorporation of isovalerate into leucine. J. Bacteriol. 83:523-532.

Allison, M.J. and M.P. Bryant. 1963. Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria. Arch. Biochem. Biophys. 101:269-277.

Allison, M.J., J.A. Bucklin and I.M. Robinson. 1966. Importance of the isovalerate carboxylation pathway of leucine biosynthesis in the rumen. Appl. Microbiol. 14:807-814.

Allison, M.J. and I.M. Robinson. 1967. Tryptophan biosynthesis from indolacetic acid by anaerobic bacteria from the rumen. Biochem. J. 102:36P-37P.

Allison, M.J. and I.M. Robinson. 1970. Biosynthesis of ∝-ketoglutarate by the reductive carboxylation of succinate in Bacteroides ruminicola. J. Bacteriol. 104:50-56.

Andrew, I.G. and J.G. Morris. 1965. The biosynthesis of alanine by Clostridium kluyveri. Biochim. Biophys. Acta. 97:176-179.

Bachofen, R., B.B. Buchanan and D.I. Arnon. 1964. Ferredoxin as a reductant in pyruvate synthesis by a bacterial extract. Proc. Nat. Acad. Sci. U.S.A., 51:690-694.

Baldwin, R.L. 1965. Pathways of carbohydrate metabolism in the rumen. P. 379 to 389. In R.W. Dougherty, R.S. Allen, W. Burroughs, N.L. Jacobson and A.D. McGilliard (ed.) Physiology of digestion in the ruminant. Butterworth Inc., Washington, D.C.

Baldwin, R.L. and L.P. Milligan. 1964. Electron transport in Peptostreptococcus elsdenii. Biochim. Biophys. Acta. 22:421-431.

Baldwin, R.L., W.A. Wood and R.S. Emery. 1962. Conversion of lactate-C^{14} to propionate by the rumen microflora. J. Bacteriol. 83:907-913.

Baldwin, R.L., W.A. Wood and R.S. Emery. 1963. Conversion of glucose-C^{14} to propionate by the rumen microflora. J. Bacteriol. 85:1346-1349.

Barton, L.L., J. LeGall and D. Peck, Jr. 1970. Phosphorylation coupled to oxidation of hydrogen with fumarate in extracts of the sulfate reducing bacterium, Desulfovibrio gigas. Biochem. Biophys. Res. Comm. 41:1036-1042.

Beppu, T. and K. Arima. 1960. Metabolism of L-alloisocitric acid in Achromobacter. J. Biochem. (Japan), 48:557-568.

Bergmeyer, HU. and E. Bernt. 1963. α-Ketoglutarate. P. 324 to 327. In Hans-Ulrich Bergmeyer (ed.) Methods of enzymatic analysis. Academic Press, New York and London.

Bessman, S.P., J. Rossen and E.C. Layne. 1953. γ-aminobutyric acid-glutamic acid transamination in brain. J. Biol. Chem. 201:385-391.

Bruno, G.A. and J.E. Christian. 1961. Correction for quenching associated with liquid scintillation counting. Anal. Chem. 33:650-651.

Bryant, M.P. 1965. Rumen methanogenic bacteria. P. 411 to 418. In R.W. Dougherty. R.S. Allen, W. Burroughs, N.L. Jacobson and A.D. McGilliard (ed.) Physiology of digestion in the ruminant. Butterworths Inc., Washington, D.C.

Bryant, M.P. and I.M. Robinson. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. 84:605-614.

Bryant, M.P., N. Small, C. Bouma and H. Chu. 1958. Species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol. 76:15-23.

Buchanan, B.B. 1969. Role of ferredoxin in the synthesis of α-ketobutyrate from propionyl coenzyme A and carbon dioxide by enzymes from photosynthetic and nonphotosynthetic bacteria. J. Biol. Chem. 244:4218-4223.

Buchanan, B.B., R. Bachofen and D.I. Arnon. 1964. Role of ferredoxin in the reductive assimilation of CO_2 and acetate by extracts of the photosynthetic bacterium, Chromatium. Proc. Nat. Acad. Sci. U.S.A. 52:839-847.

Buchanan, B.B. and M.C.W. Evans. 1965. The synthesis of ∝-ketoglutarate from succinate and carbon dioxide by subcellular preparation of a photosynthetic bacterium. Proc. Nat. Acad. Sci. U.S.A., 54:1212-1218.

Buchanan, B.B., M.C.W. Evans and D.I. Arnon. 1967. Ferredoxin-dependent carbon assimilation in Rhodospirillum rubrum. Arch. Mikrobiol. 59:32-40.

Burchall, J.J., R.A. Niederman and M.J. Wolin. 1964. Amino group formation and glutamate synthesis in Streptococcus bovis. J. Bacteriol. 88:1038-1044.

Chalupa, W., J.H. Clark, P. Opliger and R. Lavker. 1968. Enzyme systems for ammonia metabolism in the rumen. Fed. Proc. 27:557.

Chalupa, W., J.H. Clark, P. Opliger, and R. Lavker. 1970. Ammonia metabolism in rumen bacteria and mucosa from sheep fed soy protein or urea. J. Nutr. 100:161-169.

Eisenstein, K.K. and J.H. Wang. 1969. Conversion of light to chemical energy I. porphyrin-sensitized photoreduction of ferredoxin by glutathione. J. Biol. Chem. 244:1720-1728.

Emmanuel, B., M.J. Lawlor and D.M. McAleese. 1969. The rummen buffering system of sheep fed pelleted roughage- oncentiate rations. Br. J. Nutr. 23:805-811.

Evans, M.C.W. 1965. The photoassimilation of succinate to hexose by Rhodospirillium rubrum. Biochem. J. 95:669-677.

Evans, M.C.W. 1968. Ferredoxin dependent synthesis of ∝-ketoglutarate and pyruvate by extracts of the green photosynthetic bacterium Chloropseudomonas ethylicum. Biochem. Biophys. Res. Comm. 30:146-150.

Evans, M.C.W. and B.B. Buchanan. 1965. Photoreduction of ferredoxin and its use in CO_2 fixation by subcellular system from a photosynthetic bacterium. Proc. Nat. Acad. Sci. U.S.A., 53:1420-1424.

Gottschalk, G. and H.A. Barker. 1966. Synthesis of glutamate and citrate by Clostridium kluyveri. A new type of citrate synthase. Biochemistry. 5:1125-1133.

Harlan, J.W. 1966. Liquid scintillation counting of CO_2 in aqueous carbonate solutions. P. 115 to 118. In S. Rothchild (ed.) Advan. tracer methodol. Vol. I. Plenum Press, New York.

Hildebrand, J.G. and L.B. Spector. 1969. Succinyl phosphate and the succinyl coenzyme A synthetase reaction. J. Biol. Chem. 244:2606-2613.

Hopgood, M.F. and D.J. Walker. 1969. Succinic acid production by rumen bacteria. III. Enzymic studies on the formation of succinate by Ruminococcus flavefaciens. Aust. J. Biol. Sci. 22:1413-1424.

Hungate, R.E. 1966. The rumen bacteria. P. 8 to 81. In The Rumen and its microbes. Academic Press Inc., New York.

Isle, D. and R.W. O'Brien. 1967. Glutamate biosynthesis by Clostridium kluyveri. An unusual distribution of radioactivity in glutamic acid. Biochim. Biophys. Acta. 141:454-465.

Jakoby, W.B. 1962. Enzymes of γ-aminobutyrate metabolism (bacterial). P. 765 to 778. In S.P. Colowick and N.O. Kaplan (ed.) Methods in enzymology. Vol. V. Academic Press Inc., New York.

Johnson, W.M. 1971. Studies on the pathway of glutamic acid fermentation by Peptococcus aerogenes. Ph.D. Thesis, University of Alberta, Edmonton, Alberta, Canada.

Johnson, W.M. and D.W.S. Westlake. 1969. The production of α-hydroxyglutaric acid from glutamic acid by cell-free preparations of Peptococcus aerogenes. Can. J. Biochem. 47:1103-1107.

Joyner, A.E., Jr. and R.L. Baldwin. 1966. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92:1321-1330.

Kaufman, S. 1955. α-ketoglutarate dehydrogenase system and phosphorylating enzyme from heart muscle. P. 714 to 718. In S.P. Colowick and N.O. Kaplan (ed.) Methods in enzymology. Vol. I. Academic Press Inc., New York.

Knight, M. 1962. The photometabolism of propionate by Rhodospirillum rubrum. Biochem. J. 84:170-185.

Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. P. 447 to 454. In S.P. Colowick and N.O. Kaplan (ed.) Methods in enzymology. Vol. III. Academic Press Inc., New York.

Lewis, D. 1957. Blood-urea concentration in relation to protein utilization in the ruminant. J. Agric. Sci. 48:438-446.

Mandell, N. and C.F. Roberts. 1966. A piston extractor for the Hughes Press. Science 152:799-800.

Matheson, A.T., E. Tigane and C.S. Hanes. 1961. Quantitative chromatographic methods. Part 5. An improved ninhydrin-hydrindantin reagent. Can. J. Biochem. Physiol. 39:417-425.

Milligan, L.P. 1970. Carbon dioxide fixing pathways of glutamic acid synthesis in the rumen. Can. J. Biochem. 48:463-468.

Mortenson, L.E. 1964a. Purification and analysis of ferredoxin from Clostridium pasteurianum. Biochim. Biophys. Acta. 81:71-77.

Mortenson, L.E. 1964b. Ferredoxin requirement for nitrogen fixation by extracts of Clostridium pasteurianum. Biochim. Biophys. Acta. 81:473-478.

Mortlock, R.P., R.C. Valentine and R.S. Wolfe. 1959. Carbon dioxide activation in the pyruvate clastic system of Clostridum butyricum. J. Biol. Chem. 234:1653-1657.

Mortlock, R.P. and R.S. Wolfe. 1959. Reversal of pyruvate oxidation in Clostridium butyricum. J. Biol. Chem. 234:1657-1658.

Niederman, R.A. and M.J. Wolin. 1967. Arginine biosynthesis by Streptococcus bovis. J. Bacteriol. 94:1002-1006.

Niederman, R.A. and M.J. Wolin. 1969. Chemical constituents and hydrogenase binding in cell envelopes of Vibrio succinogenes. J. Bacteriol. 98:160-166.

Nordman, J. and R. Nordman. 1960. Organic acids of the citric acid cycle and related acids. P. 272 to 290. In I. Smith (ed.) Chromotographic and electrophoretic techniques. Vol. I. William Heinemann, Medical Books, Ltd., London.

Northrop, D.B. and H.G. Wood. 1969. V. Transcarboxylase. The presence of bound zinc and cobalt. J. Biol. Chem. 244:5801-5807.

Palmquist, D.L. and R.L. Baldwin. 1966. Enzymatic techniques for the study of carbohydrate utilization in the rumen. Appl. Microbiol. 14:60-69.

Peel, J.L. 1960. The breakdown of pyruvate by cell-free extracts of the rumen microorganism LC. Biochem. J. 74:525-541

Rabinowitz, J.C. 1960. Factor B and other compounds related to vitamin B_{12} in pyruvic-CO_2 exchange. J. Biol. Chem. 235:PC50.

Reitz, M.S. and V.W. Rodwell. 1969. α-hydroxyglutarate oxidoreductase of Pseudomonas putida. J. Bacteriol. 100:708-714.

Robinson, I.M. and M.J. Allison. 1969. Reductive carboxylation of succinate as a mechanism of α-ketoglutarate biosynthesis in Bacteroides ruminicola. Bacteriol. Proc. P.72.

Sager, R.D. and J.V. Beck. 1956. Studies on the formation of formate, glycine, serine, pyruvate and acetate from purines by Clostridium acidi-urici. J. Bacteriol. 72:199-208.

Shigesada, E., K. Hidaka, H. Katsuki and S. Tanaka. 1966. Biosynthesis of glutamate in photosynthetic bacteria. Biochim. Biophys. Acta. 112:182-185.

Sly, W.S. and E.R. Stadtman. 1963. Formate dependent decomposition of acetyl phosphate in Clostridium kluyveri. J. Biol. Chem. 238:2632-2638.

Somerville, H.J. 1968. Enzymatic studies on the biosynthesis of amino acids from lactate by Peptostrptococcus elsdenii. Biochem. J. 108:107-119.

Somerville, H.J. and J.L. Peel. 1964. The utilization of lactate carbon for the biosynthesis of amino acids by Peptostreptococcus elsdenii. Biochem. J. 93:19P.

Somerville, H.J. and J.L. Peel. 1967. Tracer studies on the biosynthesis of amino acids from lactate by Peptostreptococcus elsdenii. Biochem. J. 105:299-310.

Stadtman, E.R. and H.A. Barker. 1950. Fatty acid synthesis by enzyme preparation of C. kluyveri. J. Biol. Chem. 184: 769-793.

Stadtman, T.C. 1964. Methane fermentation. Ann. Rev. Microbiol. 21:121-142.

Stern, J.R. 1965. Role of cofactors in pyruvate oxidation and synthesis by extracts of Clostridium kluyveri. P. 199 to 209. In A. San Pierto (ed.) Non-heme iron proteins: Role in energy conversion. Antioch. Press, Yellow Springs, Ohio.

Stern, J.R., C.S. Herge and G. Bambers. 1966. Glutamic biosynthesis in anaerobic bacteria. II. Stereospecificity of aconitate and citrate synthesis of Clostridium kluyveri. Biochemistry. 5:1119-1124.

Tagawa, K. and D.I. Arnon. 1962. Ferredoxins as electron carriers in photosynthesis and in the biological production and consumption of hydrogen gas. Nature. 195:537-543.

Tagawa, K. and D.I. Arnon. 1968. Oxidation reduction potentials and stoichiometry of electron transfer in ferredoxins. Biochim. Biophys. Acta. 153:602-613.

Valentine, R.C. 1964. Bacterial ferredoxin. Bacteriol. Rev. 28:497-517.

Whatley, F.R. and D.I. Arnon. 1963. Photosynthetic phosphorylation in plants. P. 308-313. In S.P. Colowick and N.O. Kaplan (ed.) Methods in enzymology. Vol. VI. Academic Press Inc., New York.

White, D.C., M.P. Bryant and D.R. Caldwell. 1962. Cytochrome-linked fermentation in Bacteroides ruminicola. J. Bacteriol. 84:822-828.

Whiteley, H.R. and N.G. McCormick, 1963. Degradation of pyruvate by Micrococcus lactilyticus. III. Properties and cofactor requirements of the carbon dioxide-exchange reaction. J. Bacteriol. 85:382-393.

APPENDIX I

Pyruvate synthase

In the following experiments, the reductive carboxylation of acetyl coenzyme A to pyruvate in CFE of mixed rumen microorganisms was considered. The reaction mixture consisted of the components indicated in Appendix Table 1. Acetyl phosphate and coenzyme A were used as substrates in the presence of $H^{14}C\bar{O}_3$ and reduced ferredoxin. Phosphotransacetylase (EC 2.3.1.8), which catalyzes the conversion of acetyl phosphate to acetyl coenzyme A has been shown to be active in rumen microorganisms (Joyner and Baldwin, 1966).

The requirements for $HC\bar{O}_3$ fixation are given in Appendix Table 1. The reaction is dependent on acetyl phosphate, coenzyme A, reducing reagents and soluble enzyme. Thiamine pyrophosphate stimulates the fixation.

The effect of pH on $HC\bar{O}_3$ fixation is illustrated in Appendix Figure 1. The fixation was optimum at pH 7.5.

The dependence of fixation on enzyme and substrate concentration is demonstrated in Appendix Figure 2 and Appendix Table 2, respectively.

When synthetically prepared acetyl coenzyme A, or acetate plus ATP and coenzyme A were used as substrates, bicarbonate fixation also was shown. The activity of acetyl coenzyme A synthetase was shown in the present studies.

Alanine was formed by adding 50 umoles of NADH and 1 mmole NH_4Cl to the reaction mixture described in Appendix Table 1 and detected as outlined (Methods-VI and VII). The R_f value (0.37) obtained was identical to standard alanine.

Further experimental evidence in the present studies suggesting the possibility of occurrence of pyruvate synthase in rumen microorganisms were the exchange reaction between pyruvate and $H^{14}C\bar{O}_3$ (107 dpm/min/mg protein) and the activity of acetyl coenzyme A synthetase (0.8 umoles hydroxymate/mg protein).

Discussion

A brief review of pyruvate synthase was given in the review of literature. The data obtained in the present studies shows that the optimal pH is within the physiological range and that the reaction requires reduced ferredoxin. Thiamine pyrophosphate stimulates the reaction. In the absence of acetyl phosphate or coenzyme A the fixation was markedly decreased. These results are in accordance with the report of Evans (1968).

Pyruvate would be an intermediate of carbohydrate fermentation by rumen microorganisms. Therefore, the significance of the presence of pyruvate synthase in the ruminal microbes may be questioned. Bryant (1965) has shown that methanogenic bacteria can grow on acetate as the carbon source. The rumen is populated with numerous types of microbes (Hungate, 1966). Thus, it is quite possible that the end products of certain bacteria may serve as starting substrates for others.

Appendix Table 1

Requirements for $H\bar{C}O_3$ fixation in the presence of acetyl phosphate and coenzyme A by extracts of mixed rumen microorganisms

Treatment	$H^{14}\bar{C}O_3$ fixed (dpm)
Complete	4,750
Minus thiamine pyrophosphate	3,000
Minus coenzyme A	1,860
Minus acetyl phosphate	1,100
Minus enzyme	300
Minus reducing reagents	590

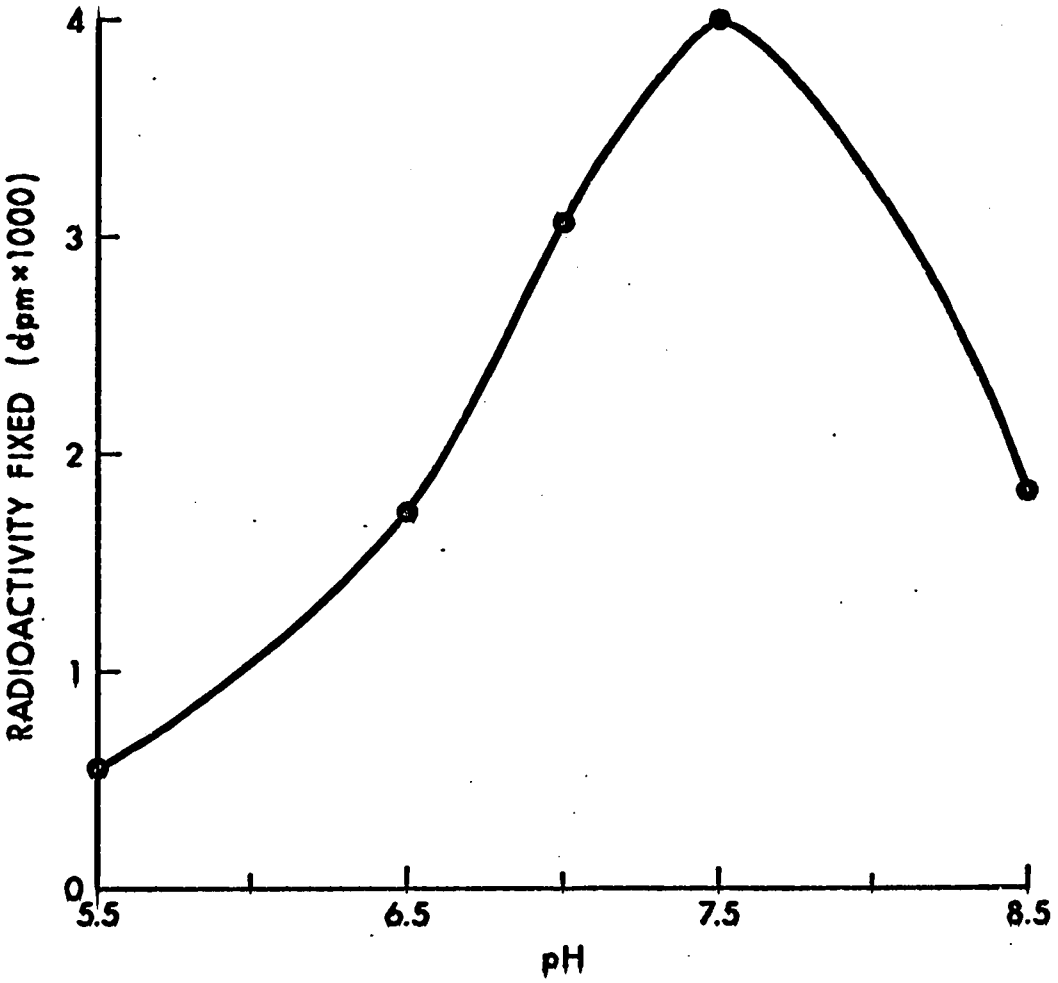
The CFE was prepared as described (Methods-I) except that Sephadex G-10 was used. The complete reaction mixture contained Sephadex-treated CFE, 0.5 mg; potassium phosphate buffer (pH 7.5), 150 umoles; ATP, 2.5 umoles; $MgCl_2$, 5 umoles; coenzyme A, 0.25 umoles; acetyl phosphate, 5 umoles, thiamine pyrophosphate, 2 umoles; sodium bicarbonate, 5 umoles (containing $NaH^{14}CO_3$, 2.5×10^6 dpm). The reducing system was composed of 0.5 ug methyl viologen, 150 ug ferredoxin and zinc powder (enough to reduce the methyl viologen). Final volume 0.8 ml. The reaction mixture was incubated at 37°C in a shaking bath for 30 min.

Appendix Table 2

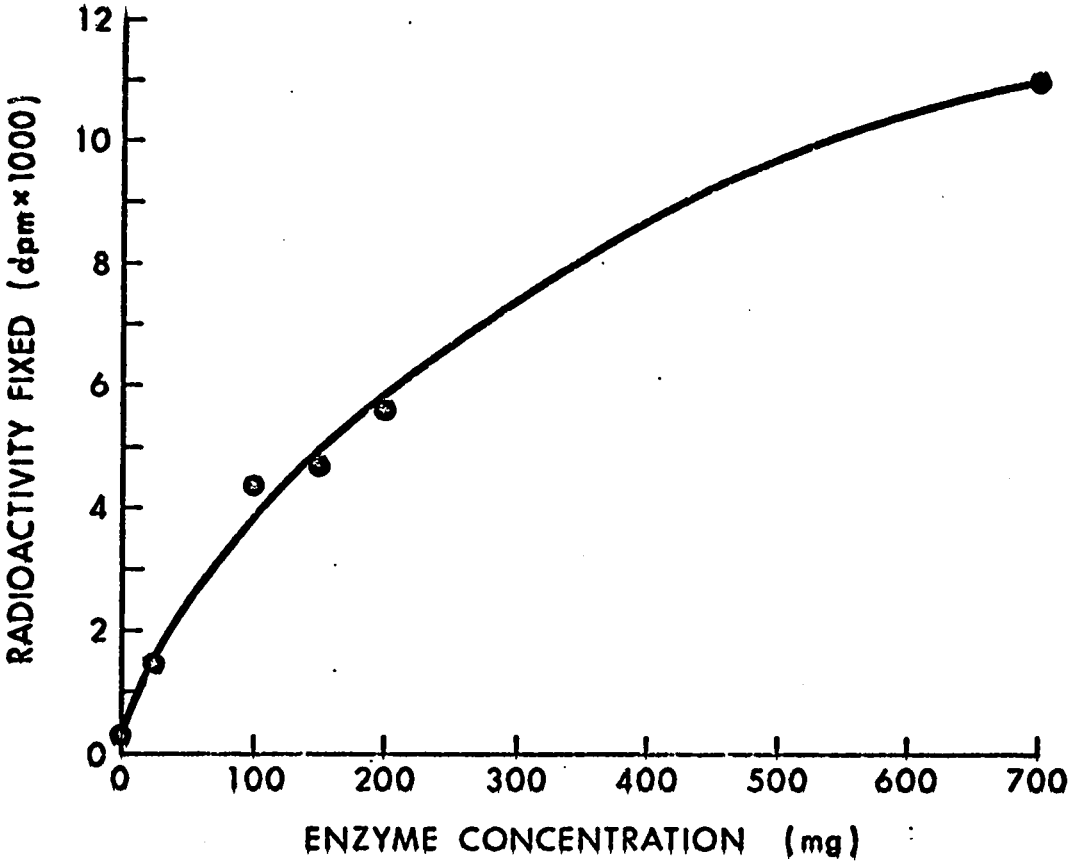
Dependence of $HC\bar{O}_3$ fixation on substrate concentration

Acetyl phosphate (umoles)	Coenzyme A (umoles)	$HC^{14}O_3$ fixed (dpm)
0.0	0.0	1,065
0.0	0.2	2,340
3.0	0.2	12,200
4.5	0.3	15,000
6.0	0.4	18,650

Experimental conditions were the same as given in Appendix Table 1 except for varying the concentrations of substrates.



Appendix Figure 1. The effect of pH on $HC\bar{O}_3$ fixation in the presence of acetyl phosphate and coenzyme A. Experimental conditions were the same as given in Appendix Table 1, except for replacing the buffer with the buffering system as described in Figure 1.



Appendix Figure 2. Dependence of $HC\bar{O}_3$ fixation on enzyme concentration in the presence of acetyl phosphate and coenzyme A. Experimental conditions were the same as given in Appendix Table 1, except for varying the concentration of enzyme.

APPENDIX II

Preparation of ferredoxin (FD)

CFE were prepared from 50g microbial paste as described (Methods-1). All the following steps were carried out at 0°C. The exudate from the press was suspended in 20 ml distilled water and centrifuged at 48,000 x g for 30 min. The supernatant was applied to a DEAE-cellulose column (50 x 30 mm) prepared similar to the method of Mortenson (1964a). The adsorbed FD was removed from the column by elution with 0.5 M Tris-HCl buffer (pH 8). Subsequently, 25 ml of the effluent at the concentration peak of FD as judged visually was applied to a Sephadex G-25 column (3 x 20 cm). Then 50 ml of the effluent at the concentration peak from the Sephadex column was treated with $(NH_4)_2SO_4$. The fraction at 60% saturation was discarded. The final preparation obtained was at 90% saturation.

Whiteley and McCormic (1963) have shown that the exchange of $H\bar{C}O_3$ with pyruvate was stimulated by FD. At the outset, experiments were done in the present studies to assess the activity of the various fractions of FD using the exchange reaction as a FD assay. The reaction mixture used was as outlined (Methods-VIII) and contained pyruvate as substrate. The results are given in Appendix Table 3.

Appendix Table 3

Purification of Ferredoxin

Fraction (mg protein/assay)	$H^{14}C\bar{O}_3$ fixed (dpm)	$H^{14}C\bar{O}_3$ fixed (dpm)/mg protein
———	4,700	———
DEAE-cellulose (1.75)	7.900	4,500
Sephadex (1.37)	9,300	6,800
$(NH_4)_2SO_4$ (2.00)	36,000	18,000

This data demonstrates that the exchange reaction is stimulated by the addition of FD. The reaction mixture containing the FD carried through 90% saturation of $(NH_4)_2SO_4$ showed 7.7 fold activity over that which had had no FD added. The specific activity of various fractions is also compared. The DEAE-cellulose fraction showed a peak absorbancy of 0.19 at 425 nm using a 1 cm light path. The solution contained 290 ug protein/ml.

Discussion

A general review on the structure, properties and biological functions of FD was given by Valentine (1964). Ferredoxin has been shown to participate in many oxidative-reductive reactions. Stadtman (1967) has reported on the role of ferredoxin in methane formation in anaerobic bacteria. Carbon dioxide in the rumen is reduced to methane (Bryant, 1965) and quite possibly ferredoxin is involved in one of the steps in this conversion. Nitrogen fixation in CFE of Clostridium pasteurianum was shown to require

ferredoxin (Mortenson, 1964b). There are some suggestions that rumen microorganisms are capable of fixing nitrogen (Allison, 1970) which may require the participation of ferredoxin. Baldwin and Milligan (1964) obtained data indicating the presence of FD-linked NAD reductase in the rumen isolate Peptostreptococcus elsdenii. The presence of FD in mixed rumen microorganisms and its role in the reduction of succinic acid to succinic semialdehyde, as well as in the carboxylation of succinic semialdehyde to 2-hydroxyglutarate were shown in the present studies. More research is needed to further elucidate the various functions of ferredoxin in the metabolism of rumen microorganisms.