

EASTMAN CHEMICALS DIVISION
EASTMAN KODAK COMPANY
KINGSPORT, TN 37662

FOOD ADDITIVE PETITION
NO. 2193

AMMONIUM SALTS OF MIXED VOLATILE FATTY ACIDS BLEND (AS-VFA)
AS A NUTRIENT SUPPLEMENT IN THE FEED OF DAIRY AND BEEF CATTLE

June 14, 1982

Heading H

sve/2967g

Heading H

Environmental Assessment ----- 621

Enclosures Under Heading H

Enclosure 1 - Letter From Food and Drug Administration Dated
January 7, 1982, Concluding No Toxicological
Concern at the Proposed Usage Levels for AS-VFA ----- 623

Enclosure 2 - "The Safety of Certain Volatile Fatty Acids as Feed
Supplements," B. D. Astill, Health and Safety
Laboratory, Eastman Kodak Company, 1975 (Unpublished
Report) ----- 627

Enclosure 3 - Texas Water Commission Discharge Permit No. 00471
Issued to Texas Eastman Company, Division of Eastman
Kodak Company ----- 685

Enclosure 4 - National Pollutant Discharge Elimination System Permit
No. TX0000949 Issued to Texas Eastman Company, Division
of Eastman Kodak Company ----- 687

HEADING H

ENVIRONMENTAL ASSESSMENT - AMMONIUM SALTS OF
MIXED VOLATILE FATTY ACID BLEND (AS-VFA): A FEED ADDITIVE
FOR DIETS OF DAIRY AND BEEF CATTLE

In accordance with 21 C.F.R. § 25.1(g), an Environmental Impact Analysis (EIA) Report relating to this petition for a food additive regulation for AS-VFA is not required unless a request for such a report has been made in writing by the Food and Drug Administration (FDA). No such written request has been received regarding this food additive petition.

In accordance with § 25.1(g), the following areas are shown.

1. Regulatory Basis for Exemption

Exemption from the requirement of an EIA Report is warranted by § 25.1(f)(1)(iv). The criteria of § 25.1(f)(1)(iv) and references to data in the petition which substantiate compliance with the criteria follow:

- "(a) The article is composed of a substance or its derivatives that naturally occurs in the environment and that may reasonably be considered to be nontoxic in the amounts used;
- (b) The article is not metabolized in its use and is excreted unchanged back into the environment or, if it is metabolized, the metabolites and the amounts excreted into the environment are naturally occurring in the environment or may reasonably be considered to be nontoxic; and
- (c) The use of the article can reasonably be expected on the basis of all available evidence, not to alter significantly the prevalence and/or distribution of the substance or its derivatives or their metabolites in the environment."

Data establishing that AS-VFA meets all of these criteria are contained in Heading E, the Safety Section of this food additive petition. Of particular relevance and attached hereto as Heading H, Enclosure 1 is a copy of an FDA letter dated January 7, 1982, concluding that there was no toxicological concern at the proposed usage levels for AS-VFA and Heading H, Enclosure 2, a review dated October 14, 1975, entitled "The Safety of Certain Volatile Fatty Acids As Feed Additives," B. D. Astill.

2. Analysis of the Environmental Effects of the Manufacturing Process

(i) Reference is made to confidential trade secret information in a flow chart enclosed as Heading A, Enclosure 6 of petitioner's food additive petition, showing the manufacturing process whereby AS-VFA is manufactured. It will be noted that no pollutants are expected to be emitted.

(ii) AS-VFA will be manufactured at the Texas Eastman Company's Production Division in Longview, Texas. The production as AS-VFA will be one small operation in a large petrochemical complex operated by Texas Eastman Company. The entire facility is operated in accordance with all applicable federal and state laws and regulations enforced by the Environmental Protection Agency (EPA), the Texas Air Control Board (TACB) and the Texas Water Commission.

(iii) Enclosed and identified as Heading H, Enclosure 3 and Heading H, Enclosure 4 are certifications that the Longview facility is operated in compliance with Discharge Permit No. 00471, issued by the Texas Water Commission and NPDES Permit No. TS0000946.


Signature

Vice President

[Title]

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EASTMAN CHEMICALS DIVISION
EASTMAN KODAK COMPANY
KINGSPORT, TN 37662

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AS A NUTRIENT SUPPLEMENT IN THE FEED OF DAIRY AND BEEF CATTLE

June 14, 1982

Heading H, Enclosure 1

sve/2967g



INAD 1956

JAN 07 1982

Mr. Peter Morison
Product Safety Staff
Eastman Kodak Company
Eastman Chemicals Division
Kingsport, Tennessee 37662

Dear Mr. Morison:

We refer to your submissions dated July 8, 1981, July 29, 1981 (2), September 8, 1981 and October 2, 1981 pertaining to your investigational new animal drug application (INAD 1956) for Mixed Ammonium Salts of Volatile Fatty Acids Blend (Mixed AS-VFA Blend) for lactating dairy cows. These five submissions either provide data in support of the safety of Mixed AS-VFA Blend or request an investigational exemption to market the milk and edible tissues for food purposes from dairy cows used in a reproductive performance trial and an acute toxicological study. A synopsis with dates of these submissions is given below.

July 8, 1981

Information was submitted to substantiate a no-effect level for leucine or isovaleric-induced hypoglycemia and to document residues of isobutyric, isovaleric, 2-methylbutyric, and valeric acids and leucine in the milk of dairy cows.

July 29, 1981

These two submissions requested an investigational exemption to market the milk and edible tissues for food purposes from dairy cows used in a reproductive performance trial and in an acute toxicity study.

September 8, 1981

The submission responded to an agency request for milk residue raw data and a discussion of chromatographic technique.

October 2, 1981

The submission requested confirmation that tissue residue studies will not be required for leucine and that no further consideration be given these studies as a possible requirement in the progress of INAD 1956.

We have completed our review of these submissions and in general our conclusions can be summarized by the following statement:

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Leucine and volatile fatty acids, i.e. isobutyric, isovaleric, 2-methylbutyric and valeric acids are not of toxicological concern because they are natural body constituents which at the proposed use levels would not present any risk to the human population. Even at 4X the recommended dosage, the unvalidated levels of leucine in milk appear not to be significantly elevated above those levels resulting from either the unsupplemented or the normally supplemented dairy cows. The AS-VFA values appear to be less than 100 ppb for all groups. Even in the most sensitive population, i.e. individuals sensitive to leucine-induced hypoglycemia, the levels in milk are about a factor of 600 less than the no-effect level.

Instead of accumulating in the tissues, excess amino acids will be catabolized and excreted by the kidney.

For these reasons, it was concluded that there was no toxicological concern at the proposed usage levels for AS-VFA. Therefore, you need give no further consideration to tissue residues or milk depletion studies as a possible requirement in the progress of INAD 1956.

SUPPORT OF SAFETY OF AS-VFA

Your submission dated July 8, 1981 presented information to substantiate a no-effect level for leucine or isovaleric acid-induced hypoglycemia.

This information included (1) documentation of absence of risk to leucine sensitive hypoglycemics who ingested milk and edible tissues from dairy cattle fed mixed AS-VFA blend (2) documentation and experimental data for the safety of Isobutyric acid-Propionic acid (9/1) (3) safety of certain volatile fatty acids as feed additives and (4) selected literature review of leucine induced hypoglycemia and isovalerate acidemia. In addition, you presented a preliminary summary of residues of isobutyric, isovaleric, 2-methylbutyric and valeric acids and leucine in the milk of dairy cows, fed Mixed AS-VFA Blend.

Conclusions

1. Although a hypoglycemic effect due to isovaleric acid was observed in one published study, this could not be substantiated. Thus, it is reasonable to assume that isovaleric acid does not produce hypoglycemia in sensitive population groups.
2. The intravenous no-effect level for L-leucine induced hypoglycemia in sensitive individuals is 25 mg/kg or about 1.5 g (1500mg) for a 60 kg person.
3. The normal human intake of leucine from protein sources is in the range of 4-6 grams per day.

4. L-leucine taken up in bovine mammary tissue is effectively utilized as a source of energy for the synthesis of non-essential amino acids and for the production of milk protein. About 70% is used for milk protein production and the remainder for energy needs. Thus, only small amounts of free leucine are expected to accumulate in tissues.
5. The milk residue study did not demonstrate carry-through of L-leucine of volatile fatty acids contained in Mixed AS-VFA Blend.
6. An estimate of the maximum amount of free leucine in edible tissues is 5 mg/kg. The human consumption of 500 g of edible tissues will lead to the absorption of about 2.5 mg of free leucine derived from isovalerate in the feed. The safety factor for a leucine sensitive hypoglycemic is approximately 600 (1500 mg/2.5 mg).
7. The increment of leucine from edible tissues is insignificant in comparison to normal human daily intake of leucine.
8. Feeding Mixed AS-VFA to dairy cattle should not lead to substantial increases of isobutyric, isovaleric, 2-methylbutyric and valeric acids and leucine concentration in the milk or edible tissues.
9. The consumption of milk or edible tissues from cattle fed Mixed AS-VFA should not pose a hazard to human safety.
10. Although tissue residue studies and further milk residue studies are not indicated at this time, we can not accept your present milk residue study without the raw data and appropriate validating information which includes GLC chromatograms, detailed procedures, representative calculations and recovery studies in milk fortified with 100 ppb levels of each AS-VFA and also 0.5 ppm leucine.

Please submit these supporting data in addition to the final report for our review.

REQUEST FOR INVESTIGATIONAL EXEMPTION

In your submission dated July 29, 1981, you requested an investigational exemption for milk and edible tissues for food purposes from ten treated dairy cows used in a reproductive performance trial and five treated dairy cows used in an acute toxicity study.

Conclusion

We continue to support the zero withdrawal period concerning both milk and edible tissues for previously authorized levels of Mixed AS-VFA Blend limited to 1.4% of the total feed under this INAD. The equivalent dosage limit would be 560 g/cow/day which would cover the 1X (120 g/cow/day) and 4X (480 g/cow/

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day) dosage levels requested in the reproductive study and the 1X level in the acute toxicity study. The 10X, 15X, 25X and 50X levels in the acute toxicity study are all above the previously authorized levels of Mixed AS-VFA Blend.

However, because of information submitted to substantiate a no-effect level for leucine or isovaleric acid-induced hypoglycemia, we have decided to allow a higher level of supplementation up to 50X the recommended dose of 120 g/cow/day, with a zero withdrawal period for both milk and edible tissues. The available toxicological data permit the establishment of a sufficient margin of safety for levels of the ammonium salts of the volatile fatty acids or their metabolites, especially leucine, in milk and edible tissues of dairy cows treated with Mixed AS-VFA Blend.

Therefore, in a separate letter the Agency will approve your request to market milk and edible tissues for food purposes from ten treated cows used in the reproductive performance trial and five treated dairy cows used in the acute toxicity study following a zero day withdrawal and thirty day observation period.

Sincerely yours,


William D. Price, Ph.D.
Chief Metabolic Products Branch
Division of Drugs for Ruminant Species
Bureau of Veterinary Medicine

EASTMAN CHEMICALS DIVISION
EASTMAN KODAK COMPANY
KINGSPORT, TN 37662

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AS A NUTRIENT SUPPLEMENT IN THE FEED OF DAIRY AND BEEF CATTLE

June 14, 1982

Heading H, Enclosure 2

sve/2967g

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THE SAFETY OF CERTAIN VOLATILE
FATTY ACIDS AS FEED ADDITIVES

Bernard D. Astill
Biochemistry Section

October 14, 1975

HEALTH, SAFETY AND HUMAN FACTORS LABORATORY
EASTMAN KODAK COMPANY, ROCHESTER, N.Y. 14650

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NOT FOR PUBLICATION

THE SAFETY OF CERTAIN VOLATILE
FATTY ACIDS AS FEED ADDITIVES

Prepared by:

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October 15, 1975

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Robert L. Raleigh, M.D., Director
Health and Safety Laboratory

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ABSTRACT

Isobutyric, isovaleric, valeric and 2-methylbutyric acids are essential nutrients for several cellulolytic rumen microorganisms, and their use as a feed supplement for cattle has been proposed. They occur widely in nature and are components of the normal intermediary metabolism in monogastrics and ruminants, where they are converted principally to acetate, propionate and/or acetoacetate. Valeric acid occurs in the rumen as a product of proline catabolism or from dietary carbohydrates. In addition, isovaleric acid is formed from leucine, 2-methylbutyric acid from isoleucine and isobutyric acid from valine in the rumen, all originating from dietary protein. All four acids are used in rumen microbial protein and lipid synthesis. They are readily utilized by a variety of aerobic and anaerobic microorganisms. They possess slight to moderate toxicity to mammals in the undiluted form and possess little physiological activity. The principal hazard in handling arises from their irritating properties.

Based on these considerations, it is expected that the suggested intake levels of these acids in use would be well within the capacity of the ruminant to utilize and would not result in any carry through into milk or flesh. If such residues did occur they would be non-hazardous to monogastrics, and the commercial use of these acids would not be detrimental to the environment.

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THE SAFETY OF CERTAIN VOLATILE FATTY ACIDS AS FEED ADDITIVES

INTRODUCTION

Isobutyric (IBA), isovaleric (IVA), valeric (VA) and 2-methylbutyric acids (MBA)* are essential nutrients for a number of cellulolytic rumen microorganisms, and play a role in the synthesis of microbial proteins and lipids (1). In consequence it is proposed that a blend of the above acids has value as a feed supplement in the diet of beef and dairy cattle. This report reviews the available information on the natural occurrence, metabolic fate, toxicity and regulatory status of IVA, VA and MBA. An account of the occurrence, metabolism and safety of isobutyric acid has previously been presented (2) in support of an exemption from a tolerance in applications to grains and grasses.

The documentation of these acids is presented in support of the claim that their use as feed supplements would be a safe practice.

* Collectively referred to as CCN (Carbon Chain Nutrient) acids.

I. OCCURRENCE OF VALERIC, ISOVALERIC AND 2-METHYLBUTYRIC ACIDS

a) Valeric acid

Valeric acid, 1-pentanoic acid, is a volatile fatty acid of very wide natural distribution, being found free, as esters, and as part of the intermediary metabolism of fatty acids in a wide range of plant and animal species. Chemical Abstracts Indices (1947-73) list valeric acid as present in the following foodstuffs: apples, beer, brandy, butter, cheeses, citrus oils, coffee, fish, grapes, meat and meat fat, milk, molasses, oysters, peas, raspberries, rum, sake, soy sauce and soy beans, tea and tea oils, tobacco, tobacco smoke and tobacco oils, vodka and a variety of wines. Of particular interest is its occurrence in the perinephric fat of the ox (3). Although it was not detected in the volatile fatty acid fraction from cooked beef (4) more recently it has been found in meat products (5). Trace amounts of valeric acid have been detected in cream (6).

Valeric acid has been reported as a bacterial fermentation product in cultures of the anaerobes *Clostridium perfringens*, *C. histolyticum*, *C. fallax*, *C. pseudofallax*, *Eubacterium nitritogens* and *Fusiformis biacutus*, along with other volatile fatty acids (7). It is metabolized by *Pseudomonas aeruginosa* to hexane (8). It is formed from norleucine by *Staphylococcus aureus* and by a micrococcus from a cheese rind (9). It is among the fatty acids formed by *Neisseria caproicum* from lactate, pyruvate and glucose (10).

A sensitive procedure developed for measuring the concentrations of volatile free fatty acids in human blood (11) did not detect valeric acid. However valeric acid occurs in the volatile fatty acids of

human saliva in a concentration of 0.12 millimolar (12). The occurrence of valeric acid in human wastes and during waste disposal has been demonstrated. Thus dialysates of the daily human fecal output contained an average of 172 meq of volatile fatty acids per l of dialysate, of which 1.7 meq/l was valeric acid (13). Anaerobic digestion of organic wastes produced a variety of fatty acids including valeric acid (14), which rapidly decomposed, principally to acetic acid. In addition, digestion of slaughter house wastes with yeasts produced volatile fatty acids, including valeric acid (15).

Valeric acid has been detected in grass and corn silage in trace amounts by a variety of workers (16), although it is not uniformly present. More commonly, butyric (grass silage) and acetic and propionic (corn silage) acids usually predominate.

b) Isovaleric acid

Isovaleric acid, 3-methylbutyric acid, 3-methylbutanoic acid, is widely distributed in nature. (In the earlier literature, isovaleric acid frequently refers to the mixture of isomeric, isovaleric and 2-methylbutyric acids which were not readily distinguished in analysis.)

It occurs free, as esters with naturally occurring alcohols, and in the intermediary metabolism of a wide range of plant and animal species as a breakdown product of the amino acid leucine. Chemical Abstracts Indices (1947-73) list isovaleric acid as present in the following foodstuffs: apples and apple products, bananas, beer, bourbon, brandy, bread dough, butter, cheese, citrus oils, coffee, hops and hop oil, meat and meat fat, milk, mushrooms, oysters, peaches, peas, peppermint oil, pineapple, raspberries, sake, sherry, soy sauce, tabasco

peppers, tea, tobacco, tobacco smoke and oils, whale meat and whale oil and a variety of wines.

Isovaleric acid occurs in the external carcass fat of sheep (3), amounting to 0.4-1.2% of mixed isomeric 'iso' acids of the C_1-C_{10} fatty acid content. It has been detected in butter, Emmental and Gruyere cheeses (17). It is among the volatile fatty acids produced from milk casein hydrolysates by resting strains of lactic Streptococci and Lactobacilli (18). In addition the action of Streptococcus diacetylactis DRCI, Lactobacillus No. 138 (18), Staphylococcus aureus and a micrococcus from Limberger cheese (17) produces isovaleric acid from leucine. Anaerobic proteolytic bacteria of the Clostridium sporogenes group effect a similar conversion, involving the so-called "Stickland Reaction" (19).

Isovaleric acid has been detected and quantitated in human blood, amounting to about 4.4% of the volatile free fatty acid content, in a concentration of $194 \pm 80 \mu\text{g}/100 \text{ ml}$ of plasma (11). Elevated levels of isovaleric acid (100 to 1000 times normal) in the blood are associated with a rare metabolic disorder involving a defect in leucine metabolism (20) (see below p. 32). Isovaleric acid also occurs in human wastes amounting to about 2.2 meq/ml of an average daily human fecal dialysate (13), and in sewage (21). As with other fatty acids in untreated sewage, waste treatment is effective in removing those above C_2 (22).

Isovaleric acid occurs in grass silage in trace amounts, although it has not so far been detected in corn silage (16).

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c) 2-Methylbutyric acid

2-Methylbutyric acid, 2-methylbutanoic acid, is fairly widely distributed in nature, occurring free, as esters, and in the intermediary metabolism of plant and animal species as a breakdown product of the amino acid isoleucine. Chemical Abstracts Indices (1947-73) list 2-methylbutyric acid as present in apples, apricots, bourbon, cheese, clams, cocoa, coffee, hops and hop oil, milk, oranges, pineapples, strawberries, tobacco, tobacco oil and smoke. It has been identified in sheep mutton fat (3) and as a product of Lactobacillus fermentation of milk casein (18). Anaerobic proteolytic bacteria effect the conversion of isoleucine to 2-methylbutyric acid in the "Stickland Reaction" (19). Trace amounts occur in grass silage but it has not been detected in corn silage (16).

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II. MAMMALIAN METABOLISM OF VALERIC, ISOVALERIC AND 2-METHYLBUTYRIC ACIDS

a) Valeric acid

The metabolism of straight chain fatty acids has long been known to follow the β -oxidation scheme proposed by Knoop, subsequently elaborated and refined to a general scheme for all organisms (23). The overall scheme (Fig. 1) indicates that valeric acid should be converted to acetate and propionate. In addition, valerylcoenzyme A is an intermediate in the β -oxidation scheme for odd numbered fatty acids.

Early in vitro studies (24) showed valeric acid to be converted to propionate by rabbit kidney homogenates. When ^{14}C - and ^{13}C -valerate was fed to fasted rats, the incorporation pattern of ^{14}C - and ^{13}C - in liver glycogen was identical with that when ^{14}C -acetate and ^{13}C -propionate were fed (25). Thus $\text{CH}_3 \cdot ^{14}\text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot ^{13}\text{CO}_2^-$ yielded respired $^{14}\text{CO}_2$ and $^{13}\text{CO}_2$ (approximate proportions 1:2). Liver glycogen-derived glucose was labelled in the 3 and 4 positions with ^{13}C and in the 1,2,5,6 positions with ^{14}C . When fed instead of valerate, 1- ^{13}C -acetate and 2- ^{14}C -propionate give the same distributions of isotopic carbon in glucose from liver glycogen.

Intermediates in the overall enzymic oxidation pathway have been identified. 3-Hydroxyvalerate, the key intermediate in the β -hydroxylation step, occurs in the metabolism of norleucine by rat liver slices, via the pathway norleucine \longrightarrow 2-ketocaproic \longrightarrow valerate \longrightarrow 3-hydroxyvalerate (26).

^{14}C -acetoacetate was detected, together with respiratory $^{14}\text{CO}_2$, as a product of the metabolism of ^{14}C -valerate (and the homologous $\text{C}_6 - \text{C}_{10}$ acids) by rat liver or kidney slices (27). The rapidity

of valerate metabolism was also indicated by the demonstration that rat liver slices converted 47% of 1-¹⁴C-valeric acid to ¹⁴CO₂ in 3 hours at 37.5° (28). Finally, studies on the well-established carboxylation pathway for propionate metabolism, showed valeric acid to be almost as efficient as propionate in fixing ¹⁴CO₂ from ¹⁴HCO₃, in the presence of soluble fractions from rat, ox, and guinea pig liver (29).

It is reasonable to conclude therefore that valerate is converted by the general β-oxidation fatty acid metabolism system to acetate and propionate, whose fates are discussed below.

b) Isovaleric acid

Isovaleric acid has been identified in the form of its coenzyme A ester as an intermediate in the metabolism of the amino acid leucine. Leucine itself has long been recognized as "ketogenic" i.e. has a high capacity for producing "ketone bodies" as biodegradation products.

Early experiments indicated that the isopropyl group of leucine was incorporated into acetoacetate as an end product of leucine metabolism (30). Subsequently the steps in leucine and isovaleric acid metabolism were worked out (Fig. II) and shown to occur in rat liver slices and pig heart muscle (23). Leucine itself is converted to isovalerylcoenzyme A by transamination and decarboxylation (31). Isovaleric acid requires activation by conversion to its coenzyme A ester. The subsequent steps in its conversion involve dehydrogenation to β-methylcrotonylcoenzyme A, carboxylation to β-methylglutaconylcoenzyme A, hydroxylation to β-hydroxy-β-methylglutarylcoenzyme A and cleavage to acetoacetate and acetylcoenzyme A. The principal differences between straight chain

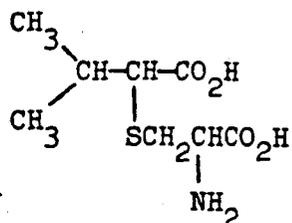
fatty acid metabolism and the isovaleric pathway lie in the carboxylation step and in cleavage of the carbon chain without prior formation of a β -keto acid (32).

Although as indicated, the transformation steps occur in mammalian tissues, detailed enzymic transformation steps have been worked out in other organisms. Thus the carboxylation step was shown to occur with β -methylcrotonylcoenzyme A rather than with β -hydroxyisovalerylcoenzyme A, using enzyme preparations from mycobacteria (33) and chicken liver (34). The carboxylation step requires biotin and ATP, forming an enzyme-biotin- CO_2 complex. The intermediate β -hydroxymethyl- β -glutarylcoenzyme A is of particular interest, since in addition to its cleavage to acetoacetate and acetylcoenzyme A, it may be converted to mevalonic acid. This is a key intermediate in the biosynthesis of steroids and polyisoprenoid substances (35). This conversion is presumably of significance in the known utilization of isotopically labelled isovaleric acid in cholesterol synthesis (36). The fates of acetoacetate and acetylcoenzyme A are discussed below.

The fate of ^{14}C -labelled isovaleric acid in rats has been reported (37). The isopropyl group was more efficiently used for cholesterol synthesis than for fatty acid synthesis, and more efficiently for both syntheses than was the carboxyl group. Isovaleric acid also enhanced the uptake of $^{14}\text{CO}_2$ into cholesterol. These observations are consistent with the above carboxylation step in isovaleric acid metabolism and the cleavage into 2 carbon fragments. The administration of isovaleric acid to rats also results in the urinary elimination of a cysteine conjugate,

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isovalthine (38), having the structure



This should arise formally from the interaction of β -methylcrotonate and glutathione. The origin of this substance is however unknown, since feeding of ^{14}C -isovaleric acid yields increased amounts of non-radioactive isovalthine (37). In addition, in vitro studies in which isovalerate and glutathione were incubated with cat, rat, liver and guinea pig slices (39), followed by treatment with guinea pig glutathionase, gave L-allo-isovalthine, whose configuration differs from naturally occurring isovalthine. At present isovaleric acid appears to stimulate isovalthine production by an unknown mechanism.

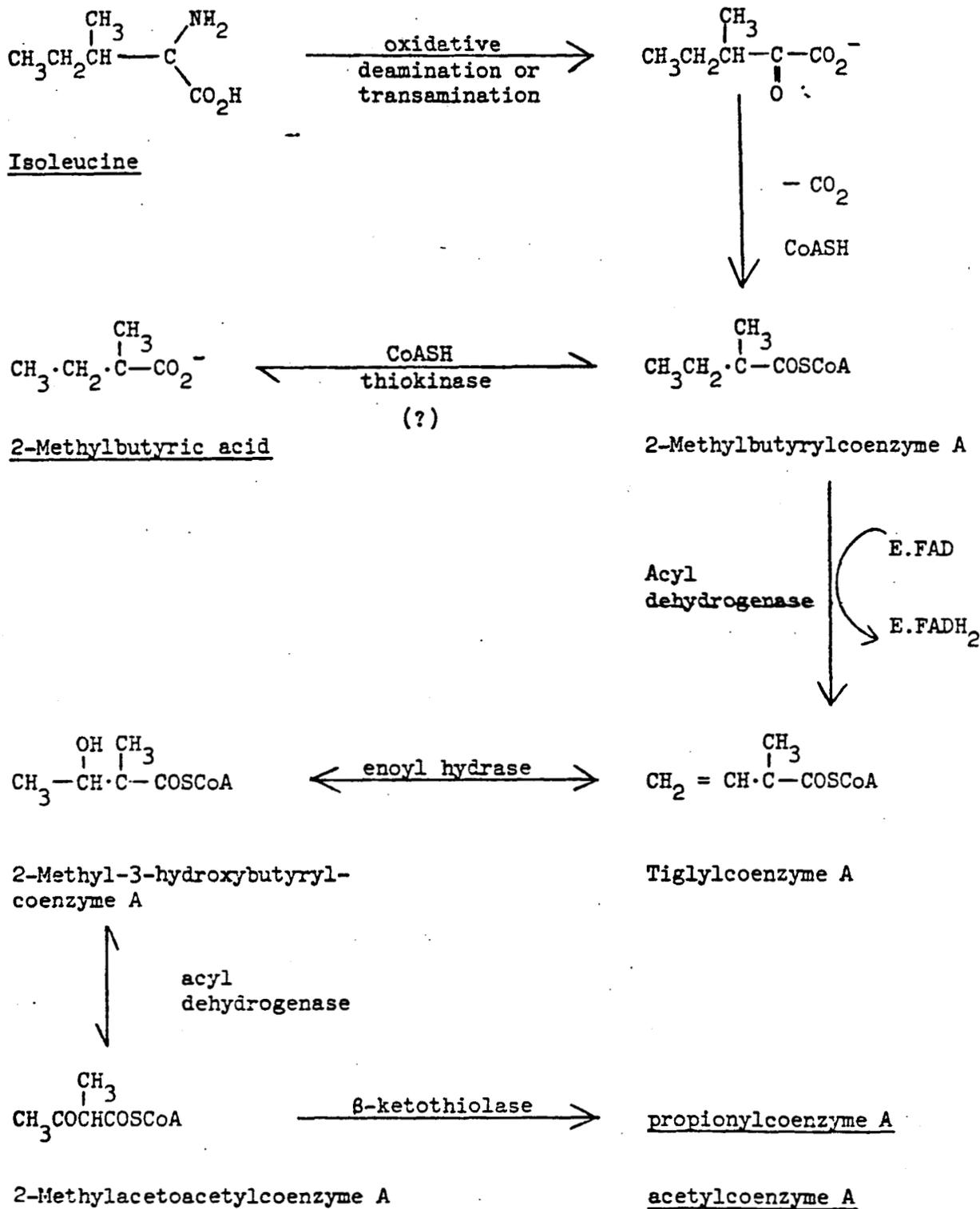
c) 2-Methylbutyric acid

2-Methylbutyric acid has been identified, in the form of its coenzyme A ester, as an intermediate in the biodegradation of the amino acid isoleucine (Fig. III). The overall process involves the formation of tiglylcoenzyme A, α -methylacetoacetylcoenzyme A, leading to the formation of acetylcoenzyme A and propionylcoenzyme A (40). All the reactions in this sequence occur in pig heart and rat liver soluble fractions. The close resemblance of several of these steps to similar steps in the β -oxidation sequence for straight chain fatty acids makes it likely that the same enzymes are involved (23).

In early studies with labelled 2-methylbutyrate incubated with rat

Figure III

Metabolism of isoleucine and 2-methylbutyric acid (after Mahler)(23)



liver tissue it was established that 3-¹⁴C-2-methylbutyrate yields labelled acetate but no labelled propionate, while 2-¹⁴C-2-methylbutyrate yields labelled propionate. Both labels gave labelled acetoacetate and ¹⁴CO₂ (41). These results indicate that 2-methylbutyrylcoenzyme A undergoes β-oxidation on the longer C chain with cleavage to acetate and propionate. The activation of 2-methylbutyric acid by conversion to its thiol ester presumably occurs in a facile manner, since the biodegradation products of isoleucine, 2-methylbutyrylcoenzyme A and 2-methylbutyric acid all appear to be identical (40,41). In addition, the degradation may terminate at 2-methylacetoacetate, since heart muscle contains a thiolase which catalyzes the transfer of the coenzyme A group of 2-methylacetoacetylcoenzyme A to succinic acid (40).

The fates of acetyl- and propionylcoenzyme A are discussed next.

d) Fates of propionate, acetate and acetoacetate

The role of both acetate and propionate in the intermediary metabolism has been well established. Propionate, as propionylcoenzyme A, occurs widely as the end product of β-oxidation of odd numbered fatty acids and certain branched chain fatty acids (23). Its further conversion is achieved by conversion to methylmalonylcoenzyme A and succinate, principally in mammals (Fig. IV), and by three pathways derived from acrylylcoenzyme A, principally in plants and microorganisms (Fig. V). In either case entry into the citric acid cycle is assured via succinate, or via pyruvate or acetylcoenzyme A, end products of the acrylylcoenzyme A routes (23).

As is well known acetylcoenzyme A is a fundamental biochemical end

Figure IV.

The enzymic oxidation of propionate: methylmalonate route
(after Mahler)(23)

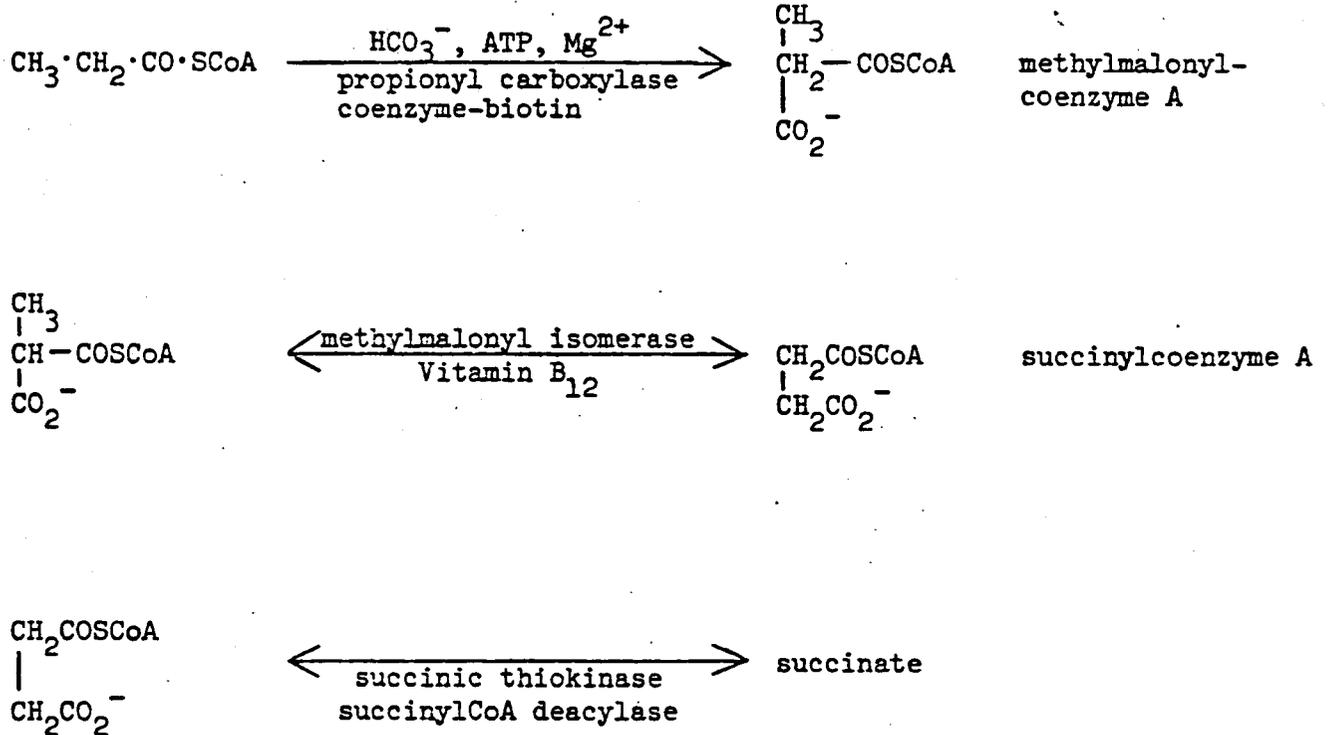
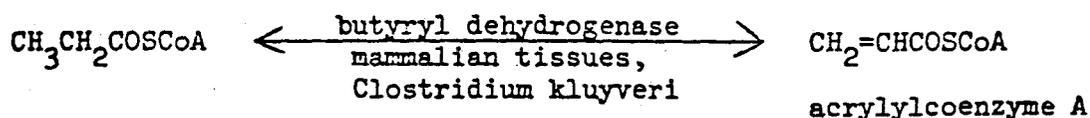
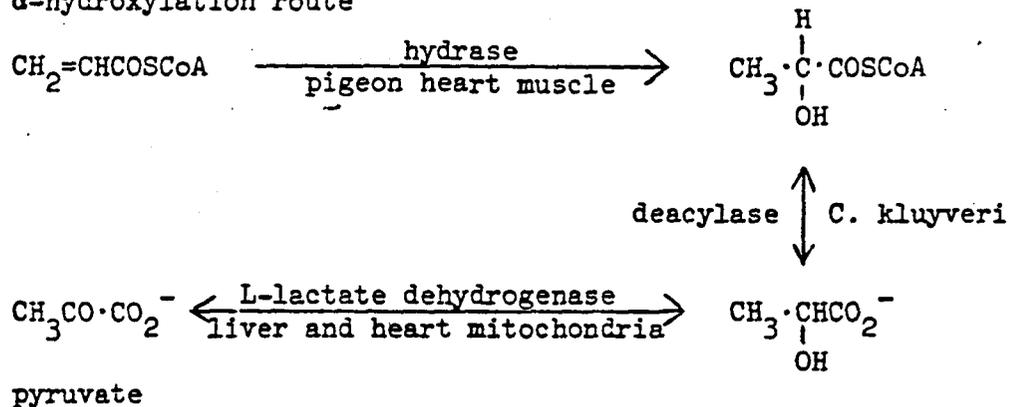


Figure V.

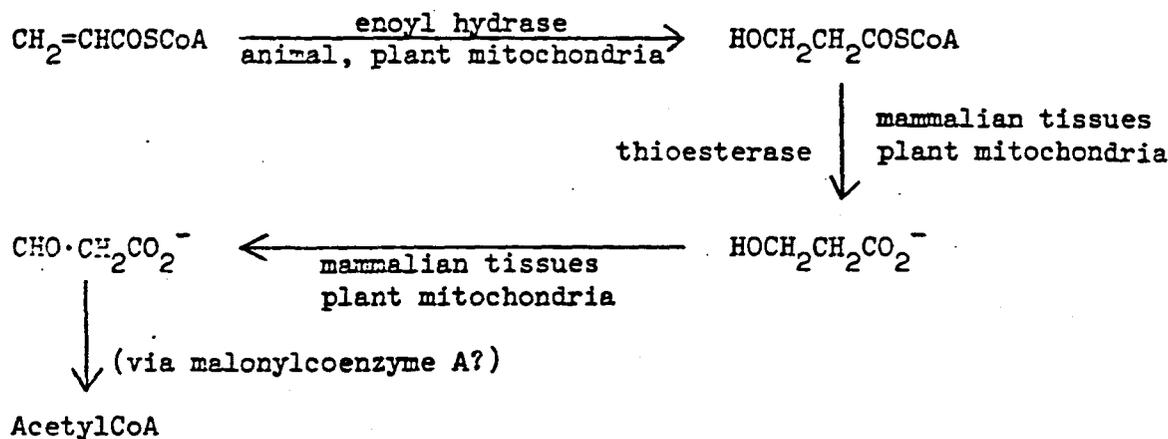
The enzymic oxidation of propionate: acrylylcoenzyme A routes
(after Mahler)(23)



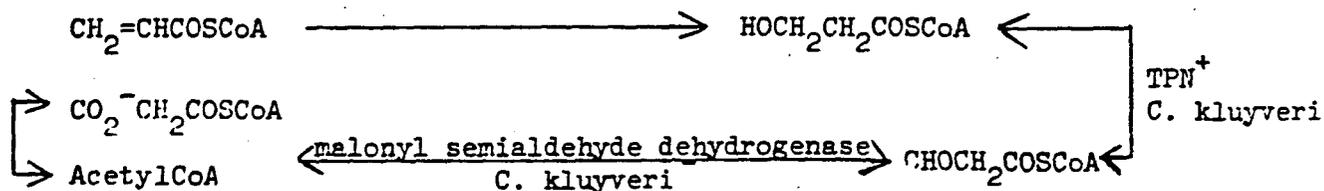
[A] α -hydroxylation route



[B] β -hydroxylation route: (a major plant route)

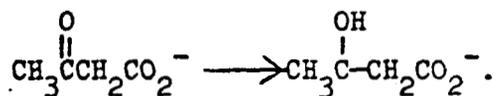


[C] Coenzyme A dependent β -hydroxylation



product and biosynthetic unit. It interconverts with the acids of the citric acid cycle, participates in lipid synthesis by virtue of its conversion to malonylcoenzyme A, participates in terpene, steroid and carotenoid synthesis by its interaction with acetoacetate to form mevalonate, and takes part in carbohydrate synthesis via phosphoenolpyruvate.

Acetoacetate may be eliminated unchanged, or converted to acetone, or to β -hydroxybutyrate or to acetoacetylcoenzyme A (23). The latter change takes place in extra-hepatic tissues in mammals, leading to acetylcoenzyme A by the action of β -ketothiolase. Free acetoacetate is metabolically inert in the liver, although liver cytoplasm contains an enzyme which catalyzes the conversion



This in turn (D form) may be activated by a thiokinase to β -hydroxybutyrylcoenzyme A, which is in turn converted to acetoacetylcoenzyme A (23). Generally in the non-diabetic free acetoacetate formed in the liver is activated and converted in extrahepatic tissue to acetylcoenzyme A.

III. MICROBIAL AND WASTE TREATMENT ASPECTS

a) Valeric acid

The microbial metabolism of valeric acid has been demonstrated by rumen microflora, waste treatment and sewage microorganisms and by a variety of other fungi and bacteria.

Extensive studies with valeric acid as an *E. coli* substrate have shown the importance of a coenzyme A transferase and a thiolase in valeric acid utilization (42), although valeric acid is a poor inducer of β -oxidation in mutants having a depressed capacity for this reaction. However valeric acid can be used as a sole source of carbon even in mutants lacking coenzyme A transferase capacity; in the latter case an acyl-CoA transferase can be induced (43).

Studies with wheat stem rust ureidospores (*Puccinia graminis tritici*) confirmed the generality of the β -oxidation pathway, in that 1, 3, or 5- ^{14}C -valerate produced $^{14}\text{CO}_2$ and labelled intermediates similar to those produced by 1- ^{14}C -acetate and 1- or 3- ^{14}C propionate (44).

Similarly *Clostridium kluyveri* converted valerate to acetate and propionate via the keto acid intermediate 3-hydroxyvalerate (45).

Valeric acid is biodegradable in waste treatment situations. Thus the VFA content of feces fell from 6700 ppm of mostly C_2 , C_3 and C_4 acids in fresh feces to 4600 ppm of mostly C_2 acids in 19 days; the influent to secondary waste treatment contained C_2 through C_5 n- and iso-acids, the effluent containing almost entirely C_2 acids (46). Warburg type respirometer studies indicated the ready biochemical oxidation of short chain fatty acids including valeric acid by activated sludge microorganisms (47). Activated sludges from three municipal sewage treat-

ment plants were active against fatty acid concentrations of 500 ppm (48). Anaerobic treatment results in a similar conversion in 6-18 hrs to lower fatty acids, principally acetic (14). Studies with the microbial population of staled sewage show the dissolved oxygen utilization of valeric acid to be almost identical with that of acetate (49). Soil microorganisms used in such dissolved oxygen utilization studies show a marked capacity to degrade valeric acid (50).

b) Isovaleric acid

The microbial metabolism of isovaleric acid occurs with rumen microflora, waste and sewage microorganisms and with certain fungi.

Certain key steps in the sequence isovaleric acid \longrightarrow acetoacetate and acetylcoenzyme A were elucidated with microbial preparations (33).

Sewage treatment microorganisms readily decompose isovaleric acid with an oxygen uptake comparable to that of acetic acid (49). Influent to secondary waste treatment contained C_2 to C_5 acids, including isovaleric, but effluents contained principally C_2 acids (46). A mixture of fatty acids including isovaleric acid treated with activated sludge, showed a 75% reduction in fatty acid content (51), and micrococci and sarcinae were isolated which utilized isovaleric acid as a sole carbon source. Finally *Aspergillus niger* in the presence of glucose converts isovaleric acid to 3-methylbutanol, presumably via an aldehyde intermediate (52).

c) 2-Methylbutyric acid

The metabolism of 2-methylbutyric acid by rumen microorganisms is discussed below. Sewage treatment microorganisms decompose 2-methylbutyric acid with an oxygen uptake comparable to that of acetic acid (49).

IV. BEHAVIOR OF VALERIC, ISOVALERIC AND 2-METHYLBUTYRIC ACIDS IN THE RUMEN AND RUMINANTS

a) Valeric acid

A considerable quantity of information is available on the role of valeric acid in ruminant nutrition. Valeric acid is found in the volatile fatty acid fraction of rumen fluid, not usually exceeding 6% of that fraction, the proportion varying with dietary composition (53). Table Ia lists valeric acid concentrations in the rumen for differing species receiving varied diets. It is of interest that the valeric acid content of sheep rumen decreased in traversing the rumen (1.42% of VFAs) omasum (1.07%) and abomasum (0.66%) (54).

It is accepted that the principal source of straight chain volatile fatty acids in the rumen is dietary carbohydrate, the conversion being carried out by anaerobic rumen microorganisms (55). Valeric acid itself appears to be produced from starches and hexoses (56) and in their absence cellulolytic rumen organisms produce little valeric acid (57). Other sources of valerate include dietary protein; the rumen valeric acid concentration has been shown to vary with protein content of the diet (58). The amino acid proline has been established as a precursor of valerate in incubations of ¹⁴C-labelled proline with rumen microorganisms (59), via a reductive ring cleavage and deamination (60). In addition, washed strains of a gram-negative coccus from sheep rumen will anaerobically convert threonine to acetate, butyrate and valerate (61). Diets relatively high in protein such as lucerne hay produce considerable increases in rumen valeric acid (62).

These various routes of formation have led to overall estimates of the rate of fatty acid formation in the rumen. The volatile fatty acid production from 350-400 ml of ingesta collected from the fistulated reticulorumen of a steer amounted to almost 3 kg in 24 hrs.

Table Ia

Valeric acid as a constituent of rumen liquor volatile fatty acids (VFAs)

Species	Dietary Characteristics	Total VFAs µmoles/l	Valeric Acid	Reference
Steers	hay and corn ration	105-181	3.9-5.6 mol %	53
Calves	milk or solids	12-23	2 mol %	63
Cattle	standard		1.3% of total	64
Cattle	purified with varying N and carbohydrate added		0.8-10 mol %	65
Sheep	wheaten hay		1.6-3.2% of total	56
Sheep	standard	rumen 7.83 meq omasum 4.04 meq abomasum 1.29 meq	1.42% of total 1.07% of total 0.66% of total	54
Sheep	lucerne, lucerne + formaldehyde, lucerne + wheat, wheat grain	48-153	0.22-0.67%	67

This was made up of 1.74 kg of acetic acid, 0.52 kg of butyric, 0.56 kg of propionic and 108 g of valeric acid (68).

As is well known, the bulk of the volatile fatty acids produced in the rumen is absorbed into the blood and serves as a major source of ruminant energy (69). Valeric acid may be absorbed by the rumen epithelium or metabolized by the rumen microflora. Valerate is converted to acetate and propionate on incubation with sheep rumen epithelial tissue, with an expected increase in "ketone bodies" (70).

The reported concentration of 5-220 micromoles of valeric acid/l of sheep blood plasma (71) indicates that absorption of valeric acid from the rumen also occurs. Valerate-¹⁴C injected intrajugularly into lactating cows is distributed in milk lipids without preference to odd or even numbered acids (72). This is consistent with an in vivo degradation to acetate and propionate followed by de novo lipid synthesis, rather than lipid synthesis starting with valerylcoenzyme A. The in vivo degradation has been observed with sheep liver and kidney slices, which carry out the conversion of valerate to acetate and propionate (73) as established for monogastrics. It is of interest that valerate is included in the short chain fatty acids which stimulate carboxylation of propionate in both ruminants and monogastrics (74).

Several routes for utilization of valeric acid by the ruminal microflora have been described. In vitro experiments indicate that labelled valeric acid is taken up into microbial protein by rumen bacteria (75). When valeric acid is added to sheep diets with urea an increase in in vivo microbial protein is noted (76). Uptake of valerate into lipids

has been reported from the action of cellulolytic rumen bacteria on ^{14}C -valeric acid, presumably from breakdown to acetate followed by resynthesis to fatty acids, phospholipids, sterols and triglycerides (77). *Selenomas rumenantium* incorporated ^{14}C -valerate to the extent of 40% in the phospholipid fraction of the cell wall. The remainder was located as bound lipids in subcellular particles, principally as C_{13} , C_{15} and C_{17} acids (78). Studies with *Thiobacillus neapolitanus* suggested that incorporation of valerate into cellular constituents took precedence over $^{14}\text{CO}_2$ production in the metabolism of valeric acid in autotrophic media (79). Valeric acid is also an effective substrate for methanogenic rumen bacteria in cattle, based on enrichment cultures from bovine rumen fluid (80). The methane pathway also produces CO_2 and propionic acid.

These observations suggest that valeric acid in the rumen will ultimately yield acetate, propionate, CO_2 , CH_4 and steroids, although the actual pathways to these end products may be complex.

b) Isovaleric acid

The occurrence of isovaleric in the volatile fatty acid fraction of the rumen liquor in cattle and sheep has been well attested to (Table 1b). The concentration is not usually in excess of 3% of the volatile fatty acids, depending on species, nature of the diet and ambient conditions. Isovaleric acid has also been identified in the cecum (3.5-4.5 mol % of volatile fatty acids), colon (2.5-3.8%) and in the feces (1.3-3.6%) of sheep receiving a diet of dry grass or barley (81), arising presumably from microbial fermentation of protein in the digesta.

Table Ib

Isovaleric acid as a constituent of rumen liquor volatile fatty acids (VFAs)

Species	Dietary Characteristics	Total VFAs mmoles/l	Isovaleric acid	Reference
Cattle	Standard	--	0.92% of total	64
Steers	Standard: ambient 12.8°C 30% Rel. Hum. ambient 35°C 25% Rel. Hum.	--	0.9 - 1.5 meq/l 2.7 - 3.0 meq/l	85
Cattle	Natural	116 ± 15.3	1.6 ± 0.1 mol %	65
	Various carbohydrate sources	87 - 114	0.9 - 1.4 mol %	
	Various nitrogen sources	69 - 92	0.2 - 0.3 mol %	
	Urea, purified	163.5 ± 14.2	0.6 ± 0.2 mol %	
Calves	Milk or solids	12 - 23	2 mol %	63
Sheep	Standard rumen	7.83 meq	1.44% of total	54
	omasum	4.04 meq	1.86% of total	
	abomasum	1.29 meq	1.01% of total	
Sheep	Various: lucerne, lucerne plus wheat, wheat grain etc.	48 - 153	0.15-1.33% of total	67

It is accepted that the principal source of branched chain fatty acids in the rumen is dietary protein (82, 83), which is biodegraded by proteolytic enzymes in the rumen microflora to the constituent amino acids. Early studies with casein hydrolysates indicated that branched chain fatty acids were produced by the action of washed suspensions of sheep rumen microorganisms (84). It was suggested that branched chain acids such as isovaleric and isobutyric were derived from leucine and valine, derived in turn from dietary protein. The amino acids were converted to fatty acids in a manner analogous to the Stickland reaction carried out by a variety of anaerobic microorganisms (86). Subsequently it was shown that *Bacteroides ruminicola* in pure culture produced a growth factor from casein hydrolysate for *Ruminococcus albus*. When leucine-2-¹⁴C was incubated with *B. ruminicola* a radioactive valeric acid assumed to be isovaleric was produced, and shown to be the growth factor (87). In addition, leucine has been shown to be ketogenic in goats in a similar manner to isovaleric acid (88), and to be converted to isovaleric acid in the rumen of a fistulated goat (89).

While the evidence suggests that dietary protein is the major source of rumen branched chain fatty acids, small quantities have been detected in the rumen when protein free diets are fed (90). The possibility of pathways involving carbohydrate fermentation may exist, particularly since acetate and acetoacetate, known to form isovalerate by reversal of the isovalerate catabolism sequence, could be produced.

It is generally accepted (83) that the branched chain volatile fatty acids in the rumen are carboxylated and aminated to amino acids con-

taining the original carbon chain. The sequence is characteristic of anaerobic rumen microorganisms and differs from any other amino acid biosynthesis. The carboxylation step is referred to as "reductive carboxylation". By analogy with a pyruvate synthetase system occurring in some non-ruminant anaerobic bacteria (91), it appears to follow the sequence

$$\begin{array}{ccccccc} \text{RCO}_2\text{H} & \xrightarrow{\text{ATP}} & \text{RCO}_2\text{PO}_3\text{H}_2 & \xrightarrow{\text{CoASH}} & \text{RCOSCoA} & \longrightarrow & \\ \text{RCOTPP-enz} & \longrightarrow & \text{RCHOH-TPP-enz} & \xrightarrow{\text{CO}_2} & \text{RCHOHCO}_2^- & \longrightarrow & \text{R}\underset{\text{O}}{\underset{\text{O}}{\text{C}}}\text{CO}_2\text{H}. \end{array}$$

Isovaleric acid has been shown to be utilized by a variety of rumen microorganisms in leucine synthesis (83), and in addition, is incorporated into bacterial lipids. Thus *Bacteroides succinogenes*, *Ruminococcus flavefaciens* and *R. albus* show a requirement for branched chain fatty acids, requiring isovaleric acid and ammonia for protein synthesis (92). In experiments with *R. flavefaciens* the uptake of $^{14}\text{CO}_2$ into protein in the presence of isovalerate was demonstrated, and most of the ^{14}C was located in the carboxyl of the isolated leucine (93). In addition a statistically significant correlation between rumen ammonia content and volatile fatty acid content was found in fasted sheep. This supports the contention that these acids are precursors of branched chain amino acids and essential to microbial protein synthesis (94). The addition to the diet of organic acids undergoing the reductive carboxylation sequence also increases nitrogen retention and decreases urinary nitrogen (95).

A detailed study of the uptake of 1 or 3- ^{14}C -isovalerate by *R. flavefaciens* showed that both protein and lipid incorporated radioactivity (96, 97). Radioactivity in protein was due entirely to ^{14}C -leucine, the isovalerate 1- ^{14}C atom becoming the leucine-2- ^{14}C atom. The iso-

valerate molecule is clearly incorporated intact. The lipid radio-activity was apparently due to branched chain 15C and 17C fatty acids, with a proportion of a branched 17C aldehyde. In addition *R. flavefaciens* showed little capacity for incorporating exogenous labelled leucine (98), and the specific activity of β -isopropylmalate dehydrogenase was very low. This enzyme is involved in leucine biosynthesis in *E. coli* (98).

The behavior of isovaleric acid in contact with tissues and organs of ruminants, as distinct from the action of rumen microflora, has been studied (70). It is slowly absorbed from the empty sheep rumen, being taken up into ruminal vein blood. When incubated with sheep rumen epithelial tissue in the presence of CO_2 there is a loss of isovaleric acid and production of ketone bodies, results closely paralleled with ox rumen epithelium. In addition acetic acid, but not propionic acid, accumulated in the medium. Sheep liver slices also catabolize isovaleric acid to acetic acid and ketone bodies with greater efficiency in the presence of CO_2 (73). The disappearance of isovaleric acid in kidney slices is enhanced by 60% in the presence of CO_2 . The conversion of leucine and isovalerate was studied with the udders of lactating cows in perfusion experiments (101). Both yielded CO_2 to a small extent, the bulk of the leucine being incorporated into milk protein, and of the isovalerate into milk lipids. The maximum incorporation of isovalerate was into the C_{10} fraction (99).

c) 2-Methylbutyric acid

The occurrence of 2-methylbutyric acid has been reported in sheep rumen fluid (100). The concentration of total isovaleric acids in sheep rumen is about 2-3 mol. % of total volatile fatty acids; since

the isovalerate concentration is usually 1-2 mol %, 2-methylbutyric is of the same order (101). As a branched chain fatty acid it is assumed that 2-methylbutyric acid is formed in the rumen from the amino acid containing the appropriate carbon chain skeleton. The formation of 2-methylbutyric acid from isoleucine (Fig. III) supports this view, particularly since proteolytic anaerobes formed an optically active isovaleric acid, presumably 2-methylbutyric, from mixed branch chain amino acids which included leucine (86).

As indicated above, branched chain fatty acids act as precursors in branched chain amino acid synthesis (82), utilizing a reductive carboxylation step carried out by anaerobes. The carboxylation of 2-methylbutyrate is apparently a particularly efficient conversion. Following $^{14}\text{C}\text{O}_2$ administration to a goat a high specific activity was found for ^{14}C -isoleucine in ruminal microbial protein (102). A wide range of rumen anaerobes carry out the reductive carboxylation of 2-methylbutyric acid (83). Ruminal ingesta and pure cultures of ruminal anaerobes, including *Bacteroides ruminicola* strain 23, incorporated ^{14}C from labelled 2-methylbutyrate mainly into lipid and protein. Isoleucine was the sole labelled amino acid in protein from pure or mixed cultures, 2- ^{14}C isoleucine resulting from 1- ^{14}C -2-methylbutyrate (103). With unlabelled 2-methylbutyric, *Ruminococcus flavefaciens* incorporated $^{14}\text{C}\text{O}_2$ into the carboxyl group of isoleucine (93). Thus the analogous steps for isobutyrate and isovalerate utilization in the rumen are also carried out with 2-methylbutyrate. In accordance with this, 2-methylbutyric acid has been identified as one of the branched chain fatty acids whose incorporation in the diet of steers

increases rumen nitrogen retention and decreases urinary nitrogen (95). Similar studies on 2-methylbutyric have been reported to those with isovaleric acid in sheep rumen, liver and kidney slices (70). 2-Methylbutyric acid is slowly absorbed from the empty rumen, and subsequently occurs in ruminal vein and carotid blood. On incubation with rumen epithelial slices it produces acetic and propionic acids, but no ketone bodies, and CO_2 has no effect on its rate of conversion. Similar results were obtained with ox rumen epithelium. Sheep liver slices utilized 2-methylbutyric acid efficiently, producing acetic acid, no propionic acid and ketone bodies, and kidney slices produced acetic and formic acids (70).

V. THE TOXICOLOGY OF VALERIC, ISOVALERIC AND 2-METHYLEBUTYRIC ACIDS

a) Valeric acid

The mammalian toxicity, physiological activity and pharmacological activity of valeric acid have been studied. The acute oral LD₅₀ in rats of mixed isomeric valeric acids (undiluted) has been reported as 1.12 ml/kg (0.45 - 2.79) (104) and 2.00 ml/kg (0.90 - 4.44) (105), and the LD₅₀ by skin application to rabbits as 0.70 ml/kg (0.38 - 1.28) (104) and 0.31 0.06 - 1.50) (105). A 1% aqueous solution of valeric acid had an oral LD₅₀ in rats of >400 mg/kg, and an IP LD₅₀ of 200-400 mg/kg, symptoms being weakness and ataxia (106). It is concluded that the undiluted acid is a moderately strong skin and eye irritant (107), and is moderately toxic by both the skin and oral routes. In other studies Egerov et al. (108) considered the C₅ - C₆ fractions the most toxic in chronic experiments, easily penetrating the skin. Thus 0.005 g/sq. cm of rabbit skin surface was a threshold dose, 5 g/kg applied to the skin produced intoxication and 10 g/kg was lethal to all animals. Effects on the nervous system were claimed in chronic experiments (108).

The inhalation toxicity of valeric acid has been described. Rats exposed to the concentrated vapors of mixed isomeric acids survived for eight hours without deaths (105). Mice exposed to 2.7 mg/m³ 1.2 ppm for 105 days showed changes in the bronchial tract, desquamation of lung tissue and occasional pneumonia (109). Caproic and butyric acids showed similar symptoms. The same group of workers considered that a maximum permissible airborne concentration of 0.006 mg/m³ (3 ppb) should be applied, since at this concentration there was no effect on O₂ consumption, p[CO₂] and conditioned

reflexes of experimental animals during 97 days (110). Experimental human exposures (111) suggested an odor threshold of 0.09 mg/cm^3 and a maximum acceptable concentration of 0.03 mg/cm^3 (15 ppb), based on eye light sensitivity and ENC changes. These suggested limits seem excessively restrictive, particularly in view of the TLVs established for acetic and formic acids (10 ppm and 5 ppm) respectively (112).

Valeric acid, presumably because of its hydroxylated intermediate (β -hydroxyvalerylcoenzyme A) exhibits neuropharmacologic activity, apparently as a CNS depressant. Behavior similar to that of γ -hydroxybutyric acid was elicited by valeric acid and other short chain fatty acids in inducing characteristic brain waves and increasing total sleep in cats (113) and in producing erratic epileptoid reactions in rats (114) similar to those in cats. Valeric acid also possesses anti-spasmodic activity against barium chloride in isolated rat and rabbit intestine but no other pharmacologically activity was reported (115). These observations appear to be consistent with findings that short chain fatty acids, including valeric acid, are implicated in hepatic coma (116). Thus allyl formate poisoned rabbits have a much higher concentration of lower fatty acids than normal rabbits in the liver, and ip injection of butyrate, valerate or octanoate produces reversible coma in rats (116, 117).

A variety of enzyme inhibitory reactions has been reported for valeric acid (e.g. 118), mostly in studies on enzyme structure and characteristics, and involving short chain fatty acids in general. These are not of physiological importance.

In vitro and in vivo studies indicate that valeric acid, along with

other straight chain fatty acids is readily absorbed by the rat intestine, being transferred against a concentration gradient (119). Humans absorb valeric acid readily from both the colon and jejunum (120).

The ready utilization of valerate by microorganisms suggests that it has a limited potential for adverse effects on non-mammalian species. Fatty acids including valeric have been tested for activity against soil nematodes, when survival (immobilization) times varied from 5 seconds in M solution to 2000 seconds in mM solution, without protoplasmic or cuticular changes (121). Fatty acids ($C_1 - C_6$) also show inhibition of L-serine and fructose in whole *B. subtilis* cells or membrane vesicles (122) and retard growth of *Chlamydomonas* and *Hematococcus* species in pure culture (123), the latter being concentration dependent (0.5 to 10 mM).

b) Isovaleric acid

The oral LD_{50} of undiluted isovaleric acid in rats is 400-3200 mg/kg, and it is a strong skin irritant in guinea pigs, based on 24 hr. applications of 1 and 10 mg/kg. These doses were not lethal. Lethal oral doses produce weakness, retraction of the abdomen and vasodilation (124). The only reported physiological activity exhibited by isovaleric acid is mild CNS depression, in company with other volatile fatty acids such as valeric and butyric. Thus paradoxal sleep was induced in the chronic pontine cat (125) by intravenously injected 1-1.2 millimolar sodium caproate, valerate, isovalerate and butyrate. Similar results were found in rats (126). As with other volatile fatty acids, this behavior is consistent with the hepatic coma induced in

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rats by allyl formate which appears to be associated with accumulation in the damaged liver of fatty acids including isovaleric (116). A number of physiological and pharmacological effects have been described in which the role of isovaleric acid has been studied. Thus leucine and isovaleric acid produced an enhanced hypoglycemia in human subjects with spontaneous hypoglycemia (127), but isovaleric acid alone did not produce hypoglycemia in normal subjects, and did not enhance hypoglycemia in subjects dosed with oral hypoglycemics (128). Leucine however produced an intense hypoglycemia in such subjects, but evidently not because of its metabolism to isovalerate. Hypoglycin A, a constituent of "ackee", produces prostration, vomiting and coma in the so-called "Jamaican Vomiting Sickness", together with a pronounced hypoglycemia and isovalericacidemia (129). While the accumulation of isovaleric acid was due to inhibition of isovalerylcoenzyme A dehydrogenase, isovaleric acid was shown not to be the causative factor, since when given to experimental rats with hypoglycin-A induced hypoglycemia it did not enhance or prolong hypoglycemia (134).

A rare genetic disease associated with an inborn error in the metabolism of leucine has been described; its principal biochemical feature is the elevation of isovaleric acid levels in the blood (131). It is associated with a peculiar body odor, episodic acidosis and slight mental retardation. The enzyme isovalerylCoA dehydrogenase is either inhibited or absent, leading to the elevation of isovalerate levels in blood by 100 - 1000 fold over normal levels (20). The disease is of interest here since it presents some guide to the long term effects of elevated blood levels of isovaleric acid in humans.

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Enzyme inhibitory effects have been associated with isovaleric acid as with other fatty acids. Thus isovaleric acid competes with the γ -carboxyl of α -glutamate in inhibiting in vitro glutamic decarboxylase of squash (132), and along with isocaproate and isobutyrate decreases rat liver mitochondrial oxidative phosphorylation, being somewhat more active than butyric, valeric and caproic acids (133).

c) 2-Methylbutyric acid

2-Methylbutyric acid, when given orally in the undiluted state to rats, has an LD₅₀ of 1600-3200 mg/kg, and to mice, less than 800 mg/kg. The rat IP LD₅₀ is 50-100 mg/kg. As a 5% solution in corn oil it has an oral LD₅₀ in rats in excess of 1600 mg/kg and an IP LD₅₀ of 200-400 mg/kg (134). It is both a skin and eye irritant, as shown by its immediate corneal irritation in the rabbit eye when administered undiluted, and the strong primary skin irritation when applied to guinea pig skin. The cutaneous LD₅₀ in guinea pigs was 5-10 ml/kg. Calculated vapor concentrations up to 2000 ppm for 6 hours produced no lethality in rats (134). It was concluded that 2-methylbutyric acid was slightly toxic orally, but possessed hazards for contact with skin or eyes, and that the vapors were irritating and could cause lacrimation.

Little activity of a pharmacologic or physiological nature has been reported for 2-methylbutyric acid. The action of hypoglycin A, in addition to producing isovalerylacidemia, also produces elevated 2-methylbutyric acid levels in the blood (129), but no mechanism or physiological consequences have been suggested.

VI. REGULATORY STATUS OF CCN ACIDS

a) As direct food additives

21CFR 121.1091 permits the use of valeric acid in a mixture with caproic, enanthic, caprylic and pelargonic acids to be used in washing or assist in the lye peeling of fruits and vegetables. Use level is not to exceed 1% in the lye peeling solution.

21CFR 121.1164 permits the use of isobutyric acid, isovaleric acid, 2-methylbutyric acid and valeric acid as synthetic flavoring substances or adjuvants added to food. They should be used in the minimum quantity to produce the desired effect or in accordance with good manufacturing practice.

b) As pesticides

40CFR 180.1030 grants an exemption from a tolerance for residues of isobutyric acid resulting from the post harvest application of isobutyric acid or ammonium isobutyrate to the following raw agricultural commodities: alfalfa, bermuda grass, brome grass, clover, corn, fescue, grains of barley, oats, sorghum and wheat, lespedeza, orchard grass and timothy.

c) GRAS Reviews: have been requested for isovaleric and 2-methylbutyric acids.

VII. GENERAL SAFETY CONSIDERATIONS IN THE PROPOSED USE OF CCN ACIDS

a) Estimated intake level in use

It is envisaged that the four CCN acids will be added to feed as a blend, and that the intake of each acid by dairy cattle or steers will be about 20 g per day (135).

b) Estimated normal rumen levels of CCN acids

As indicated above, the four acids occur normally in the rumen in fairly low concentrations. These are instantaneous values and presumably reflect an equilibrium. Of more importance is the turnover rate.

Estimates of the rumen production and consumption of each acid can be obtained from estimates of total feed and dietary protein intake (136) and from the daily intake of amino acid precursors, based on literature values (137, 138). Table 2a presents average estimates of valine, leucine, isoleucine, and proline content of typical cattle feed, and Table 2b the resulting estimated intake of each amino acid either in feed or from digestible protein in feed. Table 2c gives the resulting estimated turnover of each CCN derived from its amino acid precursor. It is assumed that only 50% of feed derived amino acids are available for conversion to the corresponding CCN acids, but that all the digestible protein amino acids are.

The resulting turnover rates are fairly close, whether derived from feed or digestible protein. In addition, the rate of formation of valeric acid has actually been determined in the fistulated rumen of a steer as indicated above (68) and amounts to about 108 g per day(68). This undoubtedly includes both the synthesis of valeric

Table 2a

Average percent amino acid content of feed and digestible protein (137,138)

	Valine	Leucine	Isoleucine	Proline
Feed	0.8 (0.53-2.2)	1.0 (0.5-1.7)	0.65 (4.5-9.3)	not given
Protein	4.0 (3.6-4.3)	6.9 (4.5-9.3)	3.5 (2.2-4.7)	5.7 (4.0-7.5)

Table 2b

Estimated CCN acid precursor intake by ruminants

Animal and Type	Weight* kg	Feed kg/day	Digestible Protein Intake* g/day	Valine		Daily intake in grams**		Isoleucine Feed	Proline protein	
				Feed	Protein	Leucine Feed	Protein			
Cattle 2 year beef	363-544	10.5-14.1	770-1043	84-113	31-45	104-141	53-72	68-92	27-37	44-59
Cattle pregnant cows	400-700	9.8-15.2	610-720	78-122	24-29	98-152	42-50	64-99	21-25	35-41
Cattle mature dairy	500-1200	7.8-15.5	450-800	62-125	18-32	78-155	31-55	51-101	16-28	26-46
Sheep yearling female	27-54	1.2-1.5	59-73	9.6-12.0	2.5-2.9	12-15	4.1-5.0	7.8-9.7	2.6-4.2	3.4-4.2
Sheep lactating	45-73	1.7-2.1	81-91	13.6-16.8	3.2-3.6	17-21	5.5-6.3	11-14	2.8-3.2	4.6-5.2

* Reference 136

** Based on average amino acid content in Table 2a

Table 2c

Estimated CCN turnover in the rumen from exogenous amino acid intake

Assumption: Fifty percent of feed amino acid or all of digestible protein amino acid is available for conversion to CCN acids.

Animal and Type	Weight	Daily turnover of CCN acid in grams						
		Isobutyric Feed*	Isobutyric Protein*	Isovaleric Feed	Isovaleric Protein	2-Methylbutyric Feed	2-Methylbutyric Protein	Valeric Protein
Cattle 2 year beef	363-544	32-43	23-34	41-55	41-56	26-36	21-29	39-52
Cattle pregnant cows	400-700	30-46	18-22	38-59	33-39	25-39	16-19	31-36
Cattle mature dairy	500-1200	24-47	14-24	30.4-60	24-43	20-40	13-22	23-40
Sheep yearling female	27-54	3.6-4.6	1.8-2.2	4.7-5.8	3.2-3.9	3.0-3.8	2.0-3.3	3.0-3.7
Sheep lactating	45-73	5.2-6.4	2.4-2.7	6.6-8.2	4.3-4.9	4.3-5.5	2.2-2.5	4.0-4.6

* Source of amino acid

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acid from carbohydrate and its formation from proline. The daily turnover of CCN acids therefore appears to be in the region of (g per day).

Animal	Valeric	Isobutyric	Isovaleric	2-Methylbutyric
Beef Cattle ^a	39 - 52	23 - 43	41 - 56	21 - 36
Dairy Cattle ^a	23 - 40	18 - 46	24 - 60	13 - 40
Steers ^b	108			

^a from dietary amino acid precursors in feed and protein (Tables 2a and b)

^b from analysis of ingesta (68)

The proposed supplements are therefore about one-fifth of the daily turnover of valeric acid, about half to a third of the turnover of isovaleric acid, and approximately the same as the turnover of isobutyric and 2-methylbutyric acids. Since these intakes would be prolonged for the period of feed consumption, the combined supplementary and feed-derived acids should be well within the capacity of the rumen to utilize. The range of variation of dietary precursors (Tables 2a,b,c) suggests that at any one time the combined additive and feed-derived CCN acid concentrations will lie within limits of normal variation for the acids in question.

c) Extra-rumen metabolic considerations

The proposed addition of CCN acids is expected to stimulate microbial protein synthesis (1). The consequences of the additional CCN acids in the rumen would be an increased concentration of microbial protein or microbial lipid. These would presumably be handled in a normal manner in the remainder of the gastrointestinal tract, representing

a nutritionally advantageous situation. The resulting formation within the animal of valine, leucine, isoleucine and other amino acids should be well within the capacity of the intermediary metabolism to handle (see Table 2b for normal digestible protein intake). Any unutilized CCN acids would presumably be absorbed via the rumen epithelium, and transferred to the blood. A proportion would be converted to acetate and propionate in the rumen epithelium as indicated above. Any absorbed CCN acids would presumably be handled by the intermediary metabolism as indicated above, the overall products being acetate or propionate. It is estimated that in cattle, depending on the diet and the analytical procedure adopted, daily normal production of rumen acetic acid is 9.6 - 40 moles, and of propionic acid 3.72 - 12.8 moles (139). Both acids are extensively absorbed from the rumen to provide a considerable proportion of the ruminant calorific requirements (55). If the total intake of the proposed dietary supplement were absorbed and converted to acetate and propionate, about 1 additional mol each of acetate and propionate would be contributed to the normal uptake. Since most of the CCN acids will be converted to protein, increased acetate and propionate formation from direct absorption of the CCN acids will be insignificant.

It would therefore be unlikely that these levels of supplementation with CCN acids would result in carry through into the milk of dairy cows or flesh of meat providing animals. Support for this view is provided by experiments with isobutyric acid added to the feed of dairy cows (140), where feeding for 10 days of 4.5% of isobutyric

acid in the diet (about 200 g per day) was required to produce detectable levels of isobutyric acid in the milk, and levels consistently above normal in the blood. Feeding at 1.5% (about 90 g per day) produced no carry through into milk and only a slight increase in blood isobutyrate in one of the three experimental cows employed.

d) Food safety considerations

The CCN acids are extensively distributed in human food and beverages, and must therefore be normally present constituents of the human diet. Since quantitative information on concentrations in foodstuffs is scanty it is not possible to estimate the normal human intake of these materials. However, they also occur in the intermediary metabolism as breakdown products of amino acids arising from dietary proteins. The three CCN precursors, valine, isoleucine and leucine, are essential amino acids, presumably because of the inability of mammalian organisms to synthesize directly the branched chain carbon moieties involved. Rough estimates of the adult human daily intake (141) suggest that about 10-20 g of each results from dietary protein, implying a turnover rate of about 7-14 g of each derived CCN acid in the intermediary metabolism. In addition the identification of serum pools of isobutyric acid and isovaleric acid indicates the existence of normal body burdens for at least two of these substances.

It would therefore be expected that (a) the low to moderate toxicity of the CCN acids, (b) the existence of a normal dietary intake, (c) the absence of appreciable physiological and pharmacological activity, (d) the occurrence of two of the acids in serum and of all four as part of essential amino catabolism, and (e) the evidence for their

ready metabolism in the free state, would all indicate an absence of hazard from any residues occurring in milk or flesh as a result of their use as feed supplements.

CONCLUSIONS

- (1) The carbon chain nutrient acids, valeric, isovaleric, 2-methylbutyric and isobutyric are widely distributed in food, feedstuffs, beverages, flavoring materials, human and animal wastes and occur as products of natural fermentation processes.
- (2) They are also components of the normal intermediary metabolism throughout the mammalian kingdom, occurring as catabolism products of naturally occurring amino acids. They are converted in the intermediary metabolism principally to acetate, propionate and/or acetoacetate, and may be wholly used as foods. Other minor pathways exist which may lead to their incorporation into lipids or excretion as conjugates in the urine.
- (3) All four acids occur naturally in the rumen. Valeric acid occurs as the product of proline catabolism, or from dietary carbohydrate fermentation. Isovaleric acid is formed from leucine, 2-methylbutyric acid from isoleucine and isobutyric acid from valine.
- (4) The proposed level of feed supplementation appears to be within the level of normal variation in the rumen resulting from dietary intake of these precursors in feed protein.
- (5) All four acids are utilized in rumen microbial protein and lipid synthesis. Any unutilized CCN acids would presumably be absorbed and converted to acetate or propionate in quantities insignificant in comparison with the normal acetate and propionate pools in ruminants.
- (6) The proposed level of supplementation is not expected to result in carry through into milk or flesh.

- (7) If residues occur they are expected to be insignificant, and to present no hazard to monogastrics. The CCN acids possess only a slight to moderate toxicity to mammals, and intake from such residues would be well within the capacity of monogastrics to handle, since pools of valerate, isobutyrate, isovalerate and 2-methylbutyrate already exist in humans by virtue of the dietary intake of the respective amino acid precursors. The widespread occurrence of these acids in foodstuffs indicates that there is already a low level exposure of humans to each acid, of indefinite duration.
- (8) The commercial use of these acids would not be detrimental to the environment in the course of waste disposal, since they have been identified in sewage and human wastes, are compatible with waste treatment microorganisms and are utilized by a variety of aerobes and anaerobes.
- (9) It is expected that the irritating properties of these substances will require caution in formulating, shipping and handling.

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0000684

EASTMAN CHEMICALS DIVISION
EASTMAN KODAK COMPANY
KINGSPORT, TN 37662

FOOD ADDITIVE PETITION
NO. _____

AMMONIUM SALTS OF MIXED VOLATILE FATTY ACIDS BLEND (AS-VFA)
AS A NUTRIENT SUPPLEMENT IN THE FEED OF DAIRY AND BEEF CATTLE

June 14, 1982

Heading H, Enclosure 3

sve/2967g



TEXAS WATER COMMISSION
Stephen F. Austin State Office Building
Austin, Texas

PERMIT TO DISPOSE OF WASTES
under provisions of Chapter 26
of the Texas Water Code

PERMIT NO. 00471
(Corresponds to
NPDES PERMIT NO. TX 0000949)
This permit supersedes and replaces
Permit No. 00471 approved November
26, 1974.

Texas Eastman Company
whose mailing address is

P. O. Box 7444
Longview, Texas 75602

is authorized to dispose of wastes from a plant manufacturing
organic chemicals

located five miles southeast of the City of Longview in Harrison
County, Texas

to Segment 0505 of the Sabine River in the Sabine River Basin

in accordance with effluent limitations, monitoring requirements
and other conditions set forth herein. This permit is granted
subject to the rules of the Department, the laws of the State of
Texas, and other orders of the Commission.

This permit and the authorizations contained herein shall expire
at midnight, March 31, 1981.

APPROVED, ISSUED, AND EFFECTIVE this 28th day of July,
1980.

ATTEST:


For the Commission

0000685

TEXAS DEPARTMENT OF WATER RESOURCES

1700 N. Congress Avenue

Austin, Texas



Harvey Davis
Executive Director

TEXAS WATER DEVELOPMENT BOARD

Louis A. Beecherl, Jr., Chairman
George W. McCleskey, Vice Chairman
Glen E. Roney
W. O. Bankston
Lonnie A. "Bo" Pilgrim
Louie Welch

TEXAS WATER COMMISSION

Lee B. M. Biggart, Chairman
Felix McDonald
John D. Stover

May 19, 1982

Mr. Thomas McAninch
Texas Eastman Company
P. O. Box 7444
Longview, Texas 75607

Dear Mr. McAninch:

Re: Texas Eastman Company
Permit No. 00471

Although the above referenced permit was scheduled to expire on March 31, 1981, your renewal application of February 13, 1981 has assured that the permit approved July 28, 1980 will remain in effect until a new permit is issued.

If you have any questions, please do not hesitate to call.

Sincerely,

A handwritten signature in cursive script that reads "Patricia Dargin Padilla".

Patricia Dargin Padilla
Permit Control & Reports

0000686

EASTMAN CHEMICALS DIVISION
EASTMAN KODAK COMPANY
KINGSPORT, TN 37662

FOOD ADDITIVE PETITION
NO. _____

AMMONIUM SALTS OF MIXED VOLATILE FATTY ACIDS BLEND (AS-VFA)
AS A NUTRIENT SUPPLEMENT IN THE FEED OF DAIRY AND BEEF CATTLE

June 14, 1982

Heading H, Enclosure 4

sve/2967g

NOTICE: This permit includes minor revisions made in accordance with the Regional Administrator's determination. Please retain this permit as your official copy.

Permit No. TX0000949
Application No.

**AUTHORIZATION TO DISCHARGE UNDER THE
NATIONAL POLLUTANT DISCHARGE ELIMINATION SYSTEM**

In compliance with the provisions of the Federal Water Pollution Control Act, as amended, (33 U.S.C. 1251 et. seq; the "Act"),

Texas Eastman Company
A Division of Eastman Kodak Company
P.O. Box 7444
Longview, Texas 75602

is authorized to discharge from a facility located at approximately 2 miles Southeast of the intersection of State Hwy. 149 and Interstate 20 in Harrison County, Texas, and also approximately 4 miles southeast of the City of Longview, Gregg County, Texas

receiving waters named Sabine River, Segment 0505 of the Sabine River Basin

in accordance with effluent limitations, monitoring requirements and other conditions set forth in Parts I, II, and III hereof.

This permit shall become effective on November 16, 1980

This permit and the authorization to discharge shall expire at midnight, June 30, 1981.

Signed this 15th day of October 1980



Diana Dutton
Director
Enforcement Division 6AE

0000687



UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

REGION VI

1201 ELM STREET
DALLAS, TEXAS 75270

8 MAY 1981

CERTIFIED MAIL: RETURN RECEIPT REQUESTED (511569)

Mr. George Pendergast
Secretary
Texas Eastman Co.
Division of Eastman
Kodak Co.
P. O. Box 7444
Longview, Texas 75607

Re: NPDES Permit No. TX0000949
Continuation of Existing Permit

Dear Mr. Pendergast:

Pursuant to 40 CFR §122.5 (45 Fed. Reg. 33425, May 19, 1980), the National Pollutant Discharge Elimination System (NPDES) Permit No. TX0000949, expiration date June 30, 1981 is hereby continued pending the issuance of a new permit.

In accordance with what is commonly referred to as the Administrative Procedure Act (5 U.S.C. §558(c)) and the above cited regulation, the terms and conditions of an expired permit are automatically continued if the permittee has submitted a timely and sufficient application for a new permit and the Environmental Protection Agency, through no fault of the permittee, is unable to issue a new permit before the expiration date of the previous permit. We have determined that you have met these requirements; consequently, your permit is continued.

We call your particular attention to Section 122.5(b) of the above cited regulations wherein it is specified that the previous permit terms and conditions remain enforceable against the permittee. Additionally, this permit shall be modified or, alternatively, revoked and reissued, to comply with any applicable standard or limitation promulgated or approved under Sections 301(b)(2)(C) and (D), 304(b)(2) and 307(a)(2) of the Clean Water Act, if the effluent standard or limitation so issued or approved: (i) contains different conditions or is otherwise more stringent than any effluent limitation in the permit; or (ii) controls any pollutant not limited in the permit. The permit as modified or reissued under this paragraph shall also contain any other requirements of the Act then applicable.

0000688

If you have any questions, please contact Mr. Ed McHam of my staff at (214) 767-4375.

Sincerely,



Oscar Cabra, Chief
Industrial Permits Section (6AEP)

cc: Texas Department of Water Resources