8-1-1971

Studies on the Mutual Influences of Substrates on Mammalian Alpha-Keto Acid Metabolism

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STUDIES ON THE MUTUAL INFLUENCES OF SUBSTRATES ON MAMMALIAN α-KETO ACID METABOLISM

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A Dissertation

Submitted to the Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Grand Forks, North Dakota

August
1971

This dissertation submitted by Wayne A. Johnson in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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Degree Doctor of Philosophy

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ACKNOWLEDGEMENTS

The author wishes to express sincere appreciation to Dr. J. L. Connelly for support and direction in the preparation of this dissertation.

A special note of thanks is extended to Dr. W. E. Cornatzer and to Dr. R. C. Nordlie for their encouragement during the entire course of graduate education.

The author also expresses gratitude to his wife, Helen, for her patience and understanding during the completion of this work.

This investigation was supported by a National Defense Education Act Predoctoral Fellowship, 69-02592, and a Grant, NSF-GB 12934.

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ABSTRACT

The branched-chain amino acids leucine, isoleucine and valine are transaminated to their respective α-keto acid analogues; α-keto-isocaproate (KIC) α-keto-β-methylvalerate (KMV) and α-ketoisovalerate (KIV) in normal mammalian tissues. Recently an interest in the genetic anomaly "branched-chain ketoaciduria" or "maple syrup urine disease", a metabolic disease involving the oxidative decarboxylation of the three branched-chain α-keto acids, has stimulated efforts to characterize the nature of the enzymic conversions leading from the α-keto acids to their acyl coenzyme A products. These studies have been extended toward a consideration of the metabolic interrelationships among these α-keto acids (KIC, KMV, KIV). Furthermore, the effects of the branched-chain α-keto acids upon the metabolism of pyruvate and of α-ketoglutarate have been investigated. In addition, there has been an attempt to determine whether, in the normal and the abnormal (branched-chain ketoaciduric) animal, such interrelationships might constitute a significant physiological regulatory mechanism.

α-Keto acid dehydrogenase activities were estimated by the quantitative measurement of $^{14}CO_2$ evolved from carboxyl-labeled α-keto acid substrates. The α-keto acid dehydrogenase enzyme activities were measured in several fractions of bovine liver homogenates prepared by differential centrifugation techniques.

Subcellular distribution studies established that the branched-chain α-keto acid dehydrogenase activities were localized principally in the mitochondria, and more specifically on the outside of the inner membrane of the mitochondria. In addition to the particulate activity, minor amounts of dehydrogenase activity with KMV and KIC were noted in the "soluble" (not sedimented at 80,000 x g for one hour) fraction.

The particulate branched-chain α-keto acid dehydrogenase activities were shown to depend upon added nicotinamide adenine dinucleotide (NAD^+) and coenzyme A for maximal activity. Also observed were apparent requirements for calcium, magnesium and inorganic phosphate. These findings lend support to the assumption that the branched-chain α-keto acids are catabolized by a mechanism analogous to that known to exist for the well-characterized pyruvate and α-ketoglutarate dehydrogenase complexes.

The apparent Michaelis constant for each of these keto acids (KIC, KMV, KIV, pyruvate, α-ketoglutarate) was determined. In general, the values for the branched-chain α-keto acids are of the order of 4×10^{-4} M, while those for pyruvate and α-ketoglutarate are slightly lower, 0.8×10^{-4} M and 2×10^{-4} M respectively.

The degree and type of influence exerted among the various α-keto acids was examined directly by following the enzymatic liberation of $^{14}CO_2$ from carboxyl-labeled substrate in the presence of an alternate unlabeled α-keto acid. A mutually inhibitory pattern among KIC, KIV and KMV was noted. In addition, each of the branched-chain keto acids exerted a marked inhibition of pyruvate

dehydrogenase and α-ketoglutarate dehydrogenase activities. Inhibitions were of the competitive type in all instances except for α-ketoglutarate dehydrogenase activity, where a "mixed" type of inhibition was seen. The apparent inhibitor constants were in a range such that a significant mutual influence among the branched-chain α-keto acids could exist *in vivo*. Also, the inhibitory effects of KIC upon pyruvate dehydrogenase could be considered to be significant at normal cellular concentrations of these metabolites. The net effect of the inhibitory action of the branched-chain α-keto acids on each other and upon pyruvate dehydrogenase in the normal animal is likely a homestatic balance, which allows for catabolism of excess branched-chain amino acids, while concurrently influencing the flow of pyruvate to acetyl coenzyme A.

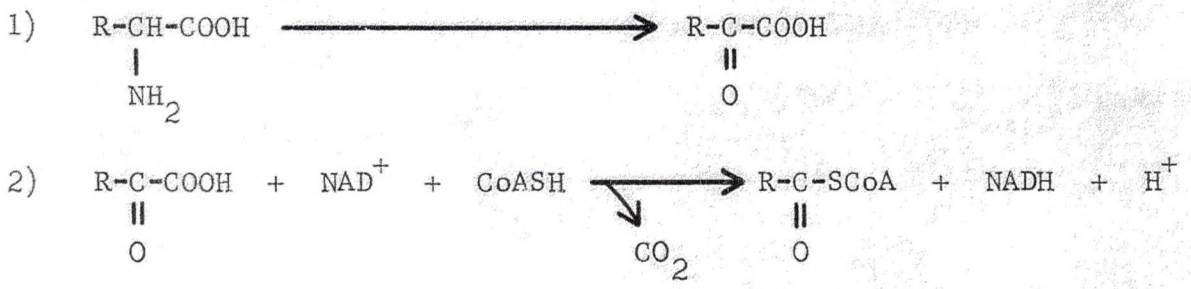
The abnormal situation, as obtained in branched-chain keto aciduria, results in extremely elevated levels of all three branched-chain α-keto acids. As these acids accumulate proximal to the metabolic block at the level of oxidative decarboxylation, they may nearly completely restrict the activity of pyruvate dehydrogenase, and exert substantial inhibitory effects at α-ketoglutarate dehydrogenase. These effects could severely limit the production of energy *via* the Krebs tricarboxylic acid cycle. In addition, production of acyl coenzyme A intermediates necessary for fatty acid synthesis would be affected.

Thus, as the result of a genetic lesion, which is manifest in the inability of the animal to catabolize the branched-chain α-keto acids, these intermediates accumulate, and in turn may exert a

deleterius influence upon cellular energy requirements, as well as on production of essential metabolic intermediates. These effects in the abnormal situation could account for many of the symptoms observed in branched-chain ketoaciduria.

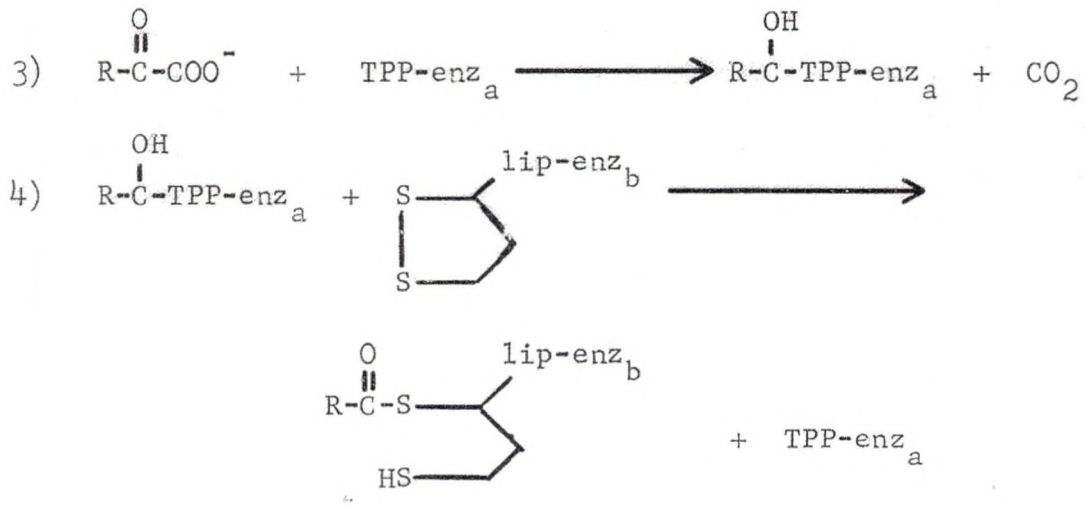
INTRODUCTION

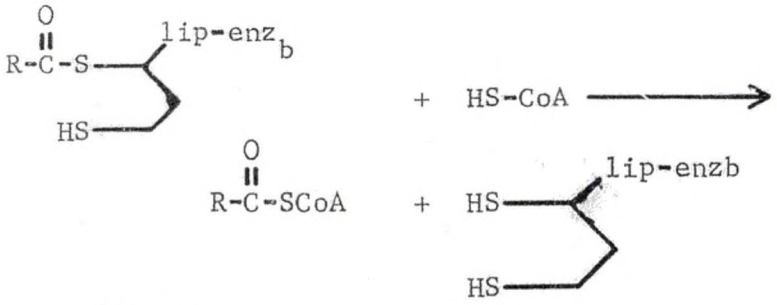
The branched-chain amino acids leucine, isoleucine and valine, which are synthesized in bacteria and plants, are essential nutrients for animals. Coon et al. (1) have studied the general pathways for the degradation of these three amino acids in mammalian tissues, and have found that the initial steps follow a similar pattern of transamination to the respective α-keto acids (α-ketoisocaproate (KIC)[1], α-keto-β-methylvalerate (KMV), and α-ketoisovalerate (KIV), followed by irreversible oxidative decarboxylation of these branched-chain α-keto acids to the corresponding acyl-CoA derivatives, as noted in the following simplified reaction scheme:

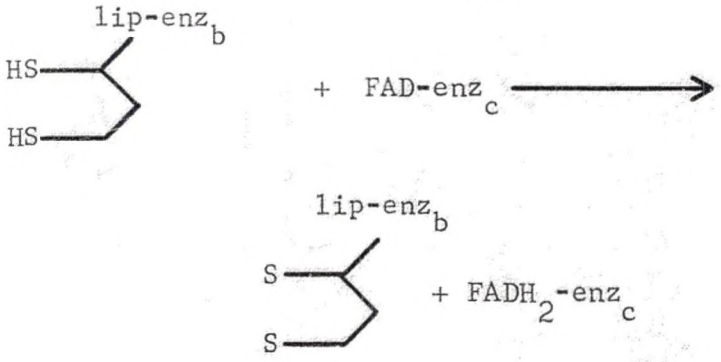


[1]The following abbreviations are used in this presentation: KIC, α-ketoisocaproate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; KB, α-ketobutyrate; KG, α-ketoglutarate; Pyr, pyruvate; KV, α-ketovalerate; KC, α-ketocaproate; NAD^+, nicotinamide adenine dinucleotide (oxidized form); NADH nicotinamide adenine dinucleotide (reduced form); CoASH, reduced coenzyme A; EDTA, ethylenediamine tetraacetic acid; $NADP^+$, nicotinamide adenine dinucleotide phosphate; α-GP, DL-α-glycerophosphate; TPP, thiamine pyrophosphate; FAD, flavin adenine dinucleotide, FMN, flavin mononucleotide; P_i, orthophosphate; g, gram; mg, milligram; g, gravity; ml, milliliter; cm, centimeter; ΔA, absorbance change, nm, nanometers, M, molar; mM, millimolar; min, minute; sec, second; $^{14}CO_2$, carbon-14-labeled carbon dioxide; cpm, counts per minute; kg, kilogram; μmole, micromole; μg, microgram.

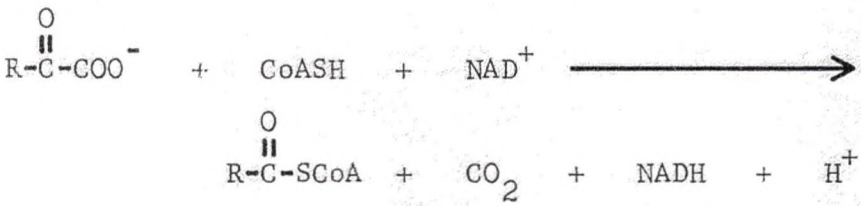
The further catabolism of the acyl-CoA compounds apparently proceeds via a sequence of reactions similar to those of fatty acid β-oxidation. Sparse information is available on the enzymes which are involved in these catabolic pathways. However, from the studies of Bloch (2) and Coon (3) it is evident that certain of the later reactions in the pathways of branched-chain amino acid degradation differ considerably from the accepted β-oxidation scheme. With respect to end-products formed from the catabolic processes involved, it is generally understood that leucine is ketogenic, valine is glycogenic and isoleucine is either ketogenic or weakly glycogenic under varying conditions (4-7). It has been postulated that the overall oxidative decarboxylation of KIC, KMV, and KIV proceeds by a metabolic conversion analogous to that proposed for pyruvate and α-ketoglutarate. Gunsalus (8) and Reed (9) have presented the following mechanistic scheme to account for the reaction sequence of the oxidation of pyruvate and α-ketoglutarate by their respective enzyme complexes:



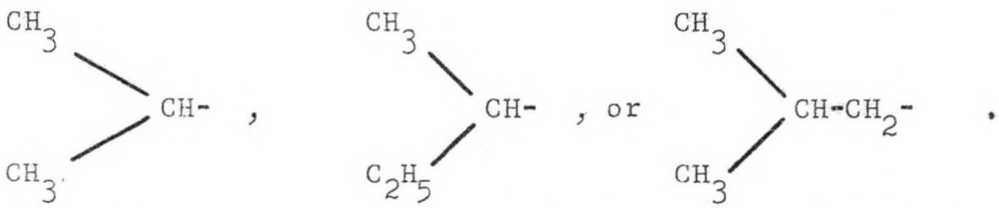
5) 

6) 

7) $FADH_2\text{-}enz_c + NAD^+ \longrightarrow FAD\text{-}enz_c + NADH + H^+$

Sum: 

In this scheme $enz_{a,b,c,}$ represent the respective decarboxylase, acyl transferase and lipoyl dehydrogenase components of the complex, lip indicates the carboxyl terminus of lipoic acid, TPP represents the prosthetic group thiamine pyrophosphate, and the various carbon skeletons of the keto acids are designated by R, where R may be as follows:



FAD and $FADH_2$ represent the oxidized and reduced forms of flavin adenine dinucleotide respectively.

Recently the obligatory participation of CoA and NAD^+ has been observed in the enzymic oxidative decarboxylation of KMV, KIC and KIV by extracts of Bacillus subtilus (10) and rat liver (11). Evidence for the active participation of the other presumptive cofactors is largely indirect. Lipoic acid was determined to be essential for the oxidative decarboxylation of KMV and KIV in Streptococcus faecalis cells harvested from a lipoic acid deficient medium (12), and added thiamine pyrophosphate resulted in slightly increased KMV dehydrogenase activity in B. subtilus extracts (10). Direct evidence for the function of lipoic acid and thiamine pyrophosphate as prosthetic groups of the branched-chain α-keto acid dehydrogenase systems of mammalian tissues has not been available.

Liver and kidney possess the highest branched-chain α-keto acid dehydrogenase activity in the rat (11); low levels were observed in heart and brain tissue, and no oxidative decarboxylation of these compounds was obtained from preparations of skeletal muscle. A general survey of branched-chain keto acid oxidative decarboxylase capacity of several mammalian species (13) indicated that bovine liver homogenates catalyzed the oxidation of KIC at a substantial rate, with a specific activity higher than that of most other tissues studied.

Several aminotransferases which will accept branched-chain amino acids as a substrate have been reported. Such an instance occurs in Escherichia coli, where one valine-specific and two leucine-accepting transaminase enzymes are indicated (14). Taylor

and Jenkins (15) have isolated an aminotransferase from pig heart cytosol which accepts leucine, isoleucine, valine or glutamic acid as the amino group donor, and the related α-keto acids, α-ketoisocaproic acid, α-keto-β-methylvaleric acid, α-ketoisovaleric acid and α-ketoglutaric acid as the amino group acceptor. Availability of amino group acceptor appears to be a possible rate-limiting step for the oxidative of L-valine (16). These transaminases are generally located in the non-particulate fraction in mammalian cells (11).

Branched-chain ketoaciduria (BCKA), also known as "Maple Syrup Urine Disease" (MSUD) because of the characteristic odor of the urine and bodily secretions, is a metabolic error of genetic origin which involves the degradation of the amino acids leucine, isoleucine and valine. Menkes (17) first described this disease in a family in which four infants died within several weeks of birth. Characteristic symptoms observed were vomiting, muscular hypertonicity, and a penetrating "maple syrup" odor to the urine. A subsequent report by Dancis (18) involved a similar event in which the infant died at 20 months of age possessing the symptoms of maple syrup odor, brain damage and generalized lethargy. Analysis of plasma and urine revealed abnormally high concentrations of the branched-chain amino acids and their α-keto acid analogues. Numerous instances of the disease have since been reported (19).

Snyderman (19) and Westall (20) have successfully treated branched-chain ketoaciduric patients with dietary therapy, the mode of which consists of reduced and carefully balanced consumption of

branched-chain amino acids. This therapy is most likely to succeed in preventing the alterations of neurophysiology, and other characteristic symptoms, if begun in the first week of life. It thus appears that the neonatal brain is particularly sensitive to high levels of branched-chain amino or α-keto acids.

Dancis (18,21,22,23,24) has proposed that the metabolic lesion effecting a block in the catabolism of the branched-chain amino acids and responsible for the genetic anomaly BCKA is a lack of, or a malfunction of, the enzyme or enzymes responsible for the catalysis of the oxidative decarboxylation of the branched-chain α-keto acids KIC, KMV, and KIV (refer to equation 2 above). Normal function of the transaminase enzymes has been observed in tissues of these individuals (25).

In an attempt to clarify further the nature of the impairment in branched-chain amino acid metabolism in BCKA, a general aim of several investigations (22,26,27) has been to determine whether three specific dehydrogenase enzyme complexes are responsible for the oxidation of KIC, KMV and KIV, or whether a single less specific enzyme complex may catalyze the catabolism of all three keto acids. This question is of general interest, since the answer could provide significant information concerning the regulation of these metabolic pathways, and give pertinent insight bearing on the comprehension of the associated genetically determined metabolic disease. Current information would indicate that one of four major possible occurrences could account for the observed branched-chain amino acid and keto acids at elevated levels as noted in the

diseased (BCKA) patient.

A. All of the branched-chain α-keto acids are oxidized by one enzyme, which is genetically altered (22).

B. Each branched-chain α-keto acid is catabolized by a separate dehydrogenase of which all are malfunctioning (26).

C. Each branched-chain α-keto acid is acted upon by a separate enzyme, only one of which is genetically altered. The resulting accumulation of intermediates acts to hamper the activity of the other two enzymes.

D. Two of the α-keto acids (KMV and KIC) may be catabolized by one enzyme, the activity of which may be affected by a genetic lesion, with a resulting increase in the concentrations of KMV and KIC in the cell acting to inhibit the metabolism of the third branched-chain α-keto acid, KIV (27).

Morris (28,29) and Dancis (23) have observed a variant form of BCKA which is not necessarily fatal and does not appear to result in significant mental retardation. They suggest that these individuals may possess a partial enzymic defect, since the peripheral leukocytes exhibited some decarboxylative activity with the branched-chain α-keto acids, as compared to the complete absence of branched-chain α-keto acid oxidative capacity in leukocytes obtained from patients with the classic branched-chain ketoaciduria. These investigators speculate that an entire spectrum of branched-chain α-keto acid dehydrogenase activities may be present in the general population; ranging from absence of the decarboxylase

activity in the classic BCKA individual to the full enzyme complement in normal tissues. Harris (30) has presented significant data with respect to a similar occurrence among certain other enzymes in the red blood cell.

Since the discovery of branched-chain ketoaciduria in humans, a number of investigators have proposed possible modes of action whereby the elevated levels of the branched-chain amino and keto acids may produce the observed physiological and neurological symptoms. Observations on patients undergoing dietary treatment have demonstrated that the acute symptoms (ataxia, convulsions) appear to be more closely related to elevated levels of leucine than of isoleucine or valine (31,32).

Tanaka, et al. (33) have recently obtained evidence for a new disorder of leucine metabolism, isovaleric acidemia, in which there is a defect in the further catabolism of isovaleryl-CoA, the oxidative product of KIC degradation. This metabolic error results in the accumulation of isovaleric acid, formed by deacylation of isovaleryl-CoA, in plasma and urine. Symptoms of the disease, in addition to the elevated plasma levels of isovaleric acid, included ataxia and loss of muscle coordination. No increase in the serum concentrations of the branched-chain amino acids or α-keto acids was noted. The fact that leucine and KIC were not significantly increased in the plasma of the patients would probably be explained by the apparent irreversibility of KIC oxidative decarboxylation.

Autopsy examinations of several diseased BCKA patients have been described (16,34) in which similarities were observed with

respect to a striking deficiency of myelination in certain primitive areas of the brain, and a spongy character to the white matter. The absence of degradation products of myelin suggested that the lack of myelination resulted from an interruption of the synthesis normally occurring after birth. Silberman, et al. (34) have suggested that increased keto acid concentrations in the developing brain may decrease the synthesis of cerebronic acids, considerable amounts of which are necessary components of myelin. However, there is no suggestion that these findings are a direct and quantitative effect of the elevated leucine, isoleucine, valine or their corresponding keto acids. Howell (35) found that KIC and KIV were able to inhibit oxygen consumption by brain slices, and postulated that this phenomenon could account for the impaired function of this tissue in BCKA. The findings of Tashian (36) lend support to these data.

There has been considerable discussion regarding a possible site of inhibition by the abnormal accumulation of metabolites proximal to the metabolic block. Among these reports are the observations that various levels of branched-chain α-keto acids tend to inhibit the activity of glutamic acid decarboxylase in brain and liver of the rat (35,36,37), as well as more recent indications that this enzyme is not significantly affected by KIC concentrations below 1.0 mM. Decarboxylation of glutamic acid produces gamma-aminobutyric acid (GABA) which may participate in the process of transmission by inhibitory neurons (36).

Another possible mechanism of toxicity of the branched-chain amino and keto acids is inhibition by these compounds at the level

of pyruvate dehydrogenase in the glycolytic pathway, or at α-ketoglutarate dehydrogenase of the tricarboxylic acid cycle. Recently Kanzaki (38) has found that the activity of α-ketoglutarate dehydrogenase purified from pig heart muscle is inhibited by KIC, KMV and KIV. Pyruvate dehydrogenase isolated from the same tissue source was inhibited by KMV and KIV; however the effects of KIC as an inhibitor of the purified pyruvate dehydrogenase complex were not discussed. Other investigators (37) have studied the inhibition of pyruvate dehydrogenase by the branched-chain α-keto acids from a semi-quantitative viewpoint, and have reported varying degrees of inhibition by KIC in rat brain and liver homogenates (13,37) and in rat liver slice preparations (39).

These preliminary observations indicate a necessity for further investigation into the mode and the extent of the effects of branched-chain α-keto acids, at the concentrations obtained during normal metabolism and in manifestations of BCKA, upon these two very important segments of intermediary metabolism. Since the glycolytic pathway and the tricarboxylic acid cycle are considered to be of fundamental importance to the provision of energy equivalents in the form of the reduced nucleotide coenzymes, a disruption of function at either site could conceivably be of serious consequence to the cell in terms of general oxidation state and in production of energy necessary for anabolic processes. Decreased pyruvate dehydrogenase activity would also result in lowered production of acetyl-coenzyme A for the tricarboxylic acid cycle and for fatty acid and steroid synthesis.

Leucine and alloisoleucine have been observed to inhibit gluconeogenesis slightly in concentrations in 1.0 mM (40,41). Instances of hypoglycemia induced by high levels of leucine have also been reported (42).

From the viewpoint of regulatory interrelationships among the keto acids the metabolism of branched-chain amino and keto acids in normal tissue has received less attention than has the pathology of the diseased tissues of the individual with BCKA.

An apparent antagonism among the branched-chain amino acids has been noted by Harper et al. (43) and by Snyderman (32). This antagonism is expressed as an imbalance in the ratios of plasma leucine, isoleucine and valine, resulting in decreased rates of growth in rats. Wohlheuter and Harper (11) have reported an increased rate of branched-chain α-keto acid dehydrogenase activity in rats fed a 30 per cent casein diet, or a diet containing nine per cent casein plus additional amino acids. Two general types of increased activity were noted, one of which could be blocked by cycloheximide. A diurnal variation in branched-chain α-keto acid dehydrogenase activity was also noted.

The above phenomena suggest that there may be mutual metabolic interrelationships among branched-chain amino acid α-keto acids which are not obvious merely from a consideration of the individual degradative pathways.

STATEMENT OF PROBLEM

The aim of the present investigation is to characterize the utilization of and the in vitro metabolic interrelationships among

the branched-chain α-keto acids in preparations of bovine liver tissue; and secondly, to ascertain whether the influences of branched-chain α-keto acids upon the metabolism of pyruvate and α-ketoglutarate could constitute a significant physiological regulatory phenomenon. Among the specific aspects of the above primary objectives considered in this study are the elucidation of the apparent location of the branched-chain α-keto acid dehydrogenase activities in the bovine liver cell and an investigation of the apparent requirements for coenzymes. Data pertaining to the determination of the number of enzymes responsible for the catalysis of the oxidative decarboxylation of α-ketoisocaproate, α-keto-β-methylvalerate, α-ketoisovalerate, α-ketoglutarate and pyruvate are presented. An attempt has been made to determine and to describe the relative extent and the apparent mode of inhibitory interactions among these metabolites.

The results of these investigations are discussed in relation to the cellular levels reported for these α-keto acids in normal tissues and with respect to the observed elevation of KIC, KMV and KIV in the pathological conditions associated with branched-chain ketoaciduria (BCKA).

METHODOLOGY

Reagents and Materials

The chemicals used in these studies were obtained from the following sources: β-nicotinamide adenine dinucleotide (NAD^+); trilithium coenzyme A; sodium salts of α-ketoisocaproate, α-ketoisovalerate, α-keto-β-methylvalerate, α-ketobutyrate, α-ketoglutarate, α-ketovalerate, α-ketocaproate, pyruvate, succinate, malate, pyruvate and D,L-α-glycerophosphate; L-amino acid oxidase; D-amino acid oxidase; Dowex 50-W (200-400 mesh) ion exchange resin; Sephadex G-25 gel beads; triethanolamine-hydrochloride; antimycin A; oligomycin; protamine sulfate; L-cysteine; nicotinamide adenine dinucleotide phosphate ($NADP^+$); para-chloromercurisulfonate; bovine serum albumin; D,L-α-lipoic acid, thiamine pyrophosphate; flavin adenine dinucleotide (FAD); flavin nonucleotide (FMN); catalase; digitonin; (Sigma Chemical Co., St. Louis, Missouri); L-leucine-1-^{14}C, DL-valine-1-^{14}C, sodium pyruvate-1-^{14}C, sodium α-ketoglutarate-1-^{14}C, (New England Nuclear, Boston, Massachusetts); L-isoleucine-U-^{14}C (Cal-Atomic Division, Calbiochem Co., Los Angeles, California); 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 2,5 diphenyloxazole (PPO), hydroxide of hyamine 10-X, low potassium glass scintillation vials (Packard Instrument Co., Downers Grove, Illinois); 2,4 dinitrophenylhydrazine, ethylene diamine tetraacetate (disodium salt), hexyl alcohol (Eastman Organic, Rochester, New York); 2,6 dichlorobenzone indophenol, urea, sodium arsenite, potassium

ferricyanide, Folin-Ciocalteau phenol reagent, monobasic sodium phosphate, dibasic sodium phosphate, monobasic potassium phosphate, dibasic potassium phosphate, calcium chloride, sodium carbonate, magnesium chloride and other analytical reagent grade chemicals (Baker Chemical Co., Chicago, Illinois); mannitol, ammonium acetate, acetone, charcoal (Mallinkrodt Chemical Works, St. Louis, Missouri); toluene, sucrose (Fisher Scientific Co., Chicago, Illinois); Triton X-100 (Rohm and Haas, Philadelphia, Pennsylvania); iso-amyl alcohol (Matheson, Coleman and Bell, Norwood, Ohio); adenosine triphosphate (P-L Biochemicals, Milwaukee, Wisconsin); EDTA-recrystallized ammonium sulfate (Mann Research Biochemicals, New York, New York); Blue Dextran 2000 (Pharmacia, Uppsala, Sweden); Bio-Gel P-2 (BioRad, Richmond, California). All other chemicals were of analytical reagent grade.

α-Ketoisocaproate-1-^{14}C (sodium salt), α-ketoisovalerate-1-^{14}C (sodium salt) and α-keto-β-methylvalerate (sodium salt) were synthesized from the appropriate labeled amino acids prior to use as described below. All water used in these studies was distilled and deionized (Corning 3508A double bed resin). Detailed methods for preparation of solutions of reagents are shown in Appendix I.

Preparation of ^{14}C-labeled α-Keto Acids

Carbon-14-carboxyl-labeled α-keto acids were prepared with modifications of the method of Meister (44). L-amino acids are oxidized to the corresponding α-keto acids by L-amino acid oxidase in the presence of catalase. After removal of protein the α-keto acids can be purified by ion exchange chromatography and isolated

as the crystalline sodium salt.

Snake venom L-amino acid oxidase (300 mg) dissolved in 20 ml of water is dialyzed against running water at 5°C for 12-24 hours. Crystalline bovine liver catalase is also dialyzed against running water at 5°C for 12 hours to remove preservative.

Two grams of the L-amino acid are suspended in 50 ml of water, to which is also added 50-100 microcuries of ^{14}C-labeled amino acid, catalase (100 units) and the L-amino acid oxidase. The solution is adjusted to pH 7.2 with 0.5 N NaOH and the volume brought to 100 ml with distilled water. One ml of hexyl alcohol is added to prevent foaming, and oxygen gas is bubbled continuously through the mixture. The reaction is allowed to continue at 37°C for 6-24 hours, or until keto acid analyses (spectrophotometric measurement of dinitrophenylhydrazone derivatives) indicate no further increase in α-keto acid formation. The pH of the solution is adjusted to pH 7.2 with 0.5 N NaOH as needed.

The α-keto acid is separated from protein by gel filtration of the reaction mixture on a Sephadex G-25 column (3 cm x 45 cm) at a flow rate of 1.0 ml per minute. Collected fractions were analyzed for radioactivity by liquid scintillation spectrometry and for protein by spectrophotometric determination of relative absorbance at 260 and 280 nm in a Beckman Model DU-2 multiple sample spectrophotometer. Complete separation of protein from radioactivity was obtained. Highest activity fractions (90-95 per cent of total radioactivity) were combined and evaporated in a reduced pressure distillation apparatus at 26 mm pressure and

35°C. The concentrated solution thus obtained was passed through a Dowex 50-W cation exchange resin (H^+ form), and the α-keto acid eluted with water at a flow rate of 0.5 ml per minute. All fractions were analyzed for amino acid with ninhydrin reagent and for radioactive label by liquid scintillation spectrometry. Fractions containing 90-100 per cent of remaining radioactivity and no amino acid contaminant were combined, adjusted to pH 4.5 with 5 N NaOH, and evaporated to a volume of less than 10 ml under reduced pressure.

Fifty to sixty volumes of ice-cold acetone were added slowly and the sodium salt of the α-keto acid allowed to crystallize at 0°C. The crystals were filtered over reduced pressure, recrystallized from concentrated aqueous solution by addition of ice-cold acetone, filtered and washed with acetone followed by dry diethyl ether on the filter. The crystalline sodium salt of the α-keto acid was then dried in a vacuum dessicator over sodium sulfate. Radiochemical purity of the compounds was ascertained by chromatographic (45) and spectrophotometric (45) determination, using the 2,4-dinitrophenyl hydrazone derivatives (45). 2,4-Dinitrophenyl hydrazine (0.1% solution in 2N hydrochloric acid) was mixed in a slight volume excess with a sample of an aqueous solution of the α-keto acid to be analyzed. The crystals of the 2,4-dinitrophenyl hydrazone derivative were allowed to form in the dark. These crystals were recrystallized from hot water, dissolved in ethanol (95%) and chromatogrammed on thin-layer silica gel plates. The R_f values were determined after development of the

plates, and compared to R_f values for the known α-keto acid derivatives.

The chromatogram spots were eluted into 0.5 N NaOH and spectra recorded from 700 nm to 350 nm on a Cary Model 15 recording spectrophotometer. These spectra were then compared with those of pure α-keto acid 2,4-dinitrophenylhydrazone derivatives.

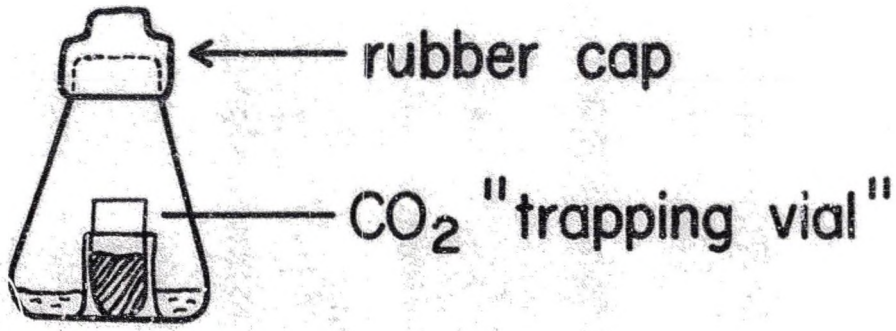
Enzyme Assay Methods

A. α-Keto acid dehydrogenase activities were estimated using modifications of the methods utilized by Wohlheuter and Harper (11) and by Snyder (46), in which the amount of labeled carbon dioxide evolved from carboxyl-labeled α-keto acids is measured after incubation with various tissue preparations. The final reaction volume of 1.0 ml usually contained the following constituents, in micromoles: β-NAD^+, 1.0; Li_3 CoA, 0.6; potassium phosphate buffer, pH 7.2, 33; mannitol, 150; $CaCl_2$, 1.0; $MgCl_2$, 1.0; Na_2CO_3, 1.0; appropriate amounts of α-keto acid-1-^{14}C substrate, enzyme (2-3 mg protein), and water to 1.0 ml. The reactions were generally initiated by addition of enzyme by pipette. Flasks were capped immediately and swirled gently to facilitate mixing.

Warburg flasks, or small Erlenmeyer flasks with a center well, served as reaction vessels (Figure 1). A small test tube (10 mm x 20 mm) containing 0.5 ml of hydroxide of Hyamine 10X was placed on the center well and the flask was tightly capped with a rubber serum cap. The reactions were allowed to proceed for 20 minutes, or other specified time interval, at 30°C in a shaking water bath, after which time 0.2 ml of 2.0 N sulfuric acid

Fig. 1. -- Diagram of Incubation Flask used for $^{14}CO_2$ Assay

Incubation flask

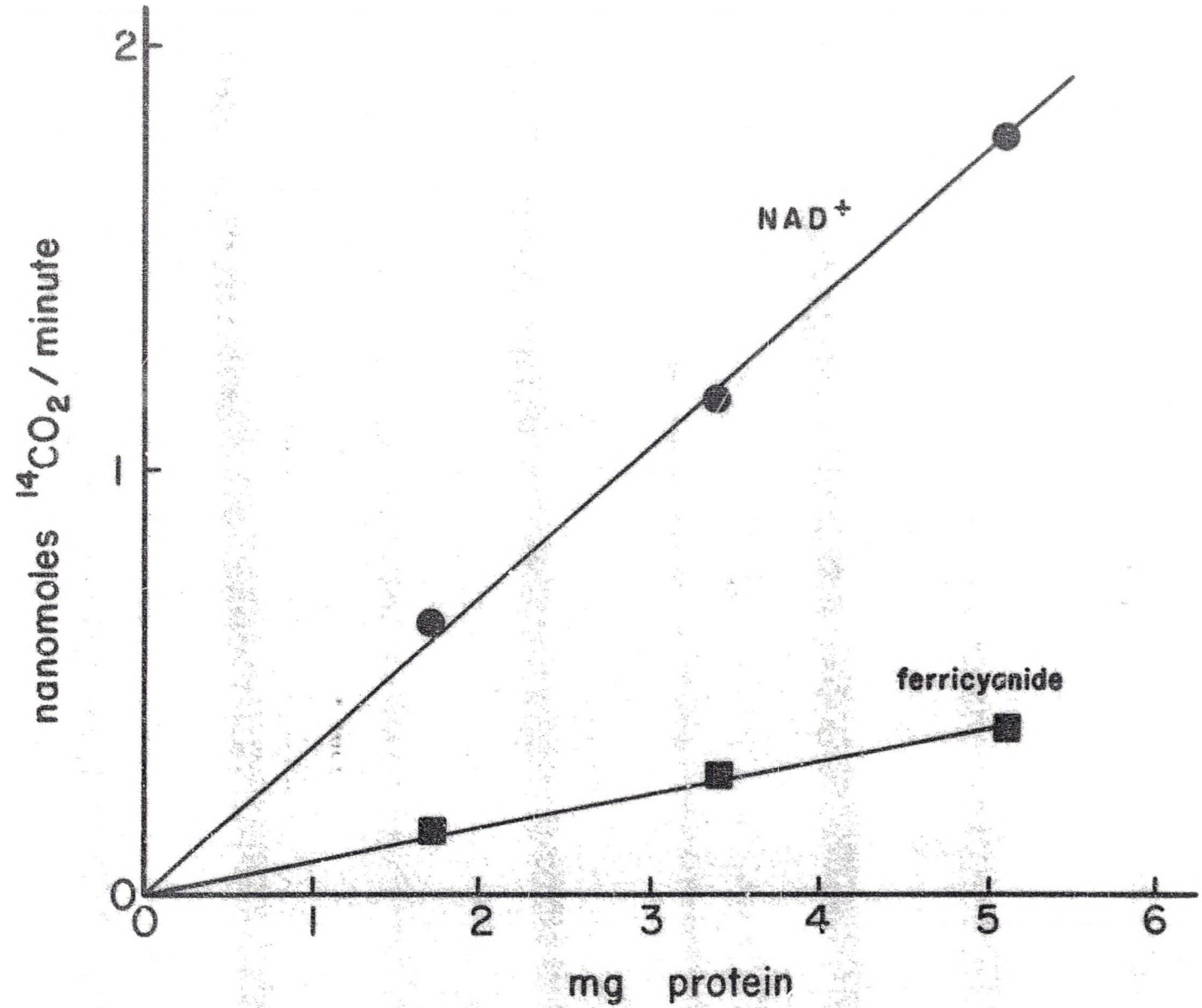


was added to the reaction mixture by syringe through the rubber cap. The acid stopped the enzymic reaction and released the dissolved $^{14}CO_2$ from solution. Distillation of $^{14}CO_2$ into the Hyamine was allowed to continue, with shaking, for one additional hour, at which time the tube and its contents was transferred to a scintillation vial containing 10 ml of a scintillation fluid /0.4 % 2,5-diphenyloxazole, 0.01 %, 1,4 bis-2-(5-phenyloxazolyl)-benzene, in toluene, w/v/, and counted three times for 10 minutes in a Packard Tri-Carb liquid scintillation spectrometer (Automatic Model 500-E). The following settings were employed: 910 volts, 100 per cent gain. The average background was 30 counts per minute (cpm). Samples were run in duplicate or in triplicate. Blank values were determined by carrying boiled enzyme preparations through an identical procedure. Blank values of 10 to 30 cpm above background were consistently observed for any one α-keto acid preparation, possibly resulting from traces of $^{14}CO_2$ in the α-keto solutions and a slow non-enzymatic decarboxylation. Typical assays measured in the range of 500 to 1500 cpm. The amount of CO_2 produced was calculated from the specific activity of the carboxyl labeled 1-^{14}C-substrate. Results are expressed in units, defined as nanomoles of CO_2 per minute; specific enzyme activity is expressed as units per mg of protein.

A modification of the above procedure, utilizing ferricyanide as an artificial electron acceptor, was also investigated and found to be a less sensitive measure of α-keto acid oxidation. A comparison of the two methods is shown in Figure 2.

Fig. 2. -- Comparison of NAD^+ and ferricyanide as electron acceptors for the $^{14}CO_2$ assay of α-keto acid dehydrogenases

The amount of $^{14}CO_2$ evolved from α-ketoisocaproate-1-^{14}C in the presence of a washed preparation of bovine liver mitochondria during a 20 min incubation period at 30° C was measured with either NAD^+ or ferricyanide as the electron acceptor for the oxidation reaction. Each reaction mixture contained, in a final volume of 1.0 ml, the following (in μmoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; NAD^+, 1.0, and Li_3CoA, 0.6; or $K_3Fe(CN)_6$, 0.05; 1-^{14}C-KIC, 2.0 (3700 cpm per μmole); and the indicated quantities of enzyme solution as shown on the figure.


nanomoles $^{14}CO_2$ / minute
2
1
0
0
1
2
3
4
5
6
mg protein
NAD+
ferricyanide

B. α-Ketoglutarate dehydrogenase purified from pig heart muscle was assayed by the modification of the method of Searles and Sanadi (47), in which the formation of NADH with time was measured at 340 nm on the Cary 15 or the Beckman Model DK-2 dual beam recording spectrophotometer. A molar extinction coefficient, E_{max340}, of 6.22×10^3 M^{-1} cm^{-1} is assumed (8) for NADH in the calculation of enzyme catalytic rates. A typical reaction mixture contained the following components, in micromoles: potassium phosphate buffer, pH 7.2, 75; β-NAD^+, 0.5; Li_3CoA, 0.6; cysteine, 5.0; α-ketoglutarate, 0.1 to 5.0; the appropriate quantity of enzyme solution, and water to a final volume of 1.5 ml in a 1 cm cuvette. A cuvette containing potassium phosphate buffer instead of enzyme, with all other reaction components present, was used as a reference. One unit of enzyme is defined as that quantity which will catalyze the formation of one micromole of NADH per minute. The stoichiometry of the reaction is such that one micromole of NADH formed is equivalent to oxidation of one micromole of α-ketoglutarate to products.

This assay system was also employed to measure oxidative decarboxylation of other α-keto acids (KIC, KIV, KMV) by partially purified enzyme preparations; however, this system is not generally satisfactory with preparations containing significant levels of NADH oxidase or for the assay of highly turbid enzyme preparations.

C. The artificial electron acceptors dichlorophenol indophenol (DCPIP) and ferricyanide have been shown to oxidize α-hydroxyethyl-thiamine pyrophosphate to the acyl-thiamine pyrophosphate

intermediate (8). Gubler (48) has described a continuous assay which involves following the reduction of ferricyanide ($Fe(CN)_6^{-3}$) with time at 420 nm as a measure of the decarboxylation of α-keto acids by suspensions of mitochondria. A typical reaction mixture contained the following components in μmoles, in a calibrated mixing tube: potassium phosphate buffer, pH 7.2, 150; ATP, 6.0; EDTA, 7.0; $MgSO_4$, 20; TPP, 0.5; $K_3Fe(CN)_6$, 3.34; a suitable amount of enzyme preparation, and 0.25 M sucrose to a final volume of 6.8 ml. These constituents were mixed by inversion and divided equally between two 1-cm matched cuvettes. The Cary Model 15 recording spectrophotometer was balanced to zero with the cuvettes in position, at which time the substrate in a volume of 0.1 ml was added to the reference cell, mixed rapidly by inversion, and the recording begun immediately. E_{max420} of 1.05×10^3 M^{-1} cm^{-1} is assumed (13) for $Fe(CN)_6^{-3}$. The slope of the line traced over a 100 second time interval is assumed to correspond to μmoles of α-keto acid oxidized in one minute.

D. Succinate dehydrogenase was measured according to the method of Bonner (49) by following ferricyanide reduction at 412 nm in a Cary Model 15 dual beam recording spectrophotometer.

E. Glycerol-1-phosphate dehydrogenase activity of washed preparations of liver mitochondria was determined by the method of Klingenberg (50), in which reduction of ferricyanide at 412 nm is followed on a Cary Model 15 recording spectrophotometer.

F. Glucose-6-phosphate phosphohydrolase activities were determined by the revised method of Nordlie and Arion (51).

"Ferricyanide Localization" of Enzyme Activity

It has been shown (50) that some mitochondrial enzymes (e.g. α-glycerophosphate dehydrogenase) are directly accessible to ferricyanide as an electron acceptor, while others (e.g. succinate dehydrogenase) are not. Those enzymes which react directly with ferricyanide are considered to be on the outside of the inner membrane, while those enzymes which lie on the matrix side of the inner membrane will not react directly with ferricyanide. Klingenberg (50) has shown that ferricyanide does not penetrate the intact inner membrane. Spectrophotometric recording of optical density (420 nm) changes due to ferricyanide reduction was carried out in matched 1-cm cuvettes in a Cary Model 15 dual beam recording spectrophotometer. For this purpose mitochondria were suspended in a 0.3 M mannitol medium at a relatively low concentration (0.5 to 1.0 mg protein per ml) with additions of 0.5 mM ferricyanide, substrates, and antimycin A as indicated in the appropriate legends.

The reduction of ferricyanide by those substrates which are oxidized outside of the inner membrane is not inhibited by antimycin A under these conditions, while reduction of ferricyanide by substrates oxidized inside the inner membrane is decreased by the presence of antimycin A in the reaction medium. The antimycin A sensitivity of ferricyanide reduction by succinate is abolished after the mitochondria have been fragmented by sonication (50,52). Where the effect of sonication was studied, the final mitochondrial suspension was sonicated for 20 seconds and used without further centrifugation in the measurement of ferricyanide reduction.

α-Keto Acid Permeation in Mitochondria

The general impermeability of the inner membrane of mitochondria toward various anions such as the branched-chain α-keto acids was estimated indirectly by following the rate of change in optical density at 515 nm of a mitochondrial suspension in the presence of high concentrations of the keto acid and of a cation which readily equilibrates across the membrane (53). Such a permeable cation with an anion which is also permeable would result in rapid influx of water with the concomitant increase in mitochondrial volume (swelling).

In the typical swelling experiments the reactions were conducted in matched 1-cm cuvettes. The suspension of mitochondria was diluted with 0.3 M mannitol so that 0.1 ml of the suspension contained approximately 0.5 mg of protein. This amount of mitochondria in a total volume of 1.5 ml gave an optical density near 0.6 at 515 nm on a Cary Model 15 recording spectrophotometer, which was balanced to zero prior to addition of mitochondria. One-tenth ml of the suspension of mitochondria was added to the reaction cuvette and mixed rapidly. The optical density change with time was followed until swelling had occurred and the optical density had leveled off, or for a convenient time intervals. This spectrophotometric determination of swelling gives qualitatively reliable measurements of mitochondrial volume changes (54).

Enzyme Preparations

Bovine liver mitochondria were prepared by a modification of the procedures utilized by Lusty and Singer (55). Fresh bovine

liver was obtained from the slaughter house, 10 to 15 gm of tissue were placed in a pre-weighed beaker containing a solution of 0.3 M mannitol, which was 10^{-4} M with respect to EDTA, and minced with a surgical scissors. The tissue was transported to the laboratory in this container, weighed and homogenized with approximately 10 volumes of 0.3 M mannitol - 10^{-4} M EDTA in a Potter-Elvehjem glass homogenizer. The crude homogenate thus obtained was then centrifuged at 600 x g for 10 minutes to remove cell debris and nuclei. The supernatant fraction was decanted and saved. The nuclear fraction (nuclei and cell debris) was resuspended in about 30 ml of 0.3 M mannitol - 10^{-4} M EDTA and recentrifuged at 600 x g for 10 minutes. The combined supernatant fractions were centrifuged at 9,600 x g for 10 minutes. The mitochondrial pellet was resuspended in 0.3 M mannitol - 10^{-4} M EDTA and centrifuged at 9,600 x g for 10 minutes. The resulting pellet was gently suspended in 0.3 M mannitol - 10^{-4} M EDTA to a final volume of 1.0 ml per gram wet liver tissue. The protein concentration of this washed preparation of mitochondria varied from 23 to 28 mg per milliliter.

Further differential centrifugation procedures were utilized to obtain microsomal and soluble fractions. The supernatant fraction of the first 9,600 x g spin was centrifuged at 80,000 x g for one hour in a Beckman Spinco Model L preparative ultracentrifuge (number 30 head). The precipitate of this spin was termed the "microsomal fraction", and the supernatant fluid was denoted as the soluble or "supernatant" fraction.

Rat liver mitochondria preparations were obtained by techniques slightly modified from those utilized by Schneider (56). All rat liver mitochondria were obtained from male Sprague-Dawley rats (200 to 300 gm); the liver was removed immediately after decapitation and bleeding of the rat, blotted, placed in a preweighed beaker containing ice-cold 0.25 M sucrose and weighed. The liver was then finely minced with a stainless steel scissors in preparation for homogenization. The liver was homogenized in a Potter-Elvehjem glass vessel with a Teflon pestle. The ten to fifteen grams of liver were homogenized in three equivalent portions of twenty-five to thirty ml of cold 0.25 M sucrose. The vessel and pestle were then rinsed in approximately ten ml of 0.25 M sucrose which was added to the homogenate to give a total volume of eighty to one hundred ml. The homogenate was centrifuged at 600 x g for ten minutes in an International Model B-20 refrigerated centrifuge with the number 870 head, to remove blood cells, cell nuclei and other cell debris. The supernatant solution was decanted and saved. The "nuclear" residue was washed and homogenized with twenty ml of 0.25 M sucrose, centrifuged at 600 x g for 10 minutes, and the supernatant decanted and saved. The combined supernatant fractions were centrifuged at 8,500 x g for 10 minutes. The supernatant fraction which contained the microsomes was decanted carefully and discarded. The mitochondrial pellet was rehomogenized in thirty ml of cold 0.25 M sucrose and centrifuged at 8,500 x g for 10 minutes. The final pellet was suspended in 0.25 M sucrose to a volume equal to 1.0 ml per gram wet liver tissue. The protein

concentration of this washed preparation of mitochondria varied from 25 to 30 mg per ml.

Solubilization by Digitonin of Outer Membrane of Mitochondria

Treatment of isolated mitochondria with 1 per cent digitonin (w/v) solution has been shown to remove outer membrane and intramembranal proteins from the inner membrane-matrix complex (57).

An aliquot of a suspension of mitochondria containing 100 to 160 mg of protein per ml is cooled in an ice bath. Cold 2 per cent digitonin (see Appendix I), in a volume equal to that of the mitochondrial suspension, is added. This suspension is incubated at 0°C for 20 minutes, then diluted with three volumes of cold 0.25 M sucrose or 0.3 M mannitol, and centrifuged for 10 minutes at 9,500 x g. The supernatant solution and the fluffy top layer of the precipitate are decanted. The solid precipitate consists mainly of inner membrane-matrix complex.

α-Ketoglutarate Dehydrogenase

Purified α-ketoglutarate dehydrogenase was prepared from pig heart muscle by the methods of Sanadi, Littlefield and Bock (58). Fresh pig heart (0.375 kg) is ground and homogenized for two minutes with 0.03 M potassium phosphate buffer, pH 7.2, in a Waring blender operating at full speed. This homogenate is then centrifuged at 2000 x g for 30 minutes and the precipitate discarded. The supernatant is adjusted to pH 5.4 with dropwise addition of 10 per cent acetic acid with continuous stirring. This suspension is then centrifuged at 200 x g for 20 minutes, the

supernatant discarded, the precipitate washed in 75 ml of water in a glass homogenizer, and recentrifuged for 10 minutes at 4000 x g. The precipitate is suspended in 25 ml of water and adjusted to pH 7.0 with 1.0 N NaOH. This suspension is then alternately frozen and thawed four times, centrifuged for 20 minutes at 18,000 x g. Solid ammonium acetate (38.5 g) are then added per 100 ml of supernatant fluid, the suspension stirred an additional 45 minutes, and then centrifuged for ten minutes at 18,000 x g. The precipitate is then dissolved in 2.5 ml of 0.02 M potassium phosphate buffer, pH 7.2, dialyzed overnight against one liter of the same buffer, and made 10^{-5} M with respect to L-cysteine. The final product is approximately three ml of fairly clear cream-colored solution which is stored frozen at -18°C. The recovery of enzyme activity is about 10 per cent of total homogenate activity with 200- to 250-fold purification.

Protein Determinations

Protein concentrations were measured by three different methods. The standard protein assay method used for these studies was the Biuret method (59) with bovine serum albumin (Armour Standard) as the protein standard. To three 0.1-ml samples of the protein preparation was added 0.1 ml of Triton-urea reagent (see Appendix I) and water to a volume of 1.0 ml. Biuret reagent (4.5 ml) was added to two of the three tubes and 4.5 ml of a 3 per cent sodium hydroxide solution was added to the third tube. Standard protein references and mannitol-EDTA blanks were assayed concurrently with the unknowns. Samples were allowed to stand for

at least thirty minutes to allow for maximum color development. The optical density of the solutions were then measured on a Klett-Summerson colorimeter equipped with the number 54 filter. Optical density readings (Klett units) of the blanks were subtracted from all readings to give actual optical density changes due to the presence of the protein. This method gives a linear relationship between absorbancy (Klett units) and protein concentration when the amount of protein added is between 0.75 mg and 6.0 mg. For preparation of solutions see Appendix I.

The Lowry phenol method (60) of protein concentration determination was utilized as follows:

Five ml of Reagent C, made up daily from 50 parts Reagent A and one part Reagent B, was added to all tubes (see Appendix I for preparation of reagents). One ml of protein solution was added to each of three tubes. The reagent blank contained water instead of protein. Color was developed by the addition of 0.5 ml of Folin-Ciocalteau phenol reagent (1.0 N with respect to sulfuric acid) to two of these tubes. The third tube served as a color blank, to which 0.5 ml of water was added rather than the Folin-Ciocalteau phenol reagent. A protein standard, containing 100 µg of bovine serum albumin (Armour Standard), was assayed concurrently with the samples in the same manner. Klett colorimeter (equipped with a number 62 filter) readings of the blanks were subtracted from both unknown and standard protein solutions. The linearity of this method is maintained between 10 µg and 150 µg of protein per ml of solution.

For rapid determination of protein content of fractions obtained during Sephadex G-25 gel filtration of the α-keto acid preparations the absorbancies at 260 nm and 280 nm were measured with a Beckman Model DU-2 spectrophotometer, and the protein concentrations calculated from these readings by the following formula:

$$\text{Protein (mg/ml)} = 1.45\ E_{280nm} - 0.74\ E_{260nm}$$

where E_{280} and E_{260} signify the molar extinction of the solution at the indicated wavelengths (61).

RESULTS

Determination of Conditions for Assay of α-Keto Acid Dehydrogenases

Prior to a kinetic evaluation of the more complex parameters of the interactions of α-keto acids in mammalian liver, it was necessary to establish baseline experimental conditions in order to ensure that subsequent studies would be conducted under standardized and optimal conditions. Since the enzymatic oxidative decarboxylation of five α-keto acids (KIC, KMV, KIV, KG, Pyr) was to be investigated, it was desirable to establish, as completely as possible, the essential components of the assay systems used to measure these enzyme activities.

The relationships between protein concentration and decarboxylase activity using carboxyl-labeled α-keto acid substrates are shown for α-ketoisovalerate (Figure 3), α-ketoisocaproate (Figure 4), α-keto-β-methylvalerate (Figure 5), pyruvate (Figure 6), α-ketoglutarate (Figure 7). The production of $^{14}CO_2$ from labeled α-keto acids was found to be linear with enzyme concentration to at least 6.0 mg of tissue preparation for the branched-chain α-keto acid substrates and for α-ketoglutarate; pyruvate gave linearity to about 4.0 mg protein. Enzyme assays were conducted as described in the legend, in the text and in the "Methods" section.

The linear evolution of $^{14}CO_2$ obtained with increasing time of incubation at 30°C is illustrated for α-ketoisovalerate

Fig. 3. -- Formation of CO_2 from α-ketoisovalerate as a function of protein concentration

The amounts of $^{14}CO_2$ produced from KIV-1-^{14}C with varied amounts of mitochondria or "supernatant" preparations was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles); mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 1-^{14}C-KIV, 2.0; and the indicated amounts of enzyme preparation. Mitochondria and "supernatant" fractions are described in the Methods section. All assays were conducted at 30°C for 10 minutes.

nanomoles $^{14}CO_2$/minute
0
1
2
3
4
mg protein
0
1
2
3
4
5
6
7
mitochondria
supernatant

Fig. 4. -- Formation of CO_2 from α-ketoisocaproate as a function of protein concentration

The amounts of $^{14}CO_2$ produced from 1-^{14}C-α-ketoisocaproate during a 10 minute incubation period were measured by the $^{14}CO_2$ assay. All reaction components and conditions are the same as those stated for Fig. 3, with the exception of α-keto acid substrate, 2.0 μmoles of 1-^{14}C-KIC, 3500 cpm per micromole, were added to each reaction mixture.

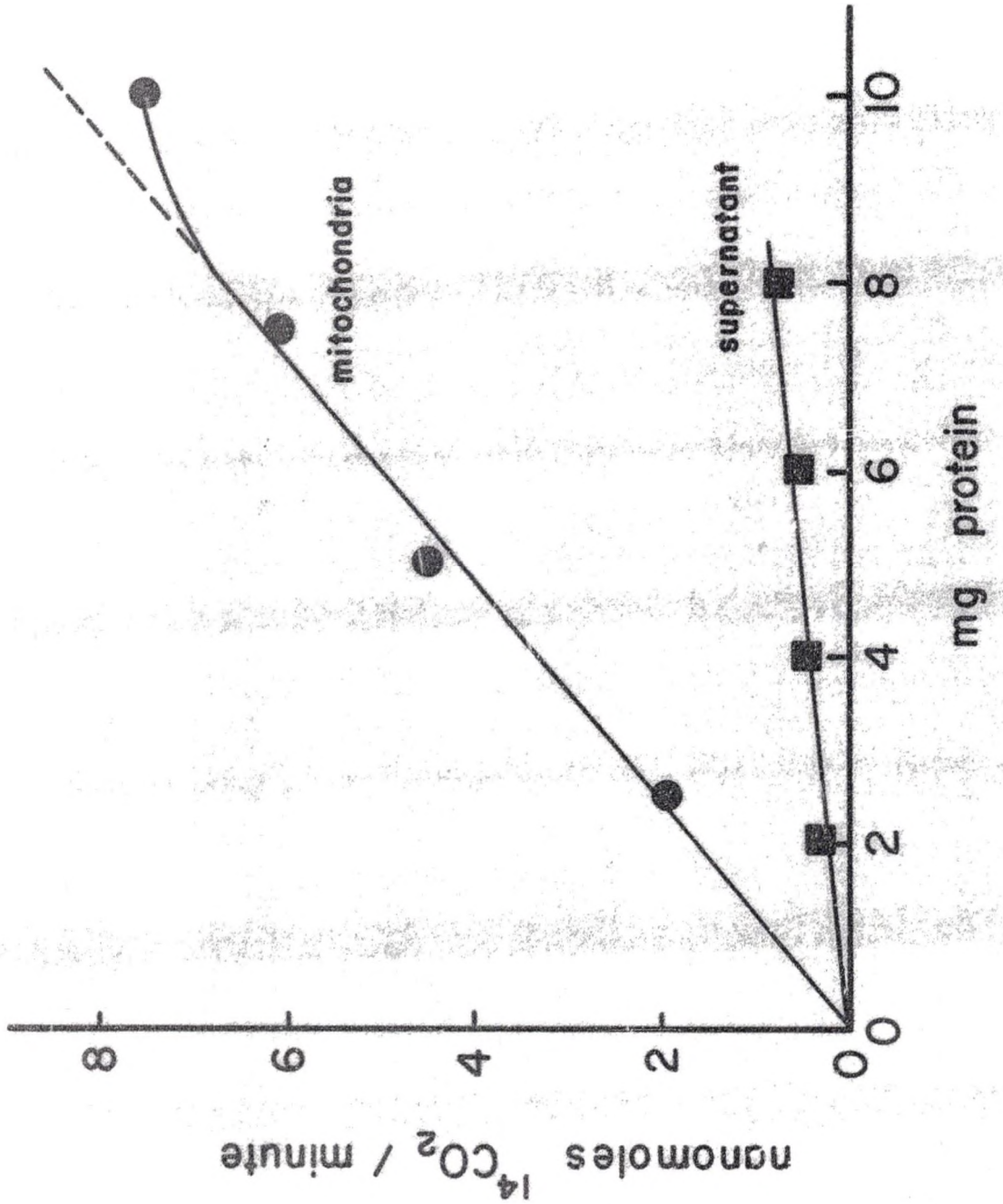

nanomoles $^{14}CO_2$ / minute
0
2
4
6
8
mitochondria
supernatant
0
2
4
6
8
10
mg protein

Fig. 5. -- Formation of CO_2 from α-keto-β-methylvalerate as a function of protein concentration

Figure 5 shows the relative amounts of $^{14}CO_2$ produced from carboxyl-labeled KMV with varied amounts of the same washed preparation of bovine liver mitochondria. The basic reaction mixture and assay procedures were as described in Figure 3 with the exception of substrate. 2.0 μmoles of carboxyl-labeled KMV (2050 cpm per micromole) were used as substrate in each flask.

5.0
4.0
3.0
2.0
1.0
0.0
nanomoles $^{14}CO_2$ / minute
0
1
2
3
4
5
6
mg protein

Fig. 6. -- Formation of CO_2 from pyruvate as a function of protein concentration

Figure 6 shows the relative amounts of $^{14}CO_2$ produced from carboxyl-labeled KMV with varied amounts of the same washed preparation of bovine liver mitochondria. The basic reaction mixture and assay procedures were as described in Figure 3, with the exception of substrate. 1.0 μmole of 1-^{14}C-pyruvate 58,600 cpm per μmole was added to each reaction flask.

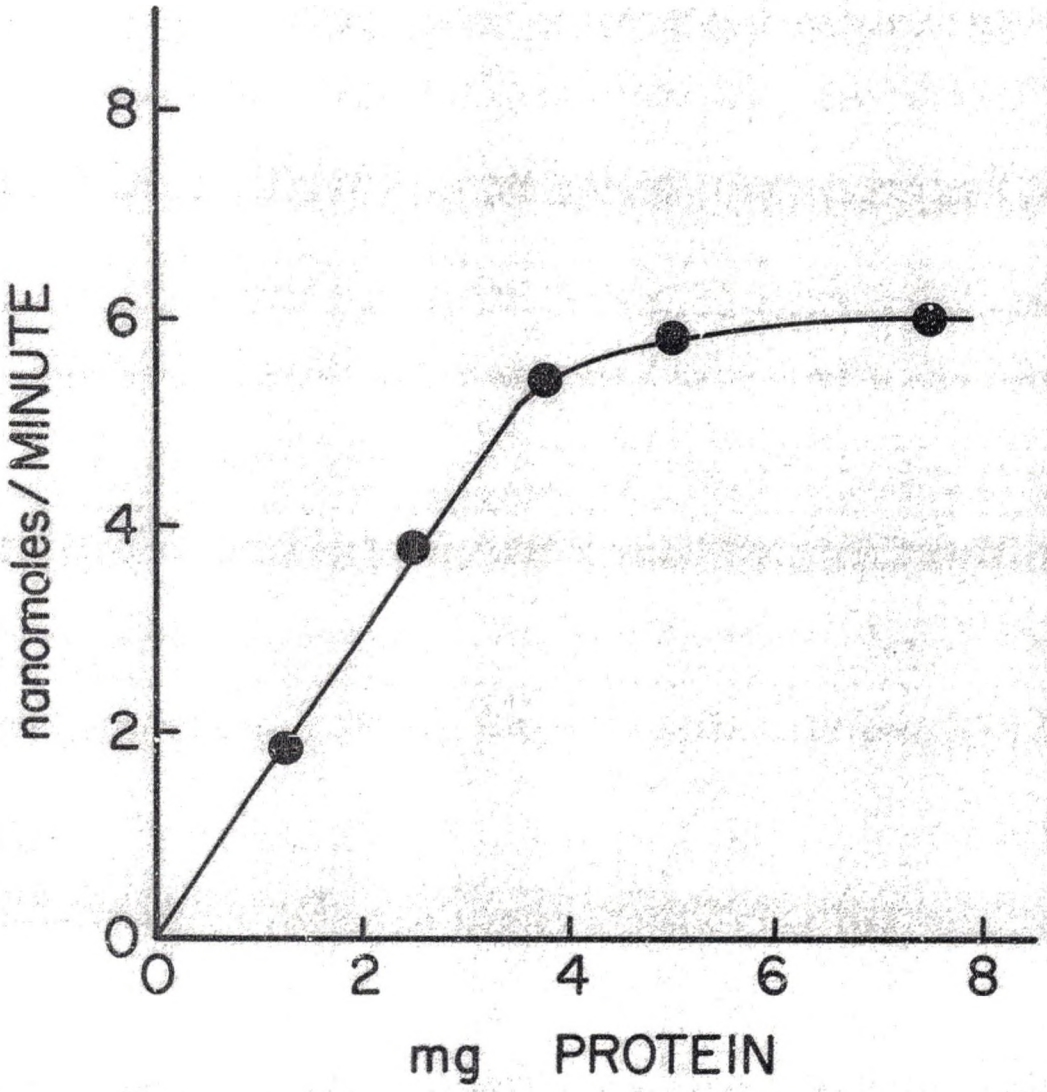
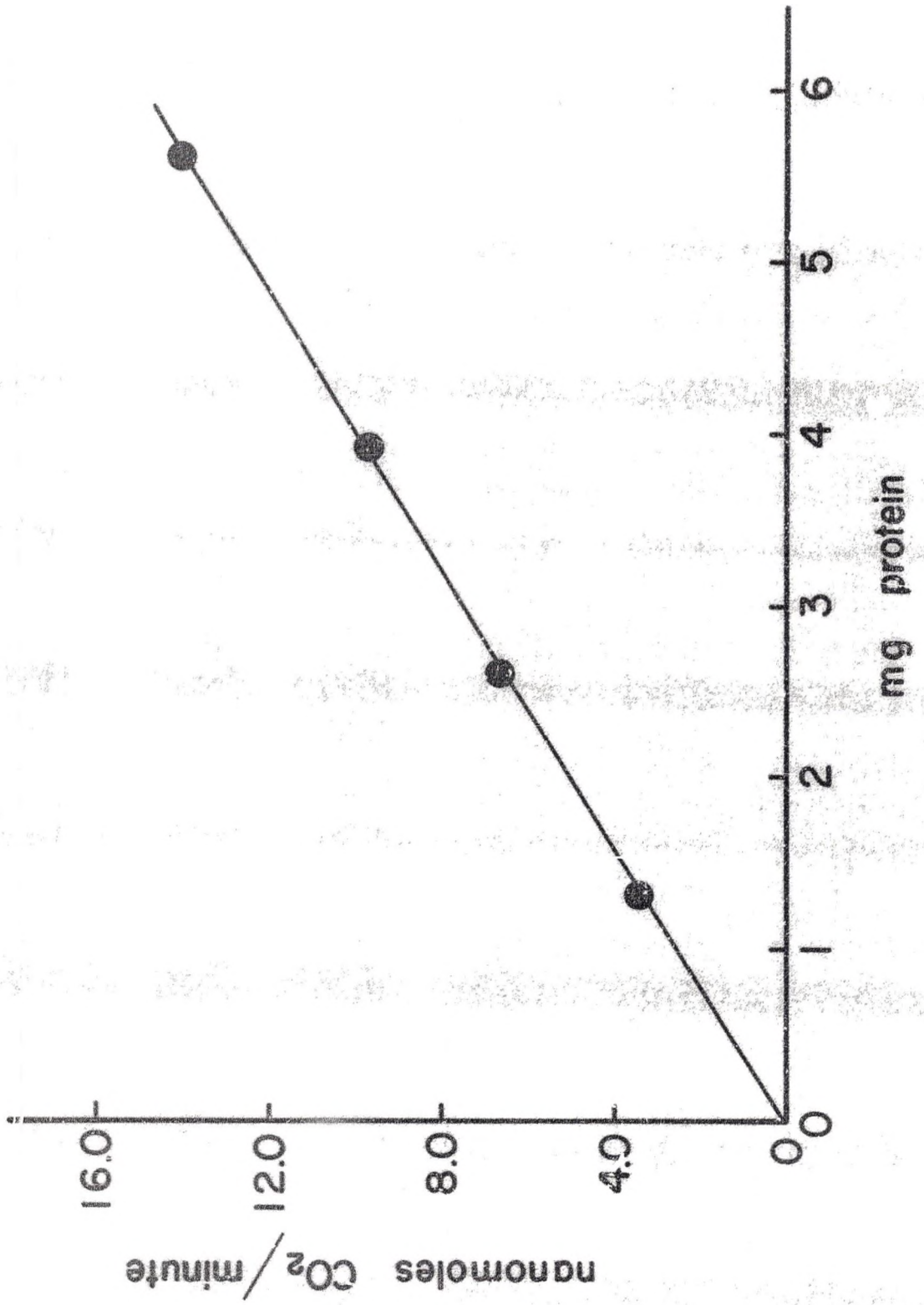

nanomoles/MINUTE
0
2
4
6
8
0
2
4
6
8
mg PROTEIN

Fig. 7. -- Formation of CO_2 from α-ketoglutarate as a function of protein concentration

The amounts of $^{14}CO_2$ produced from α-ketoglutarate-1-^{14}C with varied amounts of the same washed preparation of bovine liver mitochondria are shown in Figure 7. The basic reaction mixture and assay procedures were as described for Figure 3, with the exception of α-keto acid substrate. 1.0 μmole of carboxyl-labeled-KG (50,000 cpm per μmole) was added to each reaction flask. Incubations were for 10 minutes.


nanomoles CO_2 / minute
16.0
12.0
8.0
4.0
0
0
1
2
3
4
5
6
mg protein

(Figure 8), α-ketoisocaproate (Figure 9), α-keto-β-methylvalerate (Figure 10), pyruvate (Figure 11), and α-ketoglutarate (Figure 12). Some deviation from linearity with the longer incubation periods was noted, and for this reason appropriate linearity determinations were obtained with each set of experimental conditions preliminary to actual quantitative kinetic study.

Rates of oxidative decarboxylation of KIV, KIC, KMV, KG and pyruvate were measured at various pH values to determine the value for optimal assay conditions. The results of the effect of pH of the reaction medium upon the rate of $^{14}CO_2$ production with carboxyl-labeled KIV, KMV and KIC as substrates are shown in Figure 13. The observed activity maxima were nearly identical. Maximal reaction rates with α-ketoglutarate and pyruvate were obtained at slightly lower pH values. The optimum pH for enzyme reaction with these substrates is observed to lie in a range of 6.8 to 7.4 for bovine liver mitochondria preparations. A pH value of 7.2 was chosen for all subsequent assays, since at this hydrogen ion concentration the rate of non-enzymatic decarboxylation was minimal and the enzymic activity was satisfactory with each of the α-keto acid substrates.

The coenzymes NAD^+, coenzyme A, TPP, FAD and lipoic acid have been shown to be essential in the oxidation of pyruvate and α-ketoglutarate by their respective enzyme complexes (8,9,62,63,64). Table I shows the relative effect of these and several other cofactors on the decarboxylation of α-ketoisocaproate by washed preparations of bovine-liver mitochondria. The activity of the

TABLE 1

EFFECTS OF SEVERAL COFACTORS ON α-KETOISOCAPROATE DEHYDROGENASE REACTION[a]

Reaction medium	Relative activity
	(per cent)
Complete[b]	100.0
minus NAD^+	11.7
minus CoA	8.6
minus $MgCl_2$	69.0
minus $CaCl_2$	12.0
minus NAD^+, plus 0.5 micromole $NADP^+$	8.3
plus 0.05 μmole lipoate	103.0
plus 0.5 μmole TPP	92.6
plus 2.0 μmoles of ATP	29.0
plus 0.005 μmole of FAD	95.0
plus 0.005 μmole of FMN	89.0
plus 1.0 μmole of $CdCl_2$	2.3

[a] KIC dehydrogenase activity was measured with the $^{14}CO_2$ assay as described in the Methods.

[b] The complete reaction mixture contained in a final volume of 1.0 ml, the following, in μmoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 1-^{14}C-KIC, 2.0; and 0.1 ml of a washed preparation of bovine liver mitochondria. Subtractions from or additions to the complete media were as indicated in the Table.

Fig. 8. -- Formation of CO_2 from α-ketoisovalerate as a function of incubation time

α-Ketoisovalerate dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml the following (in micromoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.1 ml of a washed preparation of bovine liver mitochondria, and 2.0 micromoles of 1-^{14}C-KIV (3700 cpm per micromole). All assays were conducted at 30°C.

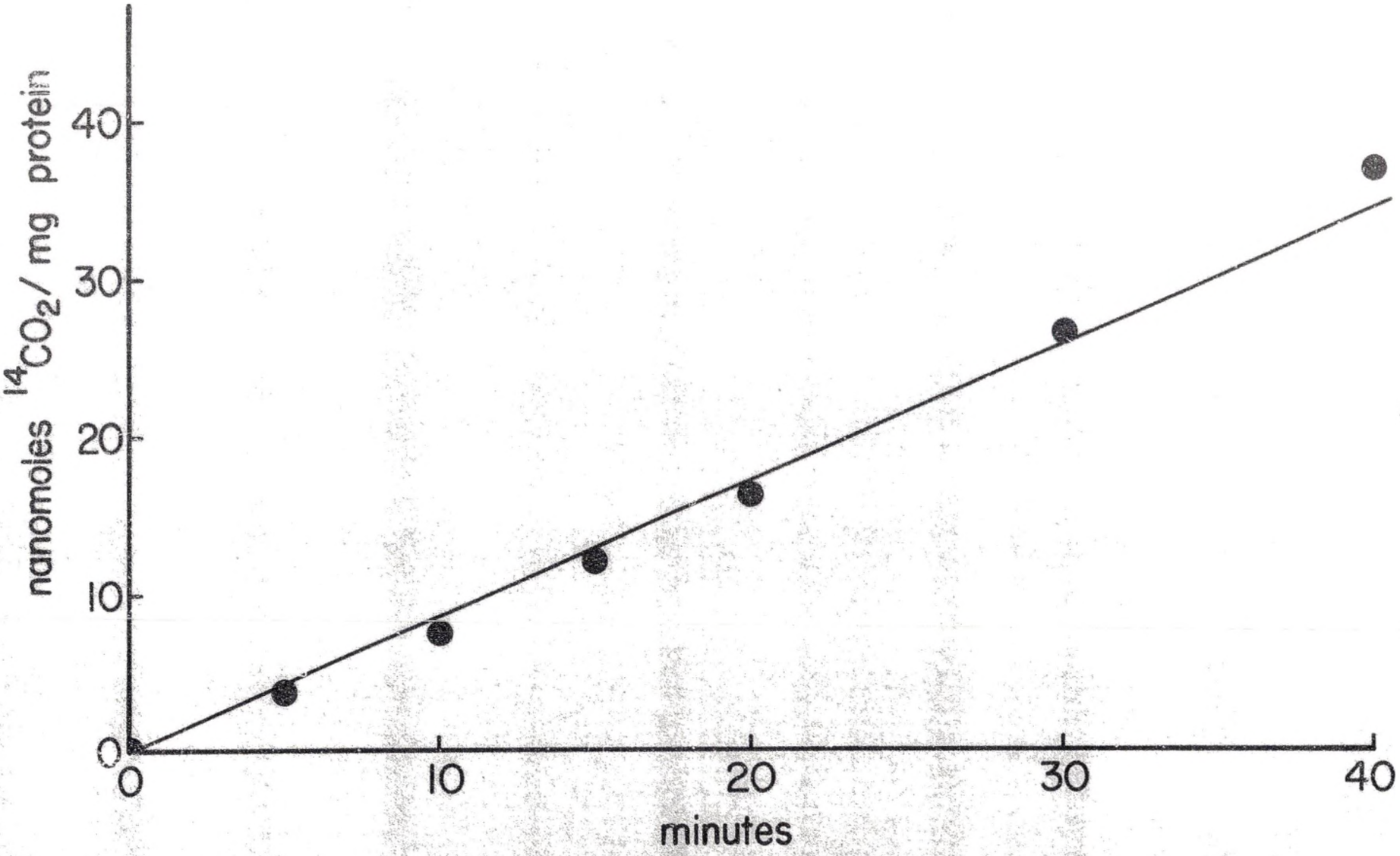

nanomoles $^{14}CO_2$/ mg protein
0
10
20
30
40
0
10
20
30
40
minutes

Fig. 9. -- Formation of CO_2 from α-ketoisocaproate as a function of incubation time

Figure 9 shows the amounts of $^{14}CO_2$ evolved after various incubation times at 30°C from 1-^{14}C-KIC. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria, and 2.0 μmoles of 1-^{14}C-KIC (3500 cpm per μmole). All other components of the reaction mixture, conditions, and assay procedures were identical with those described in Figure 8.

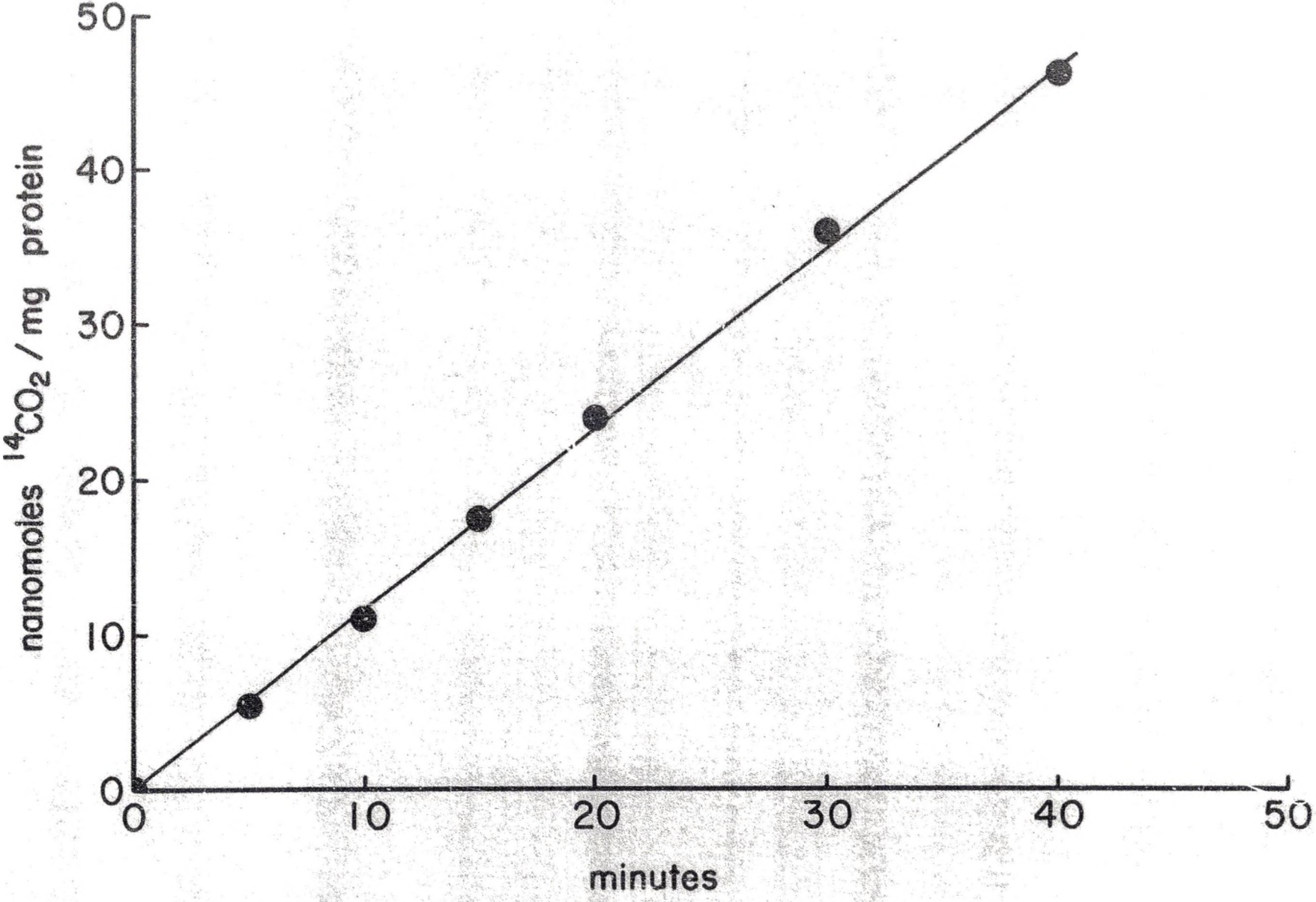

50
40
30
20
10
0
nanomoles $^{14}CO_2$ / mg protein
0
10
20
30
40
50
minutes

Fig. 10. -- Formation of CO_2 from α-keto-β-methylvalerate as a function of incubation time

Figure 10 shows the amounts of $^{14}CO_2$ evolved after various incubation times at 30°C from carboxyl-labeled ^{14}C-KMV (2050 cpm per μmole). Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria, and 2.0 μmoles of ^{14}C-KMV. All other conditions, reaction mixture components, and assay procedures were identical with those described in Figure 8.

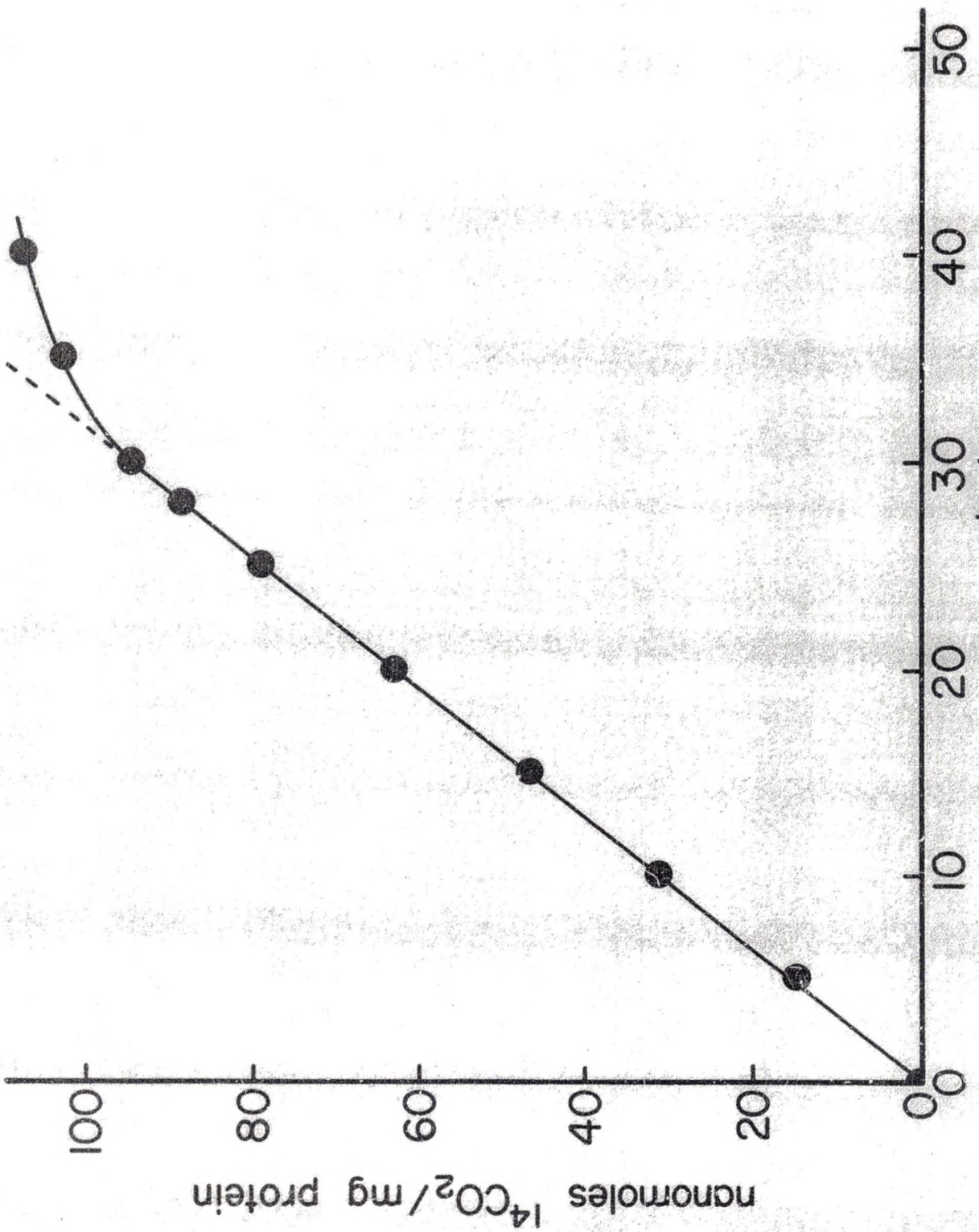

nanomoles $^{14}CO_2$/mg protein
0
20
40
60
80
100
0
10
20
30
40
50
minutes

Fig. 11. -- Formation of CO_2 from pyruvate as a function of incubation time

The amounts of $^{14}CO_2$ evolved after various incubation times at 30°C from 1-^{14}C-pyruvate (58,600 cpm per μmole) are shown in Figure 11. Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria, and 1.0 μmole of 1-^{14}C-pyr. All other conditions, reaction components, and assay procedures were as stated for Figure 8.

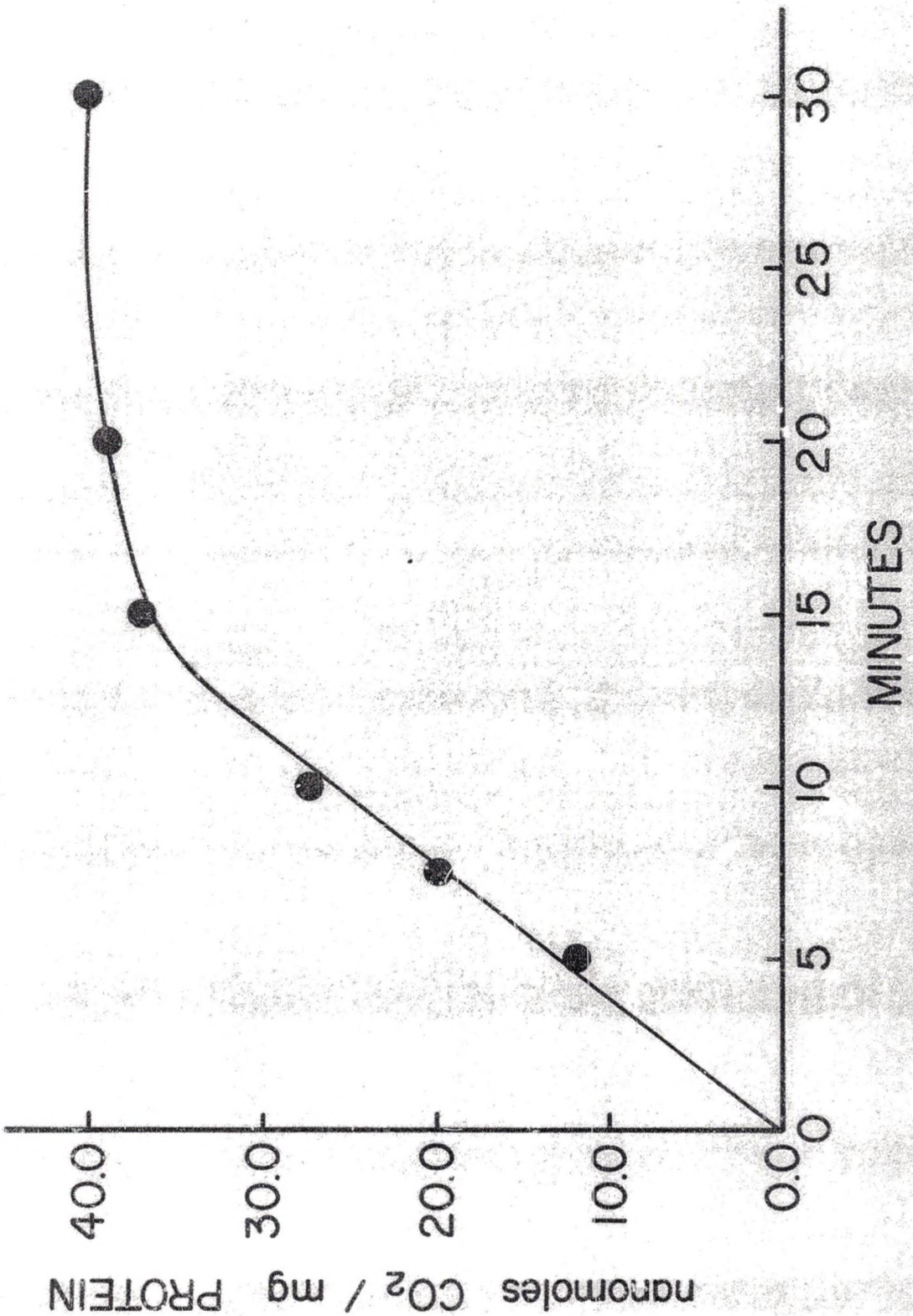

nanomoles CO_2 / mg PROTEIN
0.0
10.0
20.0
30.0
40.0
0
5
10
15
20
25
30
MINUTES

Fig. 12. -- Formation of CO_2 from α-ketoglutarate as a function of incubation time

Figure 12 shows the amounts of $^{14}CO_2$ evolved after various incubation times at 30°C from α-ketoglutarate-1-^{14}C (50,000 cpm per μmole). Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria, and 1.0 μmole of 1-^{14}C-KG. All other conditions, reaction components, and assay procedures were as stated for Figure 8.

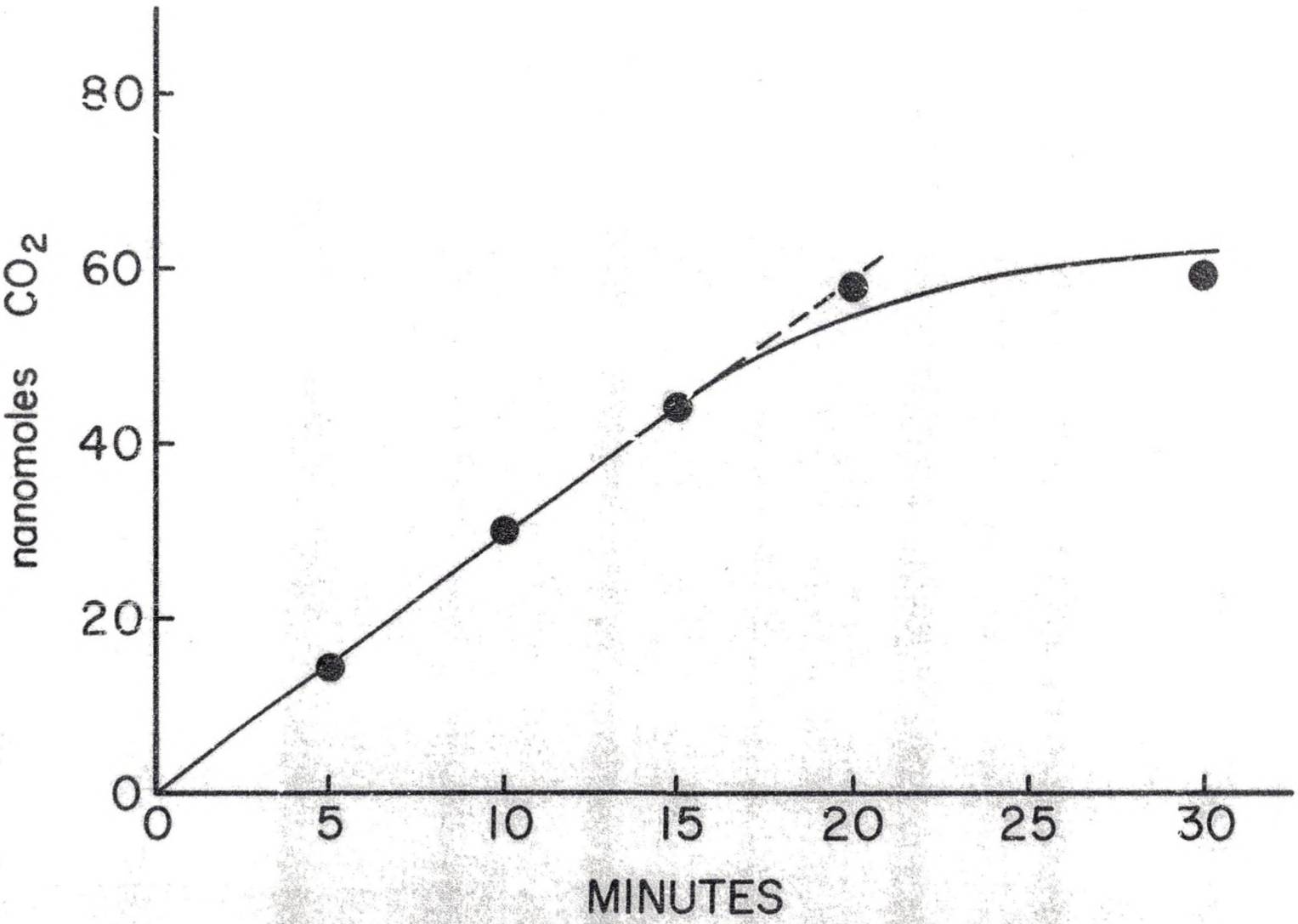

nanomoles CO_2
80
60
40
20
0
0
5
10
15
20
25
30
MINUTES

Fig. 13. -- The relationship between pH and branched-chain α-keto acid dehydrogenase activities of bovine liver mitochondria

Assays were conducted at the pH values indicated in the Figure 13. α-Keto acid dehydrogenase activities were measured with the $^{14}CO_2$ assay. Other reaction components were as stated in the legend of Figure 3 with the exception of α-keto acid substrate. Initial α-keto acid concentration in each assay was 2.0 mM. Phosphate buffers were used throughout the entire range even though some buffering capacity is sacrificed at the low and high pH values. (▲—▲) KIV, (■—■) KMV, (●—●) KIC.

nanomoles CO_2 PRODUCED
40
80
120
160
pH
6.0
6.5
7.0
7.5
8.0

mitochondrial enzyme which catalyzes this reaction is largely dependent upon the presence of added NAD^+ and coenzyme A. Other components of the standard assay medium which appear to promote optimal reaction rates are Mg^{+2}, Ca^{+2} and inorganic phosphate. The effects of P_i are shown in a later figure, and the importance of these agents will be discussed in more detail below. Attempts to demonstrate a direct stoichiometric relationship between reduction of NAD^+ and α-keto acid decarboxylation have been thwarted by the generally high degree of opacity (at 340 nm) of reaction mixtures containing the particulate enzyme preparations, and by the variance of reoxidation of NADH by the crude preparations. Data of Table 1, however, support the earlier postulation, as outlined above (equations 2-7), that all of the components of the presumed dehydrogenase complex participate in the overall oxidative decarboxylation of the branched-chain α-keto acids by mammalian liver mitochondria. It is apparent that $NADP^+$ can not replace NAD^+ as an electron acceptor in this reaction system. Addition of other presumptive cofactors such as TPP, lipoate and flavin derivatives produced no significant effect upon KIC oxidation. However, stimulation of the enzyme activity by these coenzymes would not be expected in consideration of the generally tightly-bound nature of these prosthetic groups with their protein apoenzyme moieties. Separation into free coenzyme and inactive apoenzyme usually requires rather rigorous physical or chemical procedures. These findings are in general agreement with those previously reported for the oxidation of KIV by extracts of B. subtilus (10)

and for the decarboxylation of KIC by isolated rat liver mitochondria (11).

An observation which was not entirely expected was that of the marked reduction of catalytic activity in the presence of ATP (2 mM). This apparent inhibitory effect and the stimulatory nature of inorganic phosphate in this system will be discussed in connection with the possible existence of a kinase-phosphatase activating and de-activating system for the α-keto acid dehydrogenase complexes.

The results of a more thorough investigation of the effects of CoA and NAD^+ on the branched-chain α-keto acid dehydrogenase activities are shown in Figure 14 (KIC) and Figure 15 (KIV). These requirements (with those for KMV oxidation) are summarized in Table 2. These data indicate that both NAD^+ and CoA are essential to maintain maximal branched-chain α-keto acid oxidative capacity with washed preparations of mitochondria. On the other hand, decarboxylase activity of the supernatant fluid of the 80,000 x g centrifugation procedure (preparation of enzyme, see "Methods") showed no apparent dependence on added CoA in the reaction medium. The existence of an acyl-CoA hydrolase in the soluble fraction, similar to that demonstrated by Namba et al. (10) in B. subtilus preparations, conceivably could lessen the apparent need for exogenous CoA by allowing for the continued regeneration of catalytic amounts of the free coenzyme from acyl-CoA compounds present as contaminants in the enzyme preparation.

A second possibility, which can not be excluded at present, is that the supernatant fluid may contain a decarboxylase specific for

TABLE 2

EFFECTS OF ADDED NAD^+ AND CoA ON BRANCHED-CHAIN α-KETO ACID DEHYDROGENASE ACTIVITIES OF MITOCHONDRIA AND SUPERNATANT FRACTIONS OF BOVINE LIVER[a]

Enzyme preparation	Reaction mixture			
	complete[b]	- CoA	- NAD^+	- NAD^+ - CoA
	(nanomoles CO_2/min/mg protein)			
α-Ketoisocaproate dehydrogenase:				
mitochondria---	1.74	0.16	0.26	0.06
supernatant----	0.11	0.12	0.05	0.05
α-Keto-β-methylvalerate dehydrogenase:				
mitochondria---	3.20	0.29	0.39	0.14
supernatant----	0.29	0.23	0.17	0.13
α-Ketoisovalerate dehydrogenase:				
mitochondria---	5.63	0.47	0.66	0.39
supernatant----	0.03	0.03	0.01	0.01

[a] Branched-chain α-keto acid dehydrogenase activity was measured with the $^{14}CO_2$ assay as described in the Methods.

[b] The complete reaction mixture contained the following components, in a final volume of one ml, in μmoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.1 ml of enzyme preparation and 2.0 μmoles of carboxyl-labeled α-keto acid. Subtractions from the complete media were as indicated in the Table.

Fig. 14. -- Effects of added NAD and CoA upon α-ketoisocaproate oxidation

KIC dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml, the following constituents (in micromoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 1-^{14}C-KIC, 2.0 (3600 cpm per micromole); 0.1 ml of a washed preparation of bovine liver mitochondria (2.4 mg protein), or 0.2 ml of bovine liver "supernatant" fraction (2.1 mg protein); 0.6 micromole Li_3CoA, and 1.0 micromole NAD where indicated. The "supernatant" fraction referred to is the supernatant of the 80,000 x g centrifuge spin as described in the Methods section under "enzyme preparations". Reactions were allowed to proceed for 10 minutes at 30°C.

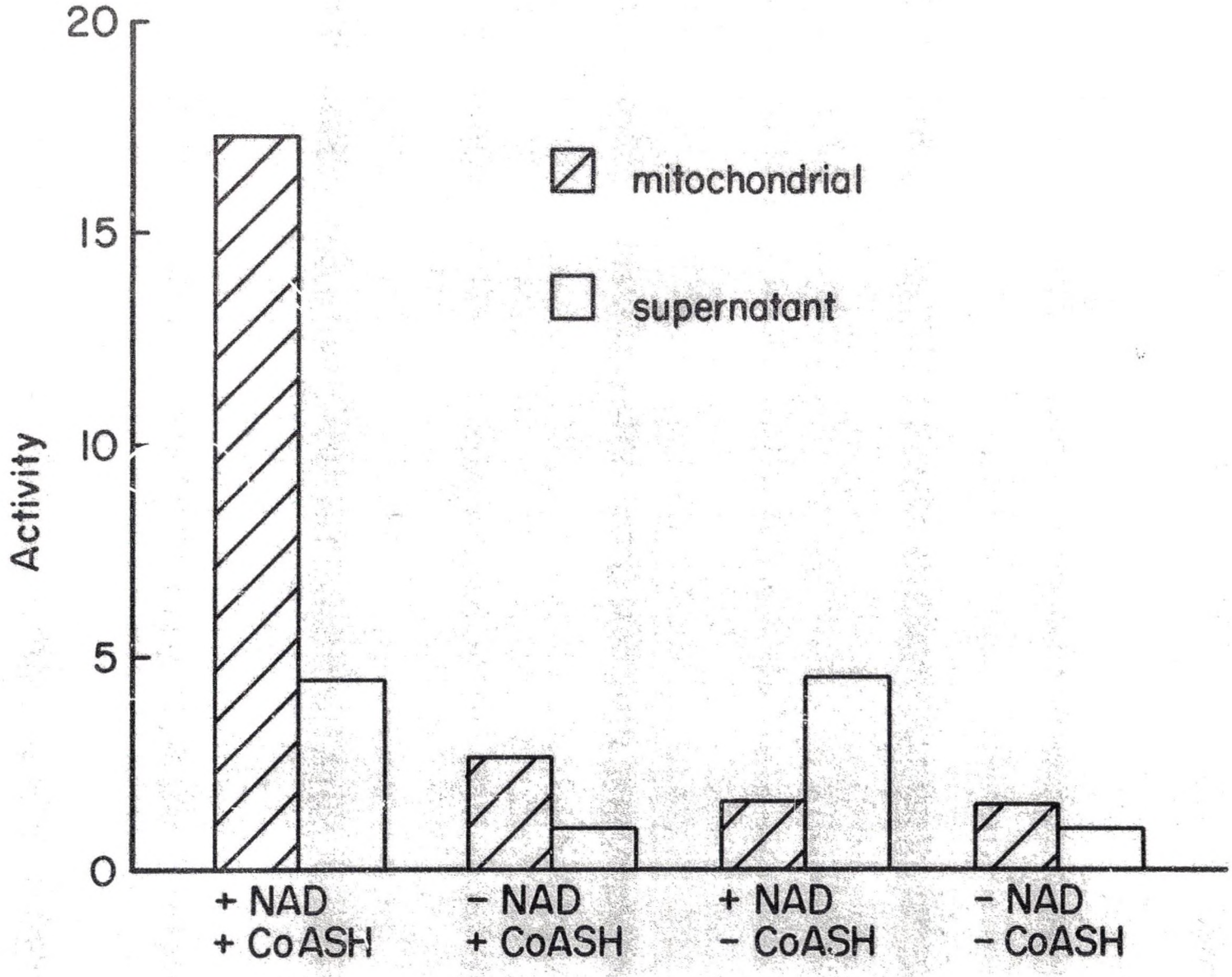
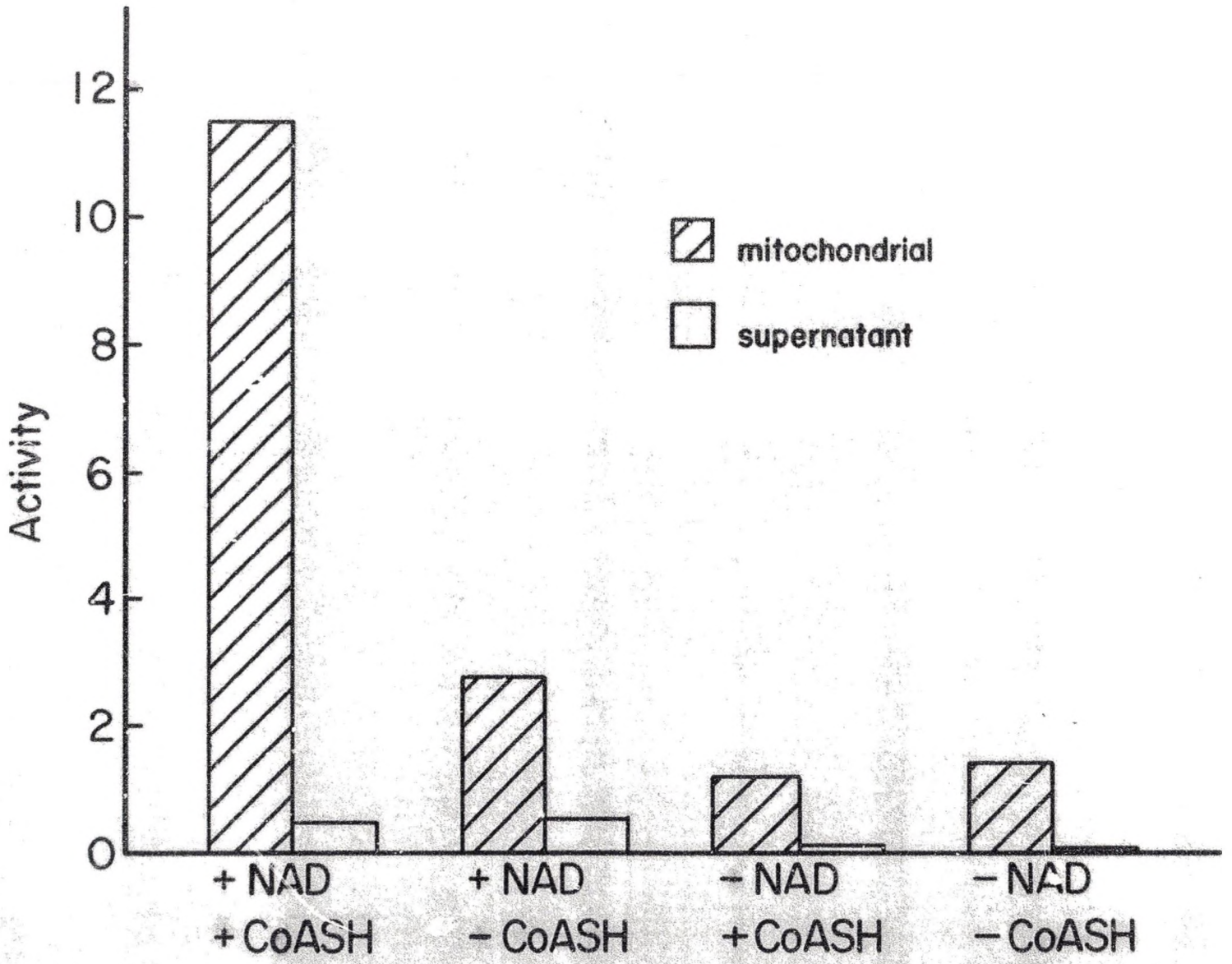

20
15
10
5
0
Activity
mitochondrial
supernatant
+ NAD
+ CoASH
- NAD
+ CoASH
+ NAD
- CoASH
- NAD
- CoASH

Fig. 15. -- Effect of added NAD and CoA on the rate of CO_2 production from α-ketoisovalerate by bovine liver mitochondria

KIV dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods. Additions of NAD, CoA and enzyme preparations were as stated for Figure 14. Each reaction mixture contained 2.0 μmoles of 1-^{14}C-KIV (3700 cpm per μmole). All other conditions, reaction components, and assay procedures were as stated for Figure 14.


Activity
0
2
4
6
8
10
12
mitochondrial
supernatant
+ NAD
+ CoASH
+ NAD
- CoASH
- NAD
+ CoASH
- NAD
- CoASH

the branched-chain α-keto acids, which does not require coenzyme A as a component of its catalytic process. The data of Table 2 also disclose that the supernatant enzyme activity is significantly higher with KMV and KIC as substrates, than for KIV. This represents a decided variance from the activity ratios observed for the mitochondrial enzyme activities, where the rate of decarboxylation was greatest with KIV as the α-keto acid substrate.

The following studies were initiated in an attempt to elucidate to what extent the rate of branched-chain α-keto acid decarboxylation was affected by varied concentrations of NAD^+ and CoA. The effects on evolution of $^{14}CO_2$ from carboxyl-labeled KIC were studied in detail. An apparent Michaelis constant for NAD^+ of 3.2×10^{-5} M was calculated from the reciprocal plots shown in Figure 16. The Lineweaver-Burk plot in Figure 17 illustrates the effect of varied CoA concentrations on KIC oxidation by bovine liver mitochondria. Apparent inhibition by CoA was observed with concentrations above 10^{-3} M, with a calculated K_m value for coenzyme A of 6.4×10^{-5} M.

Preliminary observations by this author and prior investigation by Hayakawa (65), Lusty and Singer (55) and Wohlheuter (11) had indicated that the divalent cations Ca^{2+}, Mg^{2+} and sometimes Mn^{2+} appeared to modify significantly the α-keto acid dehydrogenase activities. These effects were noted with purified enzyme preparations and with suspensions of mitochondria. Requirements of the branched-chain α-keto acid dehydrogenases for certain divalent cations are shown in Table 3. The dehydrogenase activities with each of the three branched-chain α-keto acids was strongly stimulated

TABLE 3

EFFECTS OF ADDED $MgCl_2$, $CaCl_2$ AND $MnCl_2$ IN THE REACTION MEDIUM ON THE BRANCHED-CHAIN α-KETO ACID DEHYDROGENASE ACTIVITIES OF BOVINE LIVER MITOCHONDRIA PREPARATIONS[a]

Enzyme and additions[b]	specific activity (nanomoles/min/mg protein)	relative activity (per cent)
α-Ketoisocaproate dehydrogenase:		
Ca^{+2}, Mg^{+2}	2.72	100
Ca^{+2}	1.88	69
Mg^{+2}	0.16	6
Mn^{+2}	0.13	5
Ca^{+2}, Mg^{+2}, Mn^{+2}	2.56	94
Ca^{+2}, Mn^{+2}	2.24	82
Mg^{+2}, Mn^{+2}	0.18	7
none	0.11	4
α-Ketoisovalerate dehydrogenase:		
Ca^{+2}, Mg^{+2}	3.40	100
Ca^{+2}	2.51	74
Mg^{+2}	0.35	11
none	0.21	6
α-Keto-β-methylvalerate dehydrogenase:		
Ca^{+2}, Mg^{+2}	3.31	100
Ca^{+2}	2.46	74
Mg^{+2}	0.29	9
none	0.16	5

[a] Branched-chain α-keto acid dehydrogenase activity was measured with the $^{14}CO_2$ assay as described in the Methods.

[b] Each reaction mixture contained the following, in μmoles: mannitol, 150; NAD, 1.0; Li_3CoA, 0.6; Na_2CO_3, 1.0; carboxyl-labeled α-keto acid, 2.0; 0.1 ml of a washed preparation of bovine liver mitochondria, and 1.0 μmole of each divalent cation where indicated, in a final volume of 1.0 ml.

Fig. 16. -- Lineweaver-Burk plot of KIC dehydrogenase activity as a function of NAD^+ concentration

Lineweaver-Burk plots of $^{14}CO_2$ evolution from 1-^{14}C-KIC with NAD^+ as varied coenzyme. KIC dehydrogenase activity was measured by the NAD^+- and CoASH-dependent $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained the following (in μmoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; the indicated amounts of NAD^+, and 0.1 ml of mitochondria suspension in a final volume of 1.0 ml. (v_i, nanomoles of CO_2 produced per minute per mg of protein).

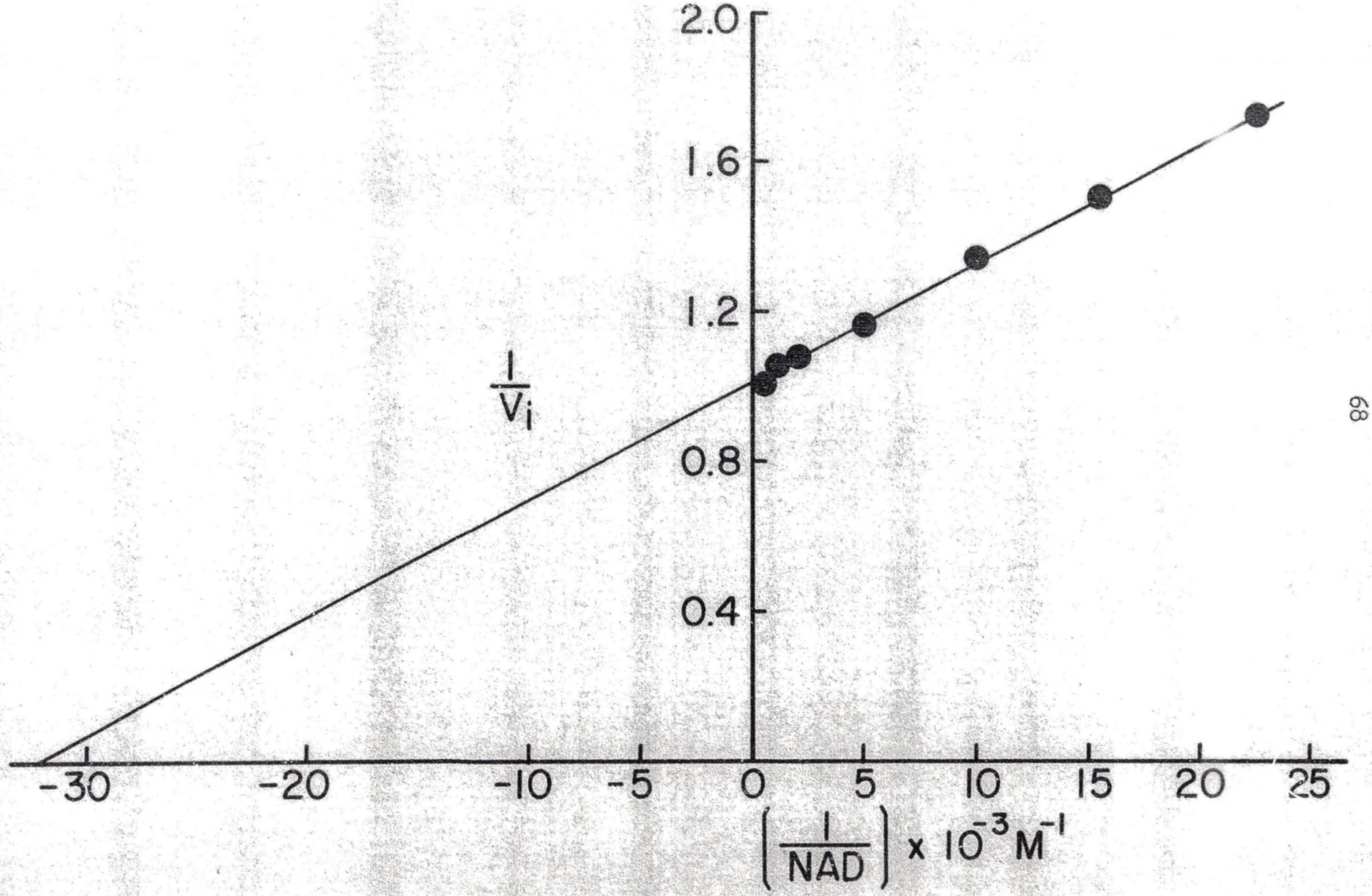
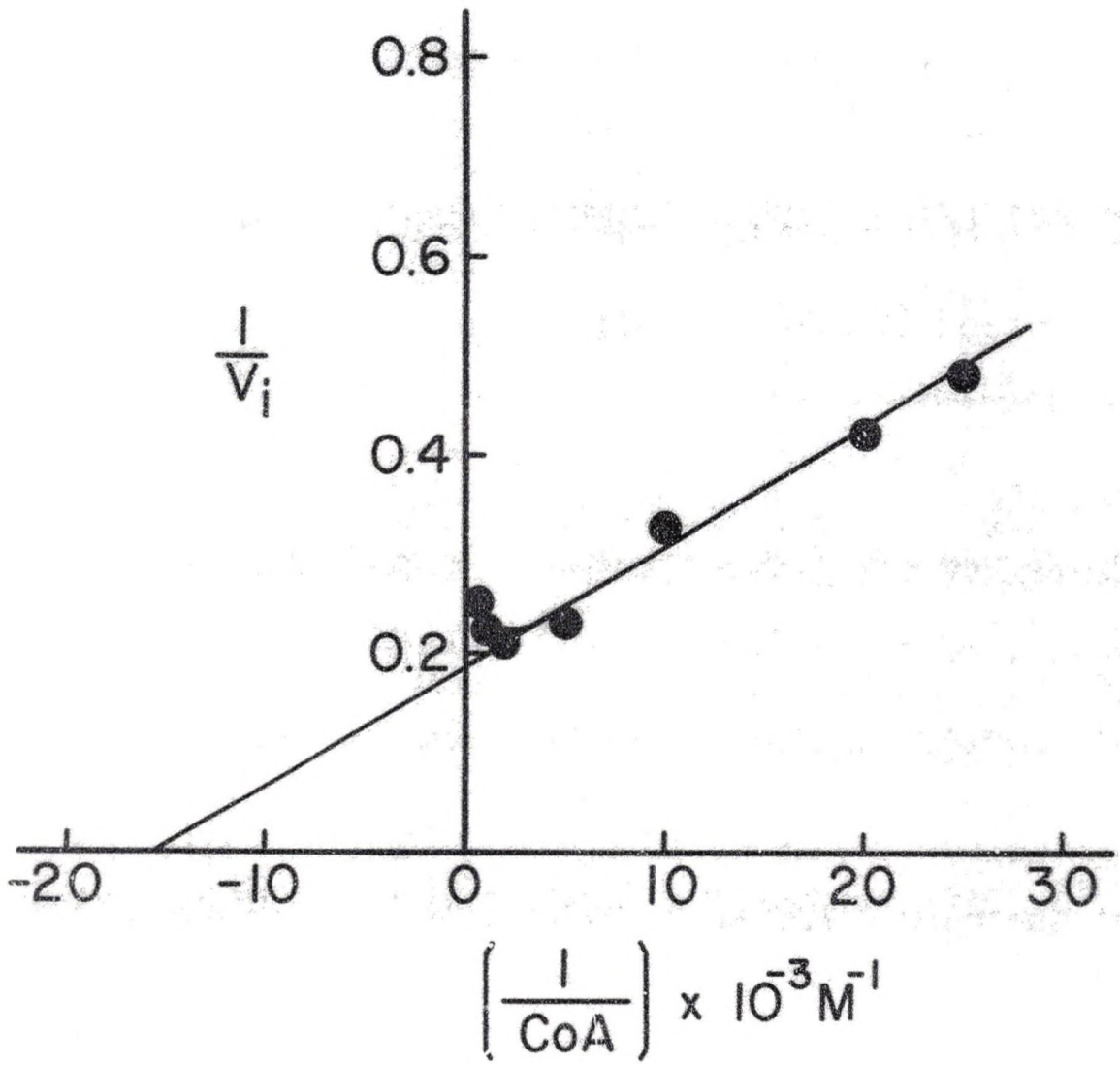

2.0
1.6
1.2
0.8
0.4
$\frac{1}{V_i}$
-30
-20
-10
-5
0
5
10
15
20
25
$\left(\frac{1}{NAD}\right) \times 10^{-3} M^{-1}$

Fig. 17. -- Lineweaver-Burk plot of the activity of α-ketoisocaproate dehydrogenase activity as a function of CoA concentration

KIC dehydrogenase activity was measured by the $^{14}CO_2$ assay. All components of the reaction medium were the same as for Figure 16, except that 1.0 μmole of NAD was used, and CoA concentration was varied as indicated in this Figure. (v_i, nanomoles CO_2 produced per minute per mg of protein).


0.8
0.6
0.4
0.2
1/Vi
-20
-10
0
10
20
30
(1/CoA) x 10⁻³M⁻¹

by added Ca^{2+}, and to a lesser extent by exogenous Mg^{2+}. The effects of Mg^{2+} and Ca^{2+} appear to be additive, suggesting that the activation by these two cations occurs by separate mechanisms. In this regard it should be noted that Mn^{2+} could apparently replace the Mg^{2+} requirement to a limited extent, but could not substitute for Ca^{2+}. The relative rates of oxidation of KIC, KMV and KIV were decreased by nearly identical amounts when exogenous Mg^{2+}, Ca^{2+} or both cations were omitted from the reaction medium. The effects of other divalent cations were not studied systematically. However, it was observed that 1.0 mM Cd^{2+} completely inhibited KIC decarboxylation.

The striking dependence of (branched-chain α-keto acid) oxidative decarboxylation on Ca^{2+} and inorganic phosphate prompted further study of their effects. Reciprocal plots illustrative of the influence of varying amounts of added Ca^{2+} (Figure 18) and P_i (Figure 19) are included. The exact nature of the non-linearity of these plots is not understood at this time. Apparent affinity constants of the order 1.0 mM for Ca^{2+} and 20 mM for P_i can be estimated from these plots. It is of some general interest to note that these values approach the physiological levels found for Ca^{+2} in mammalian liver mitochondria (66,67) and those calculated for inorganic phosphate in whole liver homogenates (68,69).

All α-keto acid dehydrogenase assays to be described in the following studies were conducted under the optimal conditions based on the information reported above: 1.0 mM NAD^+, 0.6 mM CoA; 1.0 mM $CaCl_2$; 1.0 mM $MgCl_2$; and 33 mM potassium phosphate buffer, pH 7.2. Reaction incubations were carried out at 30°C for an

Fig. 18. -- Effects of added $CaCl_2$ on the KIV dehydrogenase activity of bovine liver mitochondria

Lineweaver-Burk and Michaelis-Menten plots of the effects of varied Ca^{+2} concentration on evolution of $^{14}CO_2$ from 1-^{14}C-KIV are shown in Figure 18. KIV dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods. Reaction mixtures contained (in μmoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; Na_2CO_3, 1.0; 1-^{14}C-KIV (3500 cpm per μmole), 2.0; 0.1 ml of a washed preparation of bovine liver mitochondria, and the indicated amounts of $CaCl_2$ in a final volume of 1.0 ml. (v_i, nanomoles CO_2 produced per minute per mg of protein).

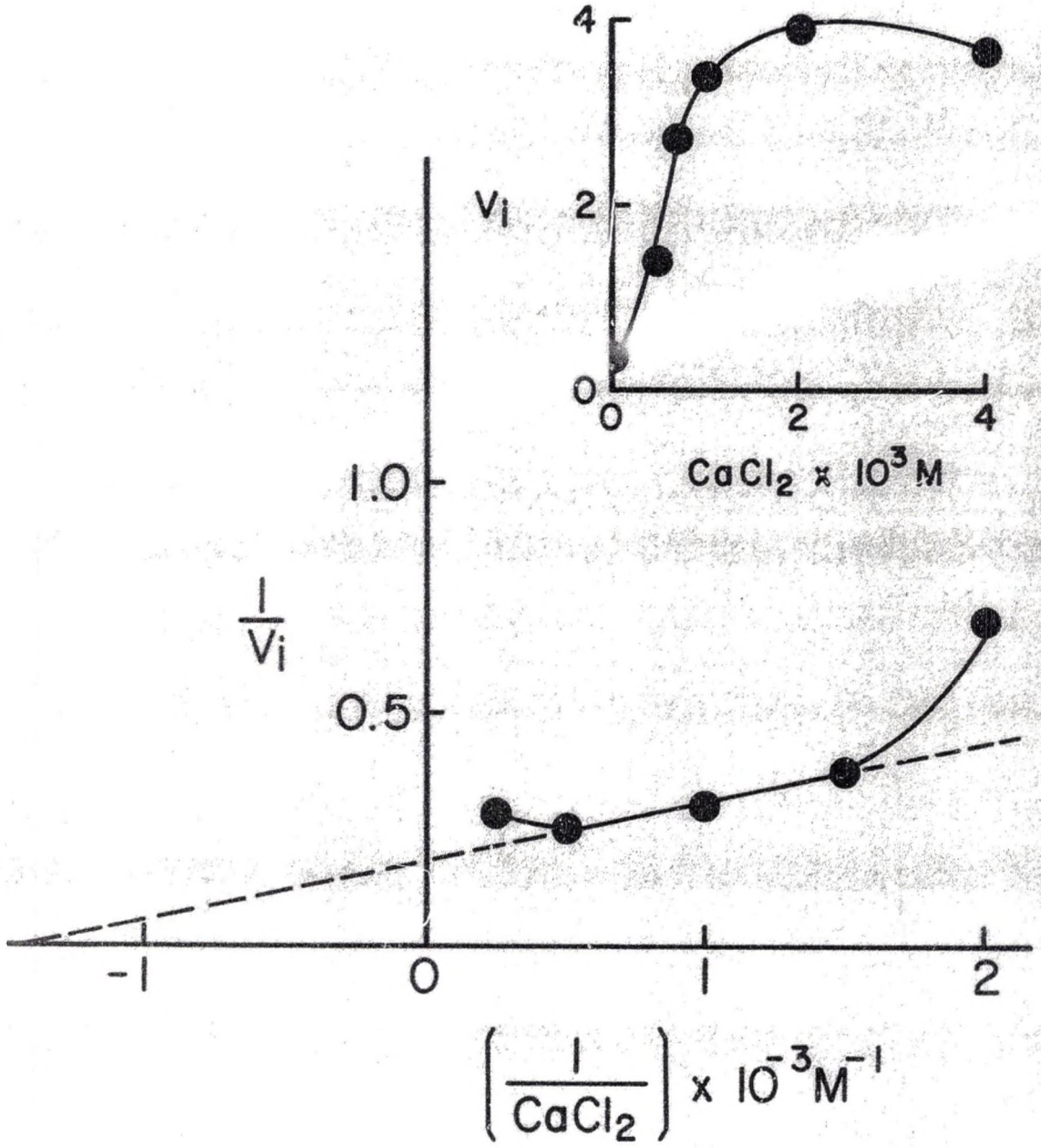
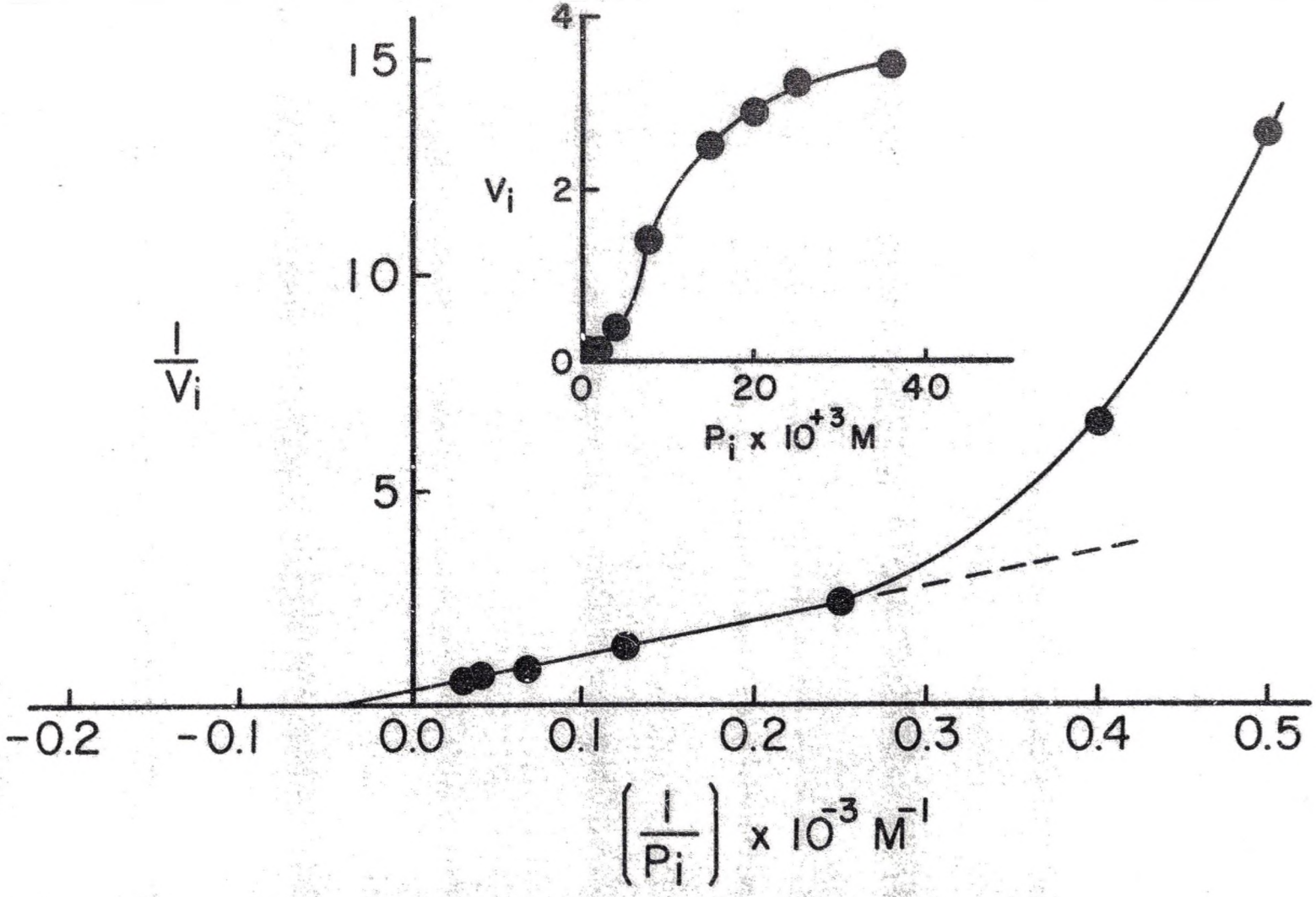

4
2
0
V_i
0
2
4
$CaCl_2 \times 10^3 M$
1.0
0.5
$\frac{1}{V_i}$
-1
0
1
2
$\left(\frac{1}{CaCl_2}\right) \times 10^{-3} M^{-1}$

Fig. 19. -- Effect of changes in orthophosphate concentration on the KIV dehydrogenase activity of bovine liver mitochondria

Lineweaver-Burk and Michaelis-Menten plots of the effects of varied P_i concentration on evolution of $^{14}CO_2$ from 1-^{14}C-KIV are shown in Figure 19. Reaction mixtures, experimental conditions, and assay procedures were as stated for Figure 18, except that P_i concentrations were varied, and 0.01 M triethanolamine hydrochloride, pH 7.2, was used to maintain buffering capacity, and 1.0 μmole of $CaCl_2$ was added to each reaction flask. (v_i, nanomoles per minute per mg protein).


15
10
5
1/Vi
-0.2
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
(1/Pi) x 10^-3 M^-1
4
2
0
Vi
0
20
40
Pi x 10^+3 M

appropriate time interval dependent upon the extent of linearity obtained for the particular set of experimental conditions of the investigation. Any variation from these conditions is noted in the legend of tables and figures, or in the text.

Effects of α-Keto Acid Substrate Concentration on α-Keto Acid Dehydrogenase Reaction Rates

Quantitative studies of the influence of substrate concentration upon the rate of oxidation of the α-keto acids were essential as a prerequisite to the investigation of mutual metabolic interactions among these compounds. Apparent K_m values were determined for KIV, KMV, KIC, KG and pyruvate. Substrate effects were investigated over at least a ten-fold variation of concentration for each keto acid. The relationship between the reciprocal of initial reaction velocity (v_i) and the inverse of KIC concentration is shown in the Lineweaver-Burk plot of Figure 20. The apparent K_m value for KIV calculated from these plots was 0.33 mM. The information represented by the reciprocal plots of Figure 21 allows for determination of the apparent K_m for KMV, which is 0.47 mM. A Michaelis constant of 0.44 mM is obtained for KIC from the data illustrated by Figure 22. The experimental results which are included in double reciprocal form in Figure 23 show the apparently linear double reciprocal plots determined by variation of α-ketoglutarate concentration between 0.05 mM and 1.0 mM. It should be mentioned that significant deviation from linearity was observed at KG concentrations below 0.04 mM. This phenomenon may be due to the existence of the presumed permeability barrier to KG

Fig. 20. -- Effect of varied α-ketoisovalerate concentration on the rate of CO_2 production by bovine liver mitochondria

Lineweaver-Burk plot of the activity of KIV dehydrogenase of bovine liver mitochondria as a function of substrate (KIV) concentration (v_i, nanomoles of CO_2 produced per minute per milligram of protein). Enzyme activity was measured with the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained, in a final volume of 1.0 ml, the following (in micromoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD^+, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.1 ml of a washed preparation of bovine liver mitochondria (about 2.5 mg of protein), and the indicated amounts of 1-^{14}C-KIV (3700 cpm per micromole). Reactions were allowed to proceed for 20 minutes at 30°C.

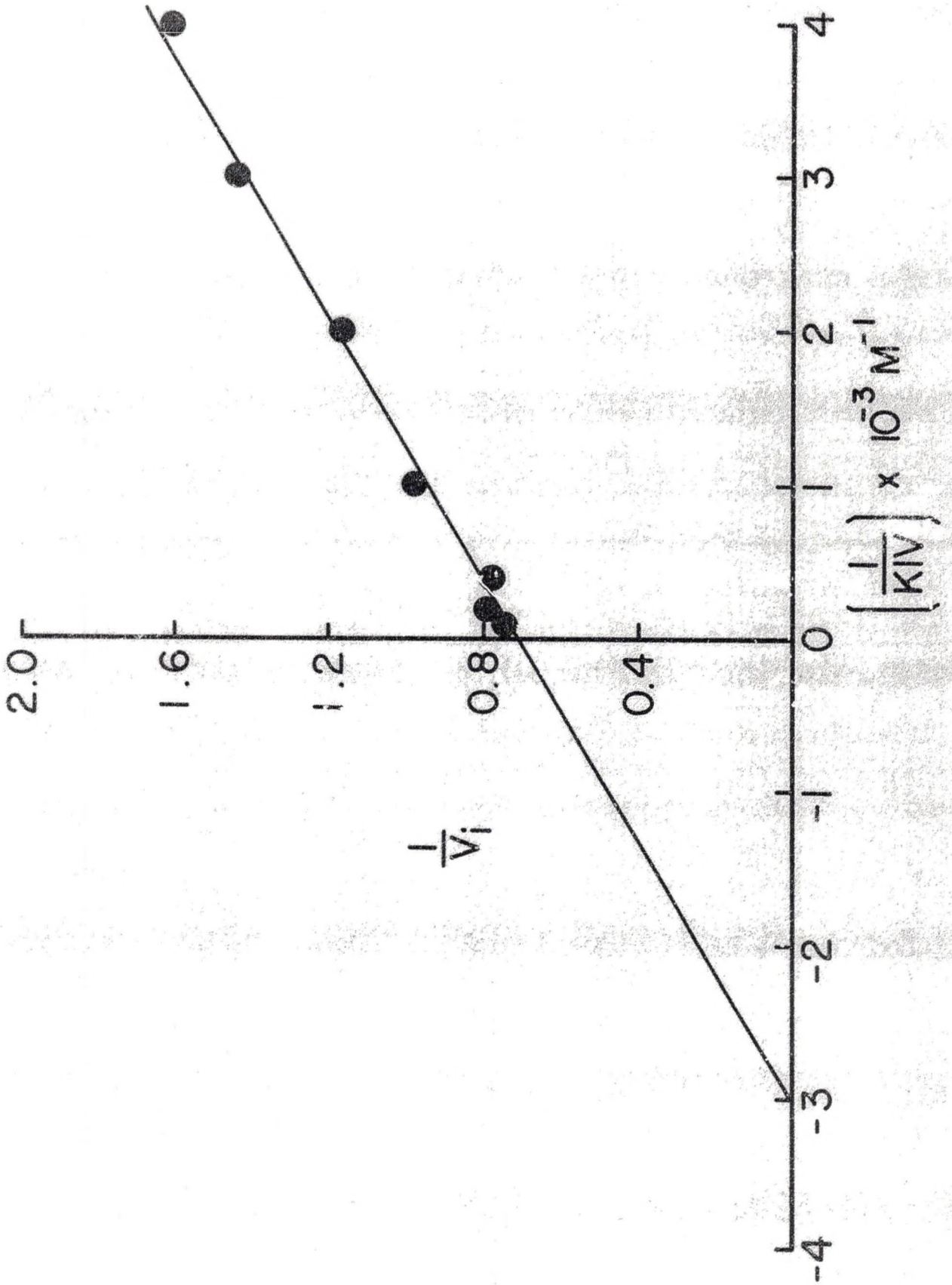

2.0
1.6
1.2
0.8
0.4
0
-1
-2
-3
-4
1
2
3
4
$\frac{1}{V_i}$
$\left\{\frac{1}{K[V]}\right\} \times 10^{-3}\ M^{-1}$

Fig. 21. -- Effect of varied α-keto-β-methylvalerate concentration on the rate of formation of CO_2 by bovine liver mitochondria

Lineweaver-Burk plot of the activity of KMV dehydrogenase of bovine liver mitochondria as a function of substrate (KMV) concentration (v_i, nanomoles of CO_2 produced per minute per mg of protein). Each reaction mixture contained 0.1 ml of a washed preparation of mitochondria and the indicated quantities of carboxyl-labeled KMV. All other experimental conditions, reaction components and assay procedures were as stated for Figure 20.

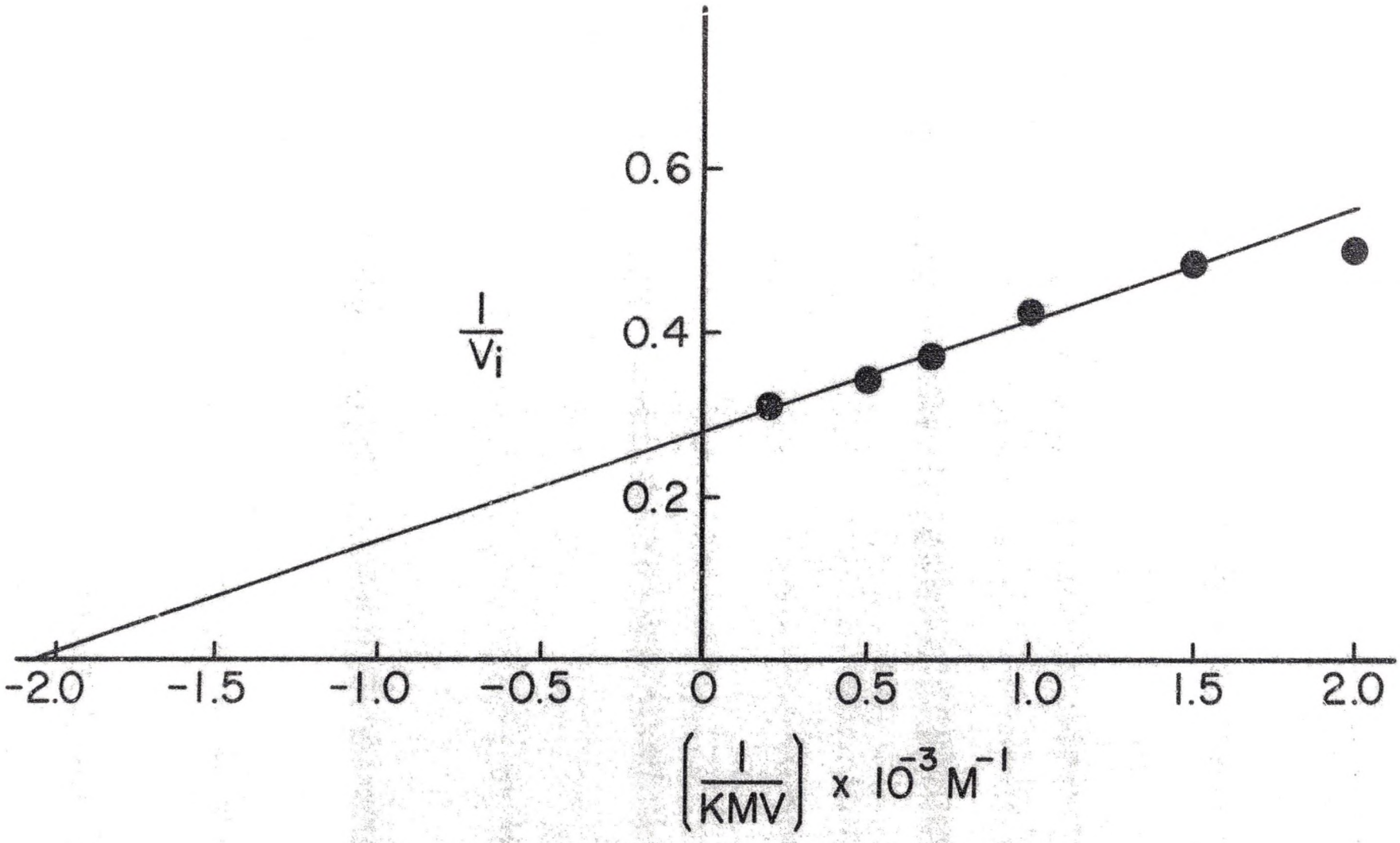

0.6
0.4
0.2
$\frac{1}{V_i}$
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
2.0
$\left(\frac{1}{KMV}\right) \times 10^{-3} M^{-1}$

Fig. 22. -- Effect of varied α-ketoisocaproate concentrations on the rate of CO_2 production by bovine liver mitochondria

Lineweaver-Burk plot of the activity of KIC dehydrogenase of bovine liver mitochondria as a function of substrate (KIC) concentration (v_i, nanomoles of CO_2 produced per minute per mg of protein). Each reaction mixture contained 0.1 ml of a washed preparation of mitochondria and the indicated quantities of 1-^{14}C-KIC (3500 cpm per μmole). All other experimental conditions, reaction components and assay procedures were as stated for Figure 20.

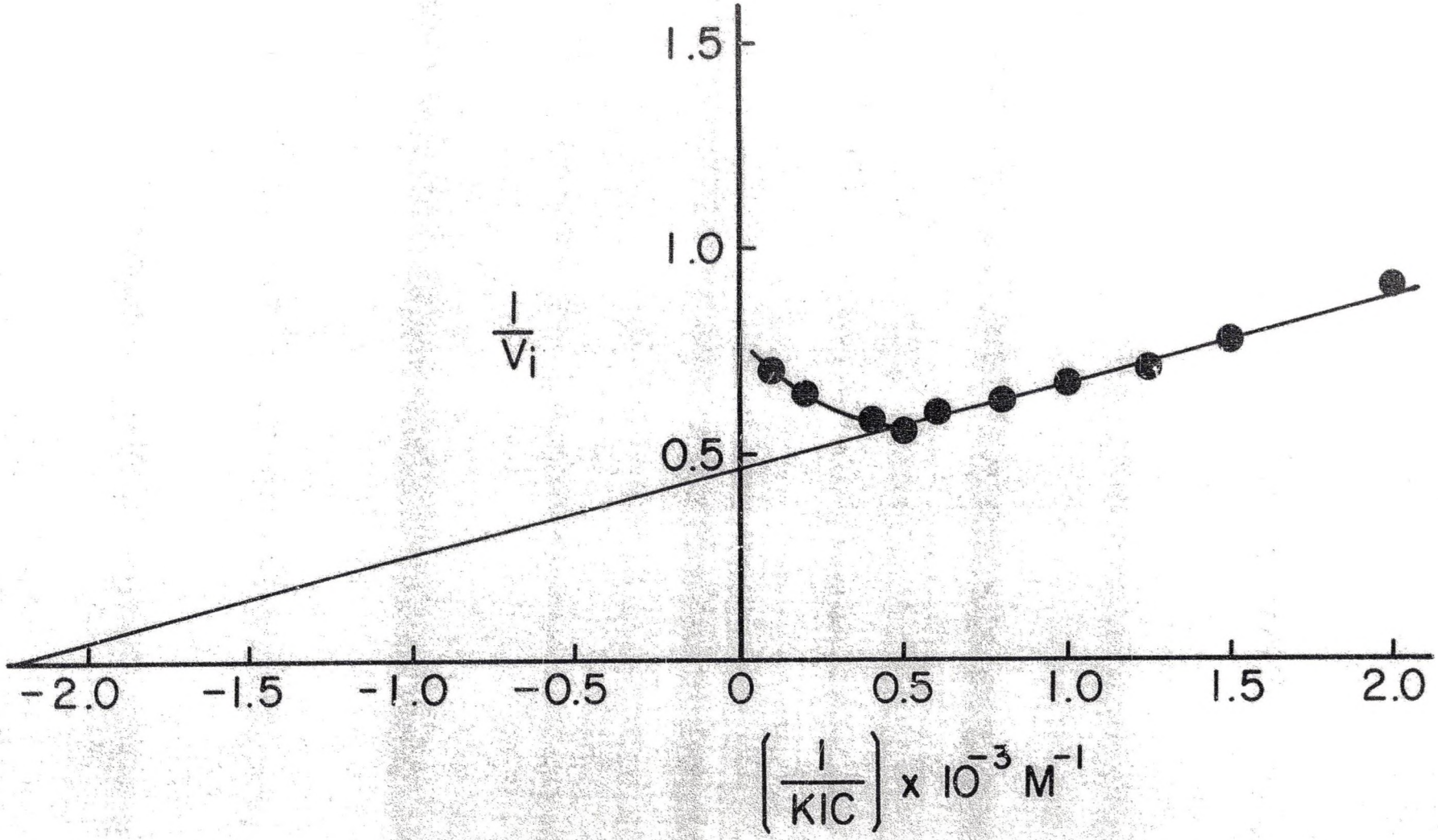

1.5
1.0
0.5
$\frac{1}{V_i}$
-2.0
-1.5
-1.0
-0.5
0
0.5
1.0
1.5
2.0
$\left(\frac{1}{KIC}\right) \times 10^{-3}\ M^{-1}$

Fig. 23. -- Effect of varied α-ketoglutarate concentration on the rate of formation of CO_2 by bovine liver mitochondria

Lineweaver-Burk and Michaelis-Menten plots of the activity of α-ketoglutarate dehydrogenase of bovine liver mitochondria as a function of substrate (KG) concentration (v_i, nanomoles of CO_2 produced per minute per mg of protein). Each reaction mixture contained 0.05 ml of a washed preparation of mitochondria and the indicated quantities of 1-^{14}C-KG (50,000 cpm per μmole). Reactions were allowed to proceed for 10 minutes at 30°C. All other experimental conditions, reaction components and assay procedures were as stated for Figure 20.

1.5
1.0
0.5
$\frac{1}{V_i}$
-10
-5
0
5
10
15
20
$\left(\frac{1}{\alpha\text{-KETOGLUTARATE}}\right) \times 10^{-3}\ M^{-1}$
V_i
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
1.0
α-KETOGLUTARATE x 10^3 M

or the effects of a translocase at the inner membrane, since this phenomenon was also observed when washed preparations of rat liver mitochondria served as the source of enzyme, but was not evident with the purified KG dehydrogenase from pig heart muscle. A K_m value of 0.08 mM KG was calculated from the data illustrated in Figure 23. The inverse plots shown in Figure 24 allow a K_m value of 0.22 mM for pyruvate to be computed. The apparent K_m values obtained from the average of several determinations are summarized in Table 4. Inhibition by KIC concentrations in excess of 2.0 mM was noted, while no substrate inhibition of the decarboxylase activity by concentrations of KIV to 10 mM was found. None of the other α-keto acids exhibited this characteristic (substrate inhibition) within the ranges of concentration utilized for these studies. The true Michaelis constants can not be determined easily for these reactions by the methods of Florini and Vestling (70), since at least three substrates (NAD^+, CoA, α-keto acid), and perhaps additional cofactors, participate in the tightly coupled enzyme reaction sequences of the α-keto acid dehydrogenase complexes.

Subcellular Distribution of Bovine Liver Branched-Chain α-Keto Acid Dehydrogenase Activities

Among the principal objectives of this entire study was the elucidation of the cellular localization of the branched-chain keto acid dehydrogenase activities. This was desirable for at least two reasons, first to obtain the highest specific enzyme activity fraction for the succeeding studies, and second, to accumulate additional information with respect to the number of

TABLE 4

K_m VALUES FOR SEVERAL α-KETO ACID DEHYDROGENASES OF BOVINE LIVER MITOCHONDRIA

α-Keto Acid Substrate[a]	K_m (moles liter^{-1})	
α-Ketoisovalerate	$3.3 \pm 0.4 \times 10^{-4}$	(5)[b]
α-Ketoisocaproate	$5.2 \pm 0.6 \times 10^{-4}$	(4)
α-Keto-β-methylvalerate	$4.7 \pm 0.8 \times 10^{-4}$	(4)
Pyruvate	$2.4 \pm 0.2 \times 10^{-4}$	(5)
α-Ketoglutarate	$0.81 \pm 0.09 \times 10^{-4}$	(4)

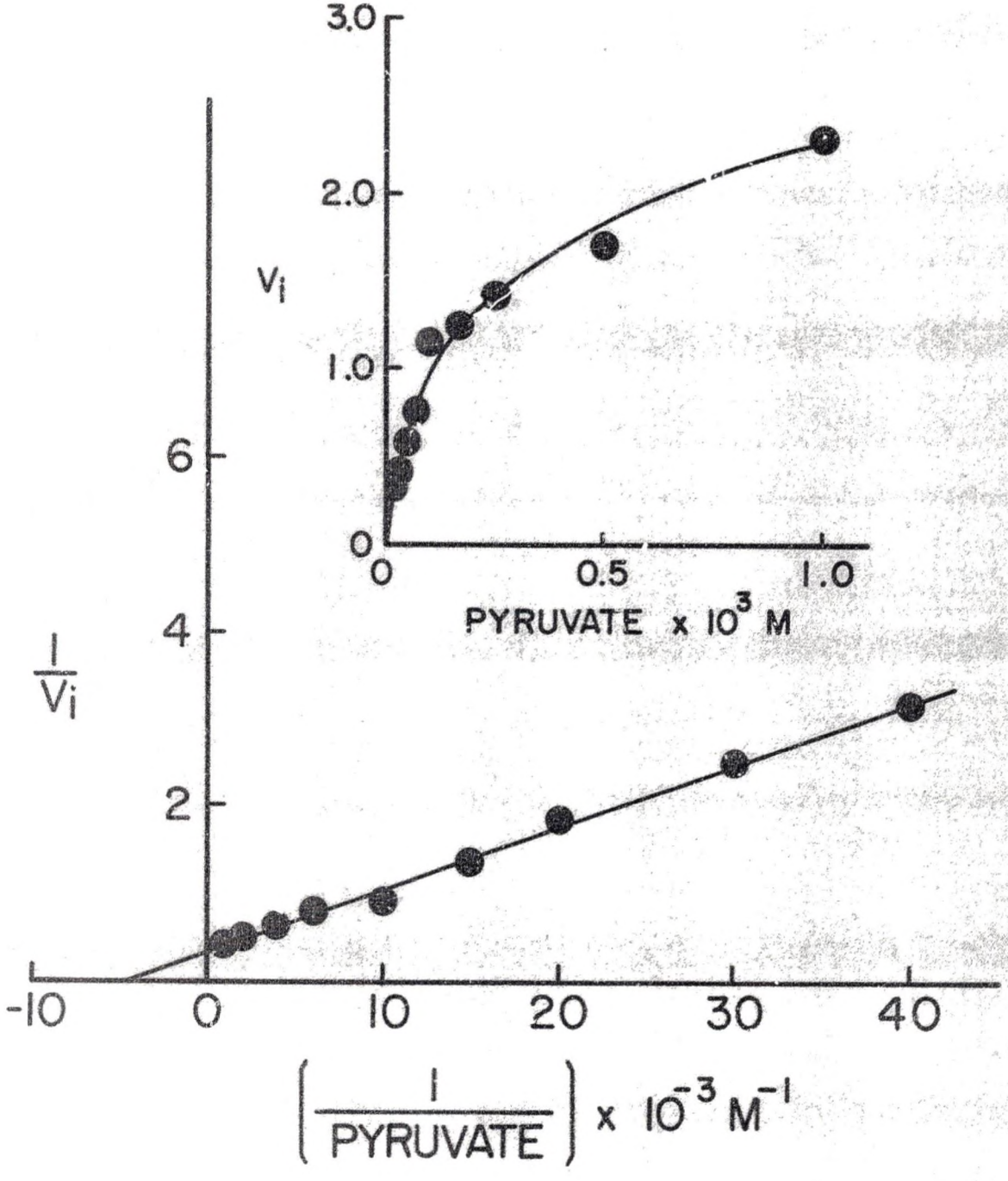
[a] Carboxyl-labeled ^{14}C-α-keto acids were used as substrates for the measurement of the enzyme activities by the $^{14}CO_2$ evolution assay as described in the text and in the Methods section.

[b] K_m values tabulated are the mean ± one standard deviation for the number of determinations indicated in parentheses.

Each reaction mixture contained the following in μmoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.05 or 0.1 ml of a washed preparation of bovine liver mitochondria, and appropriate amounts of carboxyl-labeled α-keto acids. Determinations were conducted at 30°C.

Fig. 24. -- Effect of varied pyruvate concentration on the rate of formation of CO_2 by bovine liver mitochondria

Lineweaver-Burk plot of the activity of pyruvate dehydrogenase of bovine liver mitochondria as a function of pyruvate concentration (v_i, nanomoles CO_2 produced per minute per mg of protein). Each reaction mixture contained 0.05 ml of a washed preparation of mitochondria and the indicated amounts of 1-^{14}C-Pyr (58,000 cpm per μmole). Reactions were allowed to proceed for 10 minutes at 30°C. All other experimental conditions, reaction components, and assay procedures were as stated for Figure 20.


3.0
2.0
1.0
0
V_i
0
0.5
1.0
PYRUVATE x 10^3 M
6
4
2
$\frac{1}{V_i}$
-10
0
10
20
30
40
$\left(\frac{1}{\text{PYRUVATE}}\right)$ x 10^{-3} M^{-1}

branched-chain keto acid dehydrogenase enzymes in the bovine liver cell. Figure 25 gives a general indication of the relative levels of KIC dehydrogenase activity in various bovine liver tissue preparations obtained by the centrifugation techniques described in the "Methods" section. Table 5 shows the distribution of each branched-chain α-keto acid dehydrogenase activity in these subcellular fractions. Succinate dehydrogenase (EC 1.3.99.1.) was employed as a mitochondrial marker enzyme (49), and glucose-6-phosphatase (EC 3.1.3.9.) served to identify the microsome fraction (51). The major portion of the α-keto acid dehydrogenase activity as measured by $^{14}CO_2$ production from carboxyl-labeled substrates was found in the fraction which contained the mitochondria. The origin of the smaller amounts of activity located in the supernatant (soluble) fraction is in doubt, since some of the characteristics of this activity appear to differ from those of the mitochondrial branched-chain α-keto acid dehydrogenase. The ratios of activity with KIV, KMV and KIC are different, and dissimilar coenzyme requirements were noted (see Table 2). These distinctive features will receive further comment in the "Discussion". Minor amounts of activity observed in the nuclear and microsome fractions likely result from some slight contamination with mitochondria.

Localization of α-Keto Acid Dehydrogenase Activities with Respect to the Mitochondrial Inner Membrane

Knowledge of the intra-mitochondrial location of the enzymes responsible for the catalysis of oxidative decarboxylation of KIC, KMV, KIV, pyruvate and α-ketoglutarate in bovine liver cells is of considerable general interest, and could contribute to an understanding

TABLE 5

SUBCELLULAR DISTRIBUTION OF BOVINE LIVER BRANCHED-CHAIN α-KETO ACID DEHYDROGENASE[b]

Fraction[a]	α-ketoisovalerate dehydrogenase		α-ketoisocaproate dehydrogenase		α-keto-β-methyl-valerate dehydrogenase		succinate dehydrogenase		glucose-6-phosphatase	
	% homogenate[c]	specific activity	% homogenate	specific activity	% homogenate	specific activity	% homogenate	specific activity	% homogenate	specific activity
Nuclear	8.0	0.24	6.1	0.16	9.3	0.21	8.7	0.56	N.D.*	N.D.
Mitochondria	88.2	5.54	86.8	2.98	88.7	3.51	84.6	3.60	0.72	0.002
Microsomes	2.1	0.05	0.8	0.02	0.9	0.02	5.3	0.18	83.2	0.212
Soluble	1.7	0.06	2.4	0.17	3.2	0.06	1.8	0.06	N.D.	N.D.
% recovery	95.3		98.9		99.3		100.4		N.D.	

*N.D. = not determined

[a] Subcellular fractions were prepared as described in the Methods section.

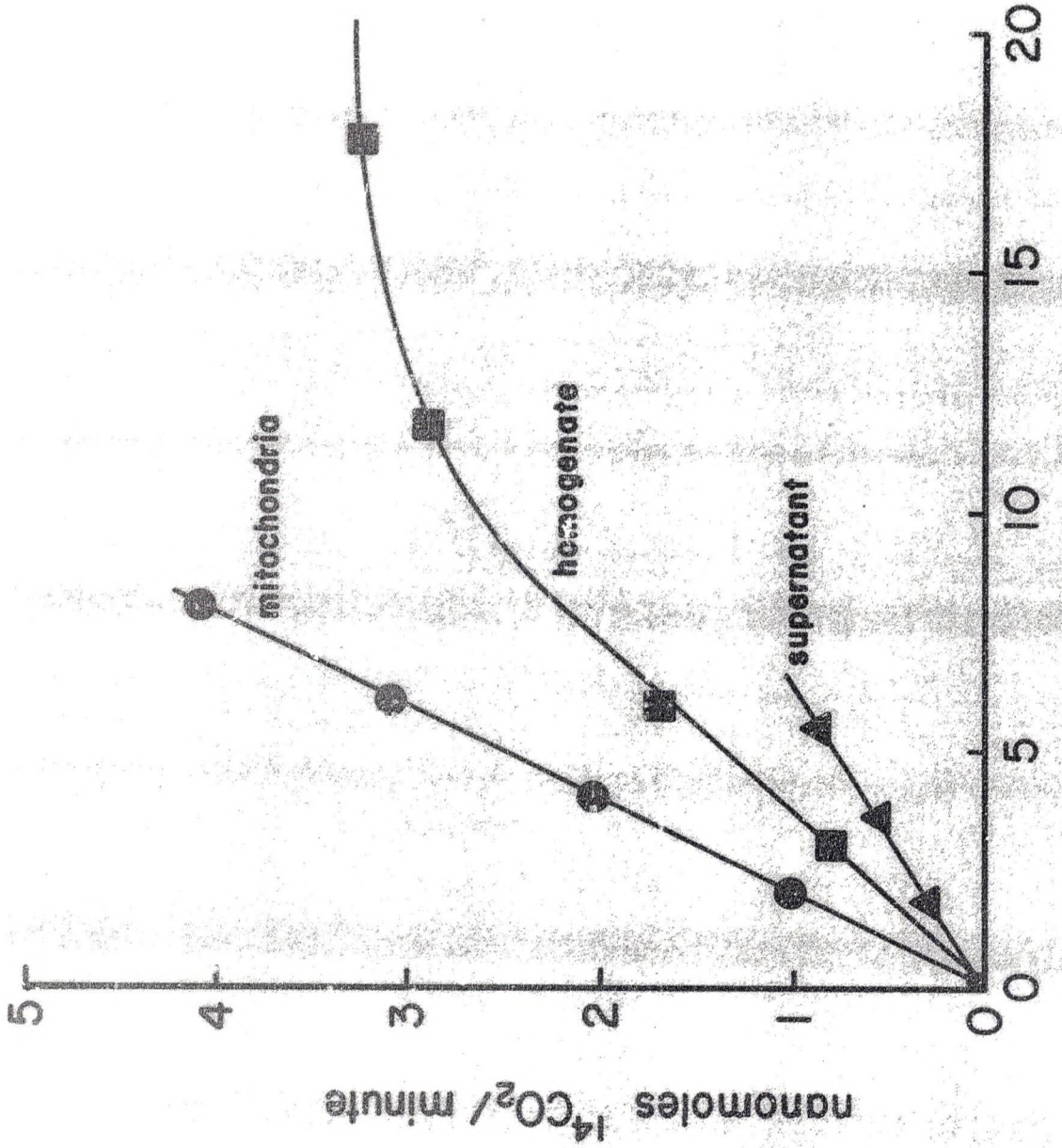
[b] α-Keto acid dehydrogenase activities were measured with the $^{14}CO_2$ (NAD, CoA) assay (see Methods). Succinate dehydrogenase activity was determined by the method of Bonner (49), and glucose-6-phosphatase by the modified method of Nordlie and Arion (51) (see also Methods).

[c] Per cent homogenate refers to the amount of activity present in a fraction compared to the total activity for that substrate in the whole homogenate.

[d] Digitonin solubilization treatment (57) (see also Methods) followed by assays for branched-chain α-keto dehydrogenase established the inner membrane-matrix as the location for these enzyme activities.

Fig. 25. -- Comparison of relative specific activity of KIC dehydrogenase in various bovine liver subcellular fractions

The amounts of $^{14}CO_2$ produced from 1-^{14}C-KIC during incubation with varying amounts of the indicated subcellular fractions of bovine liver was measured by the $^{14}CO_2$ assay as described in the Methods section. The fractions were prepared as stated in Methods. Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD^+, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; the indicated amounts of enzyme preparation, and 2.0 micromole of 1-^{14}C-KIC (3700 cpm per micromole). All reactions were allowed to proceed for 20 minutes at 30°C.


nanomoles $^{14}CO_2$/ minute
0
1
2
3
4
5
0
5
10
15
20
mg protein
mitochondria
homogenate
supernatant

of the relationships among these metabolites. Information about the spatial arrangement of these enzymes with respect to the inner membrane would provide some clarification of the question of whether the α-keto acid substrates must penetrate to the matrix prior to oxidation (see footnote d on Table 5). It is generally accepted that the outer membrane of isolated mitochondria is freely permeable to substances of low molecular weight (71,72,73,74), in contrast to the apparent selective permeability toward small molecules and charged ions characteristically exhibited by the inner membrane (50,52,75). Nucleotide derivatives such as NAD^+ (52,71), CoA (76) and most substrates which permeate the intramembranal space (10,74) do not readily penetrate the inner membrane. Klingenberg (50) and Mitchell (75) have provided direct evidence that the artificial electron acceptor ferricyanide does not penetrate the intact inner membrane. This agent has subsequently been used by several investigators to determine whether an enzyme which can reduce ferricyanide in the presence of its substrate(s) is located on the outer side or on the matrix side of the inner membrane (see "Methods" for a further discussion of this technique). A major site of ferricyanide reduction by mitochondrial preparations is at cytochrome c (50). Antimycin A, an inhibitor of respiration, is known to block electron transport between reductants inside the mitochondria through ubiquinone and cytochrome c to the non-penetrant ferricyanide which acts as electron acceptor (52). The "ferricyanide method" of enzyme localization is therefore based upon the inability of ferricyanide to react directly with those

enzymes facing toward the matrix or located in the matrical mileu. The relative ability of a substrate to reduce ferricyanide in the presence or absence of antimycin A will thus provide information pertaining to the intra-organelle location of the enzyme active site. The compartmentation of several intramitochondrial enzymes has been determined with this technique, among which are the α-glycerophosphate dehydrogenase of rat liver and locust flight muscle mitochondria (50) and the NADH dehydrogenase of yeast mitochondria (71). Figure 26 contains representations of actual spectrophotometer recordings of the rate of reduction of ferricyanide, as measured by the changes in absorbancy at 420 nm in the presence of a number of substrates. The results in this figure and in Table 6 show that antimycin A considerably decreases the transfer of electrons to ferricyanide cytochrome c with succinate or α-ketoglutarate as substrate, and has some inhibitory effect upon reduction with pyruvate. Antimycin A causes insignificant diminution of the rate of ferricyanide reduction with α-glycerophosphate, KIC, KMV or KIV as the oxidizable substrate. The results indicate that ferricyanide can apparently interact directly with the enzymes catalyzing oxidation of the branched-chain α-keto acids, an observation which locates the sites of these dehydrogenase activities on the outside of the inner membrane. Such a situation obviates the need for these compounds to cross the inner membrane for oxidation and appears to eliminate the need of a specific translocase mechanism for KIC, KIV and KMV. In contrast to these activities, the observation that reduction of ferricyanide

TABLE 6

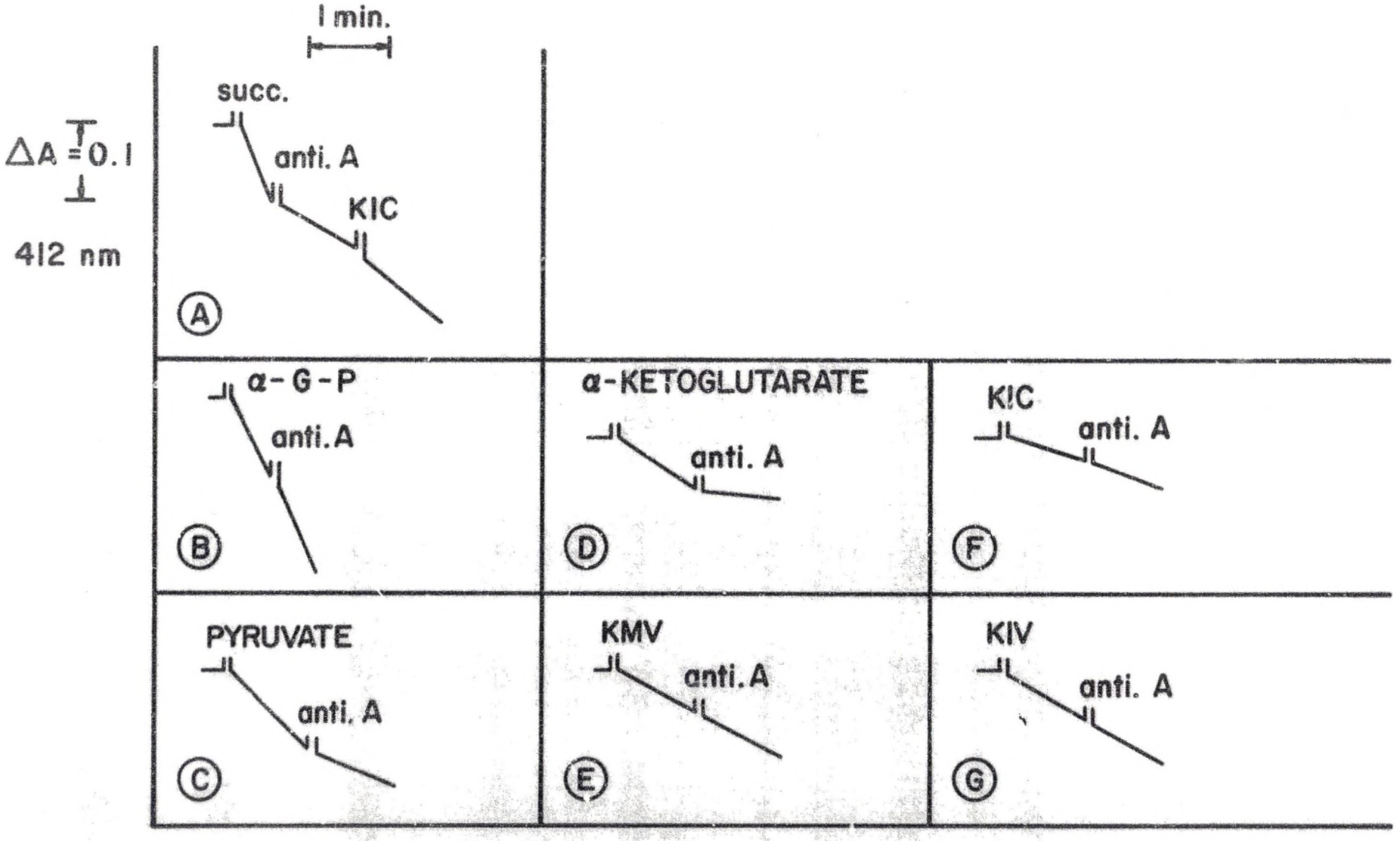
SENSITIVITY OF FERRICYANIDE REDUCTION TO ANTIMYCIN A

Substrate	(S), mM	enzyme activity[a]		
		w/o antimycin	+ antimycin A	per cent inhibition
α-Glycerol-P	5.7	0.20 ± 0.02	0.22 ± 0.04	0
Succinate	5.7	0.200 ± 0.003	0.006 ± 0.003	70
Pyruvate	5.7	0.100 ± 0.01	0.04 ± 0.01	60
α-Keto-glutarate	2.85	0.065 ± 0.01	0.010 ± 0.005	86
α-Ketoiso-caproate	7.1	0.03 ± 0.01	0.03 ± 0.01	0
α-Keto-β-methyl-valerate	5.7	0.05 ± 0.01	0.05 ± 0.01	0
α-Ketoiso-valerate	7.1	0.05 ± 0.01	0.048 ± 0.005	4

[a] Enzyme activities were estimated by spectrophotometeric measurement of ferricyanide reduction in the presence of substrate, at 420 nm on a Cary 15 recording spectrophotometer, as described in detail in the Methods section. Antimycin A (2 μg/ml) was added to assay mixtures where indicated.

Fig. 26. -- The accessibility of ferricyanide to several α-keto acid dehydrogenases compared with its accessibility to succinate dehydrogenase and α-glycerol-phosphate dehydrogenase

Spectrophotometric recording of ferricyanide reduction at 420 nm catalyzed by bovine liver mitochondria with the indicated substrates was measured as described in the Methods section. Each reaction mixture contained in a final volume of 3.5 ml, the following (in micromoles): mannitol, 525; potassium phosphate buffer, pH 7.2, 75; ATP, 3.0; EDTA, 3.5; $MgSO_4$, 10; TPP, 2.5; $K_3Fe(CN)_6$, 1.67; 0.1 ml of a washed preparation of bovine liver mitochondria, and where indicated, succinate, 20 micromoles; α-glycerophosphate, 20 μmoles; KIV, 25 μmoles; KMV, 20 μmoles; KIV, 25 μmoles; KG, 10 μmoles; or pyruvate, 20 micromoles. Enzyme activities are calculated as described in the Methods section.

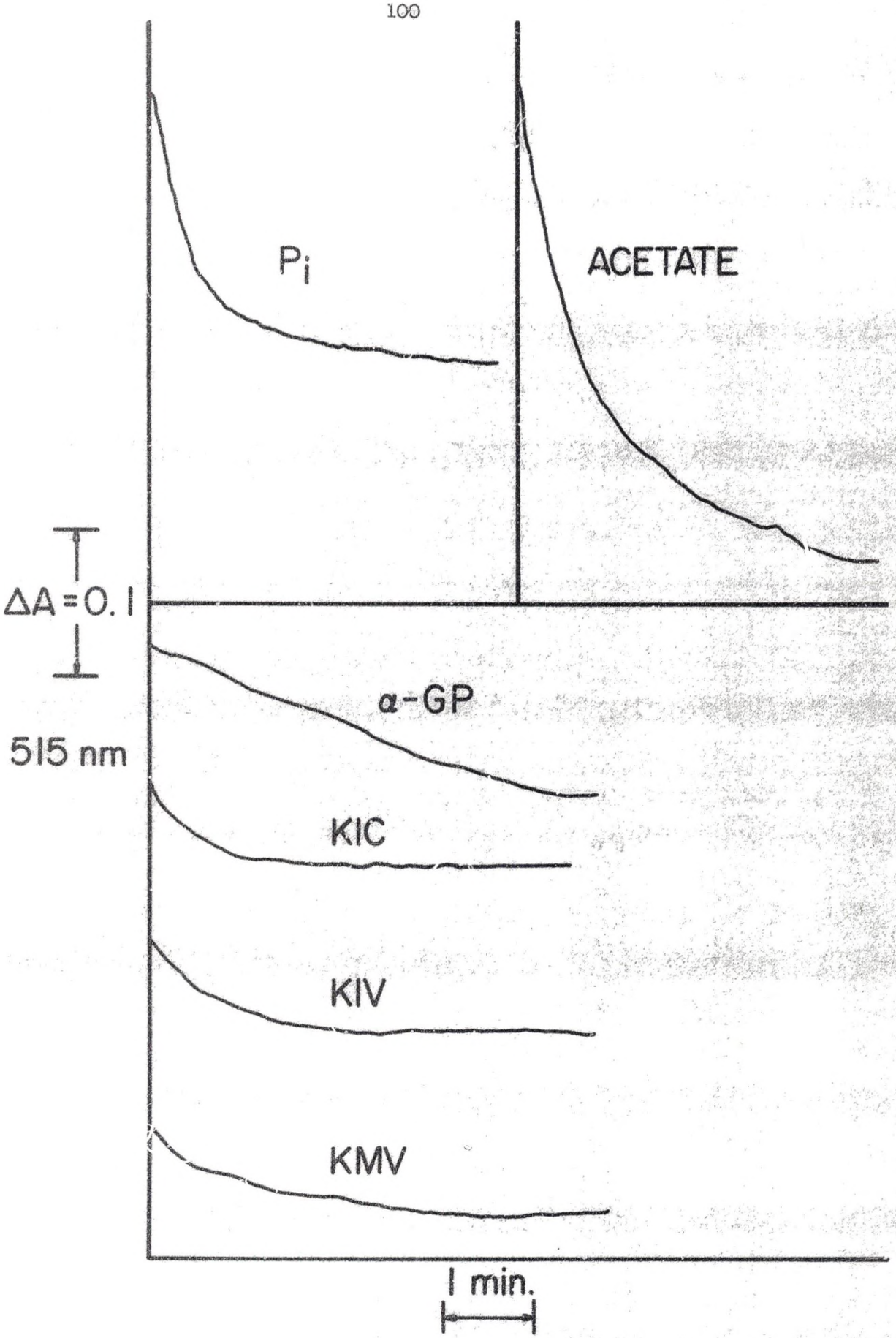

1 min.
ΔA = 0.1
412 nm
succ.
anti. A
KIC
A
α-G-P
anti. A
B
α-KETOGLUTARATE
anti. A
D
KIC
anti. A
F
PYRUVATE
anti. A
C
KMV
anti. A
E
KIV
anti. A
G

in the presence of succinate or α-ketoglutarate is severely limited by added antimycin A is in agreement with earlier evidence supporting the supposition that the dehydrogenase enzymes for these compounds are located on the inside face of the inner membrane or in the matrix (77). The observed ability of antimycin A to partially inhibit the reduction of ferricyanide by pyruvate implies that the pyruvate dehydrogenase complex also faces the inner surface of the inner membrane. The results with α-glycerol phosphate agree with those obtained by Klingenberg (50), who postulated that the mitochondrial α-glycerolphosphate dehydrogenase faces the intermembranal space.

The relatively slow rate of penetration of the inner membrane by the branched-chain α-keto acids is also inferred indirectly from the recordings of the spectrophotometric absorbance of a suspension of mitochondria in the presence of high concentration of the sodium salt of the acid and a permeant cation (e.g. NH_4^+) as an indicator of osmotic swelling (73). (See Methods for further theoretical and experimental detail). Figure 27 shows that the inner membrane of bovine liver mitochondria is apparently less permeable to the branched-chain α-keto acids (KIC, KMV, KIV) and α-glycerolphosphate than toward acetate or inorganic phosphate. The apparent existence of a permeability barrier to these α-keto acids is reminiscent of that previously reported for α-ketoglutarate with rat liver mitochondria (77,78,79). These data also confirm those of Klingenberg (50) for the low rate of permeation of α-GP through the inner membrane of rat liver mitochondria.

Fig. 27. -- Osmotic response of bovine liver mitochondria to α-keto acids and other permeant anions

Turbidity was recorded after addition of mitochondria to an isosmotic solution of various anions. A decrease of absorption indicates swelling of mitochondria due to the uptake of anions and NH_4^+. Addition of mitochondria (0.5 mg protein per ml) to a mixture of the following (in micromoles): triethanolamine, pH 7.2, 15; NH_4Cl, 75; and KIC, KMV, KIV, α-glycerol-P, sodium phosphate or sodium acetate, 75; in a final volume of 1.5 ml. All reagents were adjusted to pH 7.2 and 25°C prior to use. Other experimental conditions and assay procedures were as described in the Methods.


Pi
ACETATE
ΔA=0.1
515 nm
α-GP
KIC
KIV
KMV
1 min.

Investigation into the Number of Branched-Chain α-Keto Acid Dehydrogenases in Bovine Liver Mitochondria

The ideal approach to the question of enzyme specificity is to isolate the enzyme, which then can be characterized with respect to its ability to utilize a variety of substrates. Attempts to purify the branched-chain α-keto acid dehydrogenases from mammalian liver have been largely unsuccessful (11,13). A considerable number of physical and chemical procedures were utilized, with generally undesirable effects on the enzyme activities (11,13). Connelly, et al. (79) have reported a partial purification of a semi-specific enzyme from bovine liver supernatant fraction which was able to decarboxylate KMV and KIC. However, this preparation appeared to be unstable to dilution, and thus was unsuitable for extensive study.

Dixon and Webb (81) present a kinetic approach, which can be used when the physical separation of two or more enzyme activities is not possible, useful for analysis of the question of whether one or more dehydrogenases catalyze the oxidative decarboxylation of the three branched-chain α-keto acids KIC, KMV and KIV. According to the relationship shown in Equation 10 (below), if one enzyme is involved in a semi-specific conversion of two substrates, the observed rate of activity is dependent upon the affinity of the enzyme for each of these substrates and thus upon the degree of competition between these substrates for the common active center. In this case, if A and B are the substrates, the rate of conversion of A (v_a) in the presence of B can be expressed by the equation;

$$v_a = \frac{V_a}{1 + \frac{K_a}{[A]}\left(1 + \frac{[B]}{K_b}\right)} \qquad (8)$$

and the rate of conversion of B (V_b) in the presence of A is expressed by;

$$v_b = \frac{V_b}{1 + \frac{K_b}{[B]}\left(1 + \frac{[A]}{K_a}\right)}. \qquad (9)$$

The total velocity (v_t) of the reaction of the enzyme with varying amounts of the substrates A and B may be denoted by the sum of equations eight and nine.

$$v_t = v_a + v_b = \frac{V_a \frac{[A]}{K_a} + V_b \frac{[B]}{K_a}}{1 + \frac{[A]}{K_a} + \frac{[B]}{K_b}} \qquad (10)$$

K_a and K_b refer to the respective Michaelis constants for A and B, while V_a and V_b are the maximal velocities extrapolated at infinite substrate concentration. Equation 10 can be expressed in terms of the relative concentrations, $\alpha = A/K_a$, and $\beta = B/K_b$, in the following manner if the two substrates are being attacked by the same enzyme:

$$v_t = \frac{v_a (1 + \alpha) + v_b (1 + \beta)}{1 + \alpha + \beta} \qquad (11)$$

This equation is useful to calculate the theoretical total velocity (v_t) from a knowledge of the initial velocities (v_a and v_b) and the K_m value for each substrate, without having to compute the maximum

velocity for each instance. The results of a comparison of the total velocities in the presence of each possible paired combination of the keto acids, KIV, KIC, KMV, KG and Pyr are presented in Table 7. The specific enzyme activity (v_t, observed) was determined for each keto acid separately at two levels of substrate, and with the indicated substrate combinations. The columns headed $v_a + v_b$ represent the summations of the individual keto acid activities when present separately in the reaction mixture. This additive effect would be expected to result from the influence of two separate enzymes catalyzing the conversion of the two substrates, and no mutual inhibition. The middle column under each substrate concentration heading (v_t, calculated) gives the values calculated with the aid of Equation 11. Thus, agreement between the observed (v_t, observed) and the calculated (v_t, calculated) total velocities is considered to be indicative of the participation of a single enzyme. The only exception to this pattern could occur for two enzymes E_a and E_b, where each is inhibited by the substrate of the other enzymes, and then only in the incidental case where K_{m_a} for E_a is equal to K_{i_a} for E_b and K_{m_b} for E_b is equivalent to K_{i_b} for E_a.

The results of these combined substrate experiments, utilizing the predetermined K_m value for each keto acid and initial velocity measurements for the substrates separately and in combination, suggest the existence of a single enzyme complex catalyzing the oxidative decarboxylation of KIC, KMV and KIV. The data with bovine liver mitochondria also indicate that pyruvate and α-keto-glutarate dehydrogenase activities are separate from those of the

TABLE 7

RATES OF DECARBOXYLATION OF KIV, KIC, KMV, Pyr AND α-KG INDIVIDUALLY AND COMBINED[a]

Substrate	specific activity					
	(substrate)$_1$[b]			(substrate)$_2$		
	v_t observed	v_t[c] calculated	$v_a + v_b$	observed	calculated	$v_a + v_b$
KIV	4.38			5.55		
KIC	3.08			4.39		
KMV	3.57			4.66		
KG	0.88			1.96		
Pyr	2.43			4.01		
KIV + KIC	5.26	5.39	7.46	6.16	6.39	9.94
KIV + KMV	5.32	5.75	7.95	5.86	6.59	10.21
KIC + KMV	4.03	4.90	6.65	5.42	5.93	9.05
KIC + Pyr	4.86	3.87	5.51	6.93	5.28	8.40
KIV + Pyr	6.21	4.69	6.21	7.42	5.89	9.56
KMV + Pyr	5.83	4.20	6.00	8.11	5.45	8.67
KIV + KG	4.97	3.82	5.26	7.04	4.88	7.51
KIC + KG	3.64	2.85	3.96	5.88	4.07	6.35

TABLE 7 -- Continued

Substrate	specific activity					
	(substrate)$_1$ [b]			(substrate)$_2$		
	v_t observed	v_t [c] calculated	$v_a + v_b$	v_t observed	v_t [c] calculated	$v_a + v_b$
KMV + KG	4.39	3.19	4.45	6.13	4.23	6.62
KG + Pyr	3.17	2.39	3.31	5.49	3.86	5.97

[a] α-Keto acid dehydrogenase activities were measured by quantitation of the $^{14}CO_2$ produced from carboxyl-labeled α-keto acids in the presence of NAD and CoA (see Methods for assay). Each assay mixture contained the following, in μmoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.05 or 0.1 ml of a washed preparation of bovine liver mitochondria, and the indicated quantities of carboxyl-labeled α-keto acids adjusted to equivalent specific activities, in a final volume of 1.0 ml. All incubations were conducted at 30°C.

[b] Two combinations of α-keto acid concentrations were used. (substrate)$_1$ values for each keto acid, in μmoles: KIV, 0.25; KIC, 0.25; KMV, 0.25; KG, 0.05; Pyr, 0.10. (substrate)$_2$ values, in μmoles: KIV, 0.50; KIC, 0.50; KMV, 0.50; KG, 0.10; Pyr, 0.30.

[c] Competitive interaction among substrates was assumed for these calculations. This assumption is only partially correct for those combinations in which α-ketoglutarate and a branched-chain α-keto acids were paired.

branched-chain keto acid dehydrogenase, which is consistent with the specificity studies on the purified pyruvate and α-ketoglutarate dehydrogenase complexes from pig heart (38,63), E. coli (64) and bovine kidney mitochondria (82).

The assumption of a single dehydrogenase with equal affinities for each substrate[1] predicts that "cold" (non-radioactive) keto acid (inhibitor) in equal concentration with labeled α-keto acid (substrate) will result in 50 per cent observed inhibition, based upon measurement of $^{14}CO_2$ evolved. Production of $^{14}CO_2$ will be inhibited to the extent of seventy-five per cent when the ratio of "cold" to radioactive keto acid is three to one. Each of the branched-chain α-keto acids inhibited the in vitro oxidation of the other two acids (Table 8). The pattern of these inhibitory effects approximates the general hypothesis of one catalytic site for all pairs of the branched-chain α-keto acids. The rate of oxidation of pyruvate was substantially reduced by α-ketobutyrate, a compound which also inhibited decarboxylation of the branched-chain acids, but to a lesser degree. α-Ketocaproate and α-ketovalerate, straight chain analogues of the branched-chain keto acids, KIC and KIV, were able to inhibit significantly the $^{14}CO_2$ production from the carboxyl-labeled keto acids. A more extensive and quantitative study of mutual inhibition patterns is presented in a later section.

Parallel responses to various physical perturbing techniques is sometimes used as supporting evidence for the existence of a single enzyme, while differential effects of identical treatment

[1] Michaelis constants were similar, but not identical for KIC, KMV and KIV (see Table 4).

TABLE 8

INHIBITION OF α-KETO ACID DEHYDROGENASES BY α-KETO ACIDS[a]

Inhibitor	carboxyl-^{14}C-labeled α-keto acid, (S) = 1 mM				
	KIV	KIC	KMV	KG	Pyr
	% inhibition				
^{12}C-α-Ketoisovalerate					
1.0 mM--------		44	54	24	33
3.0 mM--------		76	71	43	39
^{12}C-α-Ketoisocaproate					
1.0 mM--------	56		53	20	36
3.0 mM--------	78		74	32	42
^{12}C-α-Keto-β-methyl-valerate					
1.0 mM--------	52	56		24	22
3.0 mM--------	79	77		34	31
^{12}C-α-Ketobutyrate					
1.0 mM--------	29	26	27		47
3.0 mM--------					69
^{12}C-α-Ketocaproate					
1.0 mM--------	66	73			
3.0 mM--------		84			
^{12}C-α-Ketovalerate					
1.0 mM--------	67				
3.0 mM--------	92				

[a] α-Keto acid dehydrogenase activities were measured by quantitation of the $^{14}CO_2$ produced when ^{14}-carboxyl-labeled α-keto acids were incubated at 30°C with washed preparations of bovine liver mitochondria. 1-^{12}C-α-keto acids were used as inhibitors. Each reaction mixture contained, in a final volume of 1.0 ml, in μmoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.05 ml of enzyme preparation and the indicated amounts of α-keto acid substrate and inhibitor.

upon two activities may indicate separate enzymes. Figure 28 illustrates the nearly parallel pattern of variation of the branched-chain α-keto acid dehydrogenase activities during storage of isolated mitochondria at 4°C. All three activities (KIC, KMV, KIV) increased during the first 24 hours, followed by a gradual decrease in the succeeding 72 hours. The slightly different response of KIV dehydrogenase activity as compared to KMV and KIC activities, could be a result of experimental variance, or may suggest the possibility of multiple enzymes. α-Ketoglutarate dehydrogenase activity, measured concurrently, did not vary significantly over the entire 96 hour interval.

The data of Figure 29 show that KIC dehydrogenase and pyruvate dehydrogenase respond differently to preincubation of isolated mitochondria for varied time intervals at 45°C. KIC dehydrogenase activity appears to increase, relative to the initial measurement, subsequent to a short exposure to the 45°C temperature. Longer periods of incubation at this temperature resulted in rapid inactivation of KIC dehydrogenase, while pyruvate dehydrogenase activity showed a steady decrease for all incubation intervals. The response of KMV and KIV dehydrogenase activities closely mimics that observed for KIC. Danner (83) had previously noted a similar response with KIC dehydrogenase of rat liver mitochondria.

When washed preparations of bovine liver mitochondria were incubated with various concentrations of sodium arsenite the branched-chain α-keto acid dehydrogenase activities declined at nearly identical rates (Figure 30). Since arsenite is known to form

Fig. 28. -- Effect of incubation at 4°C on α-keto acid dehydrogenase activities

Bovine liver mitochondria (25 mg/ml) were stored at 4°C for the indicated time periods. The mitochondria were prepared as described in the Methods. α-Keto acid dehydrogenase activities were measured by the ferricyanide reduction assay (see Methods section for procedure) at each of the stated time periods. One-tenth ml of mitochondria was used for each determination. Activity (in micromoles per minute) was calculated as described in the Methods.

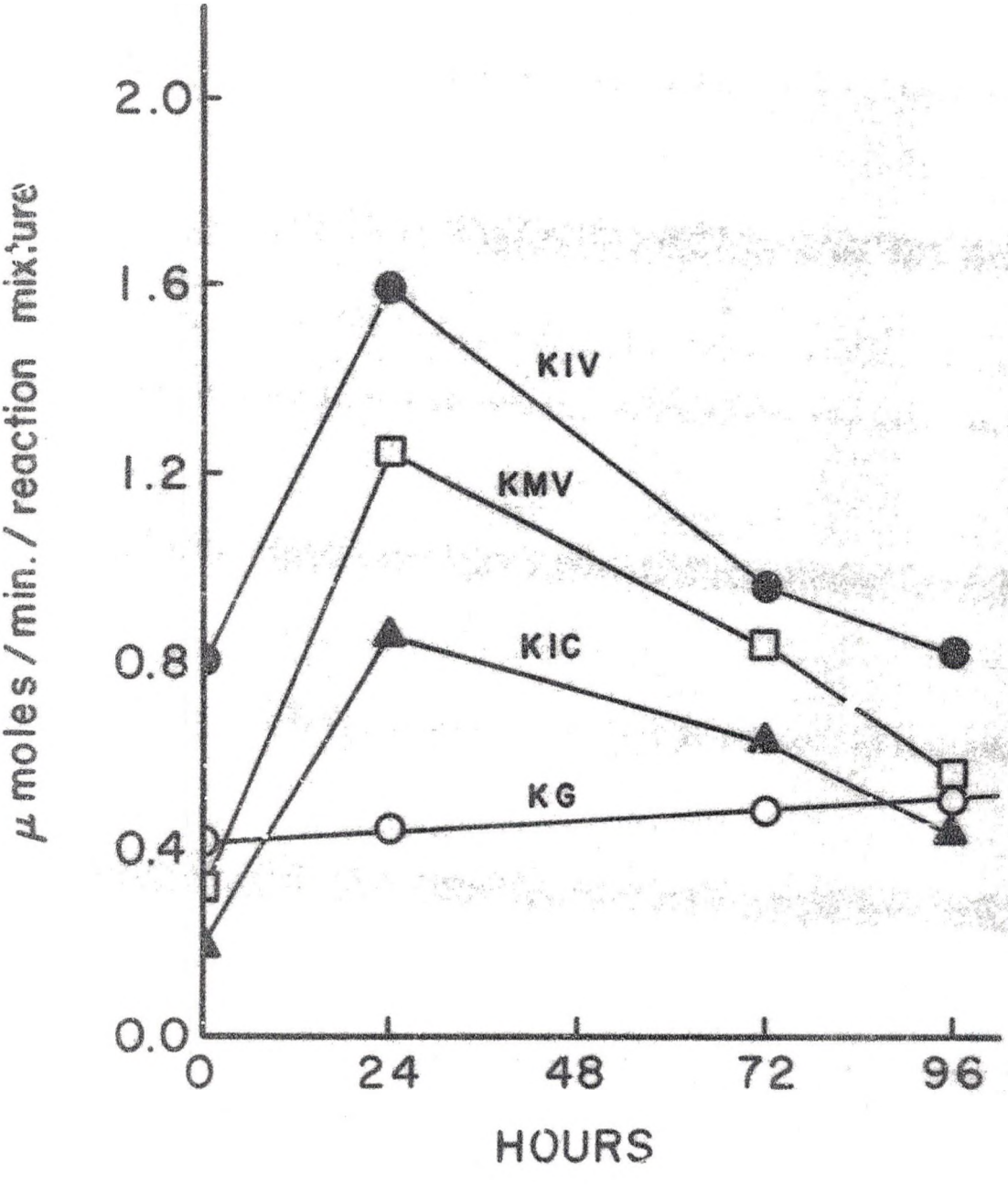

μ moles / min. / reaction mixture
2.0
1.6
1.2
0.8
0.4
0.0
KIV
KMV
KIC
KG
0
24
48
72
96
HOURS

Fig. 29. -- Inactivation of α-keto acid dehydrogenases with time of incubation at 45°C

Washed liver mitochondria were suspended in 0.30 M mannitol at a concentration of 23 mg of protein per ml. The suspension was incubated in a 45°C bath and 0.1 ml samples withdrawn and assayed immediately for dehydrogenase activity with KIC (○-○) and Pyr (✕—✕) (see text or Methods for details). Zero time assays and blanks were in triplicate, other points in duplicate. Activities are expressed as percentage of activity in samples not incubated at 45°C. The $^{14}CO_2$ assay method was utilized. Each reaction mixture contained the following in a final volume of 1.0 ml (in μmoles) mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; and either 1-^{14}C-KIC (3700 cpm per μmole), 2.0; of 1-^{14}C-Pyr (58,700 cpm per μmole), 1.0.

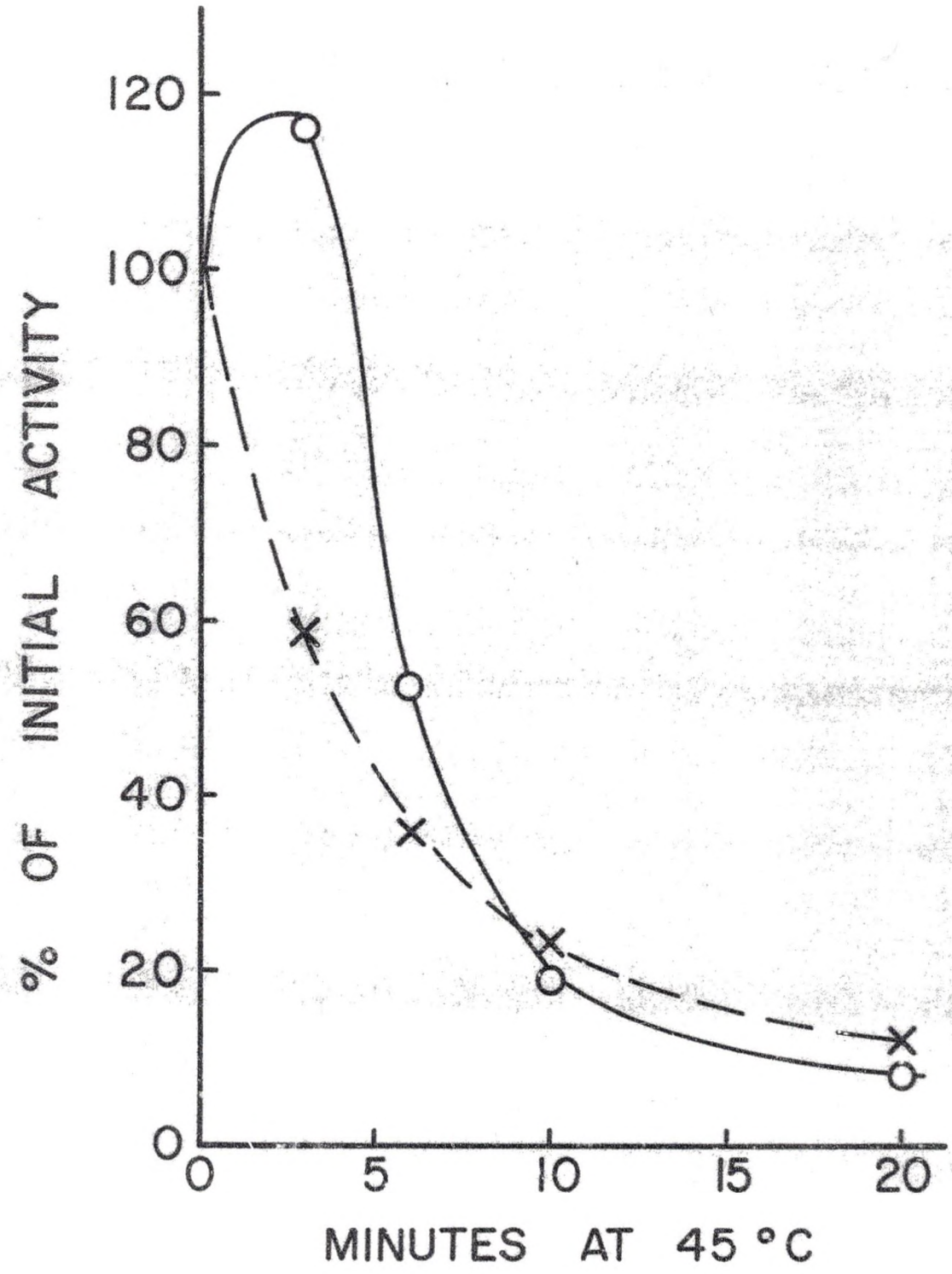
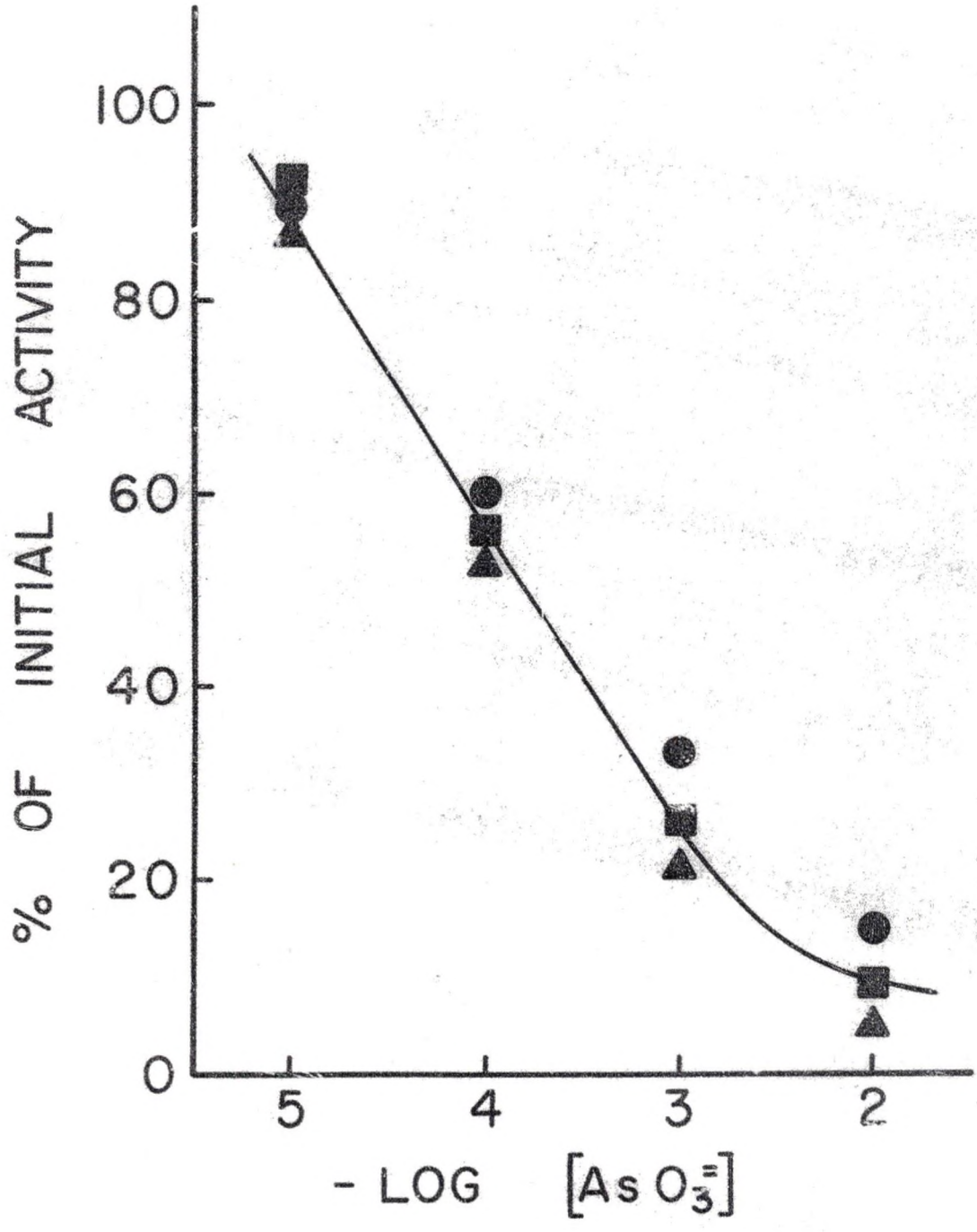

% OF INITIAL ACTIVITY
120
100
80
60
40
20
0
0
5
10
15
20
MINUTES AT 45 °C

Fig. 30. -- Inhibition of branched-chain α-keto acid oxidative decarboxylation by arsenite

Plots of per cent of original activity (in absence of arsenite) vs. arsenite concentration. A diluted suspension of fresh mitochondria (1.25 mg protein/ml of suspension) was incubated for 10 minutes at 25°C in the presence of the indicated concentration of sodium arsenite. Branched-chain α-keto acid dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained 0.1 ml of the treated or the untreated mitochondrial suspension and 2.0 μmoles of 1-^{14}C-keto acid; (● , KMV), (■ , KIC), (▲ , KIV). Reactions were allowed to proceed for 10 minutes at 30°C. All other components of the basic reaction mixture were the same as for Figure 28.


% OF INITIAL ACTIVITY
100
80
60
40
20
0
5
4
3
2
- LOG [As O3=]

mercaptides with thiol groups, these data imply the presence of a sulfhydryl group (or groups) functional in the oxidative decarboxylation of the branched-chain α-keto acids. A logical suggestion, in view of the proposed similarity of the oxidative pathways of these keto acids to those for pyruvate and α-keto-glutarate, would be that the observed inhibition by arsenite occurs through oxidation of the dithiol of reduced lipoic acid.

Mutual Inhibition In Vitro Among α-Keto Acid Substrates

A thorough investigation of the relative inhibitory interactions among several α-keto acids, including the branched-chain acids KIV, KIC and KMV, was initiated. Two major objectives were first to determine whether these interrelationships could constitute a possible physiological regulatory mechanism among the keto acids under conditions of "normal" α-keto acid levels in mammalian tissues, and second, to ascertain to what extent the in vitro metabolism of α-ketoglutarate and pyruvate is affected by the levels of KIV, KIC and KMV reported to occur in untreated branched-chain ketoaciduria.

α-Keto acid dehydrogenase activity was estimated by measurement of the amounts of radioactive $^{14}CO_2$ evolved from carboxyl-labeled α-keto acids as described under Methods. Non-radioactive α-keto acid was utilized as the inhibitor in each of the studies. Variation of substrate concentration at each of several inhibitor concentrations generated the values for the double reciprocal plots shown in Figures 31 and 32, which illustrate the apparent competitive nature of the inhibition by KMV and KIV of KIC oxidation. Reciprocal plots in Figures 33 and 34 illustrate that the oxidative

Fig. 31. -- Lineweaver-Burk plots of the inhibition of α-ketoisocaproate dehydrogenase activity by α-keto-β-methylvalerate

Lineweaver-Burk plots with 1-^{12}C-KMV as inhibitor and 1-^{14}C-KIC as varied substrate. (●——●) no inhibitor, (◪——■) 2.5×10^{-4} M KMV, (▲——▲) 5×10^{-4} M KMV, (○——○) 7.5×10^{-4} M KMV. KIC dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the following (in μmoles): mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; the indicated amounts of 1-^{14}C-KIC (3500 cpm per μmole) and inhibitor, and 0.1 ml of a washed preparation of bovine liver mitochondria in a final volume of 1.0 ml. All reactions were allowed to proceed for 10 minutes at 30°C. (v_i, nanomoles CO_2 per minute per mg of protein).

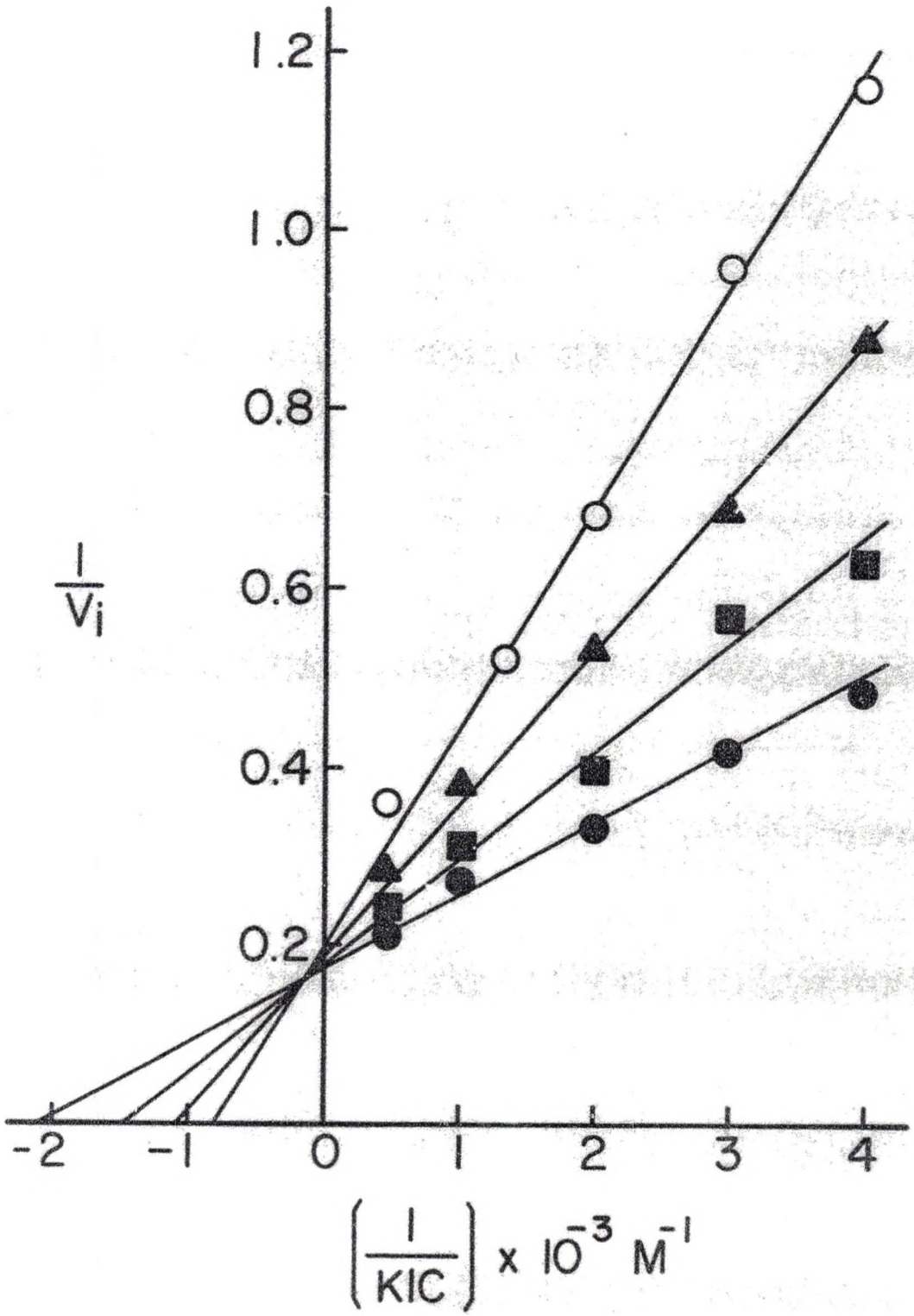

1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-2
-1
0
1
2
3
4
(1/KIC) x 10^-3 M^-1

Fig. 32. -- Lineweaver-Burk plots of the inhibition of α-ketoisocaproate dehydrogenase activity by α-ketoisovalerate

Lineweaver-Burk plots with 1-^{12}C-KIV as inhibitor and 1-^{14}C-KIC as varied substrate. (●——●) no inhibitor, (■——■) 2.5×10^{-4} M KIV, (▲——▲) 5×10^{-4} M KIV, (○——○) 7.5×10^{-4} M KIV. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria, and the indicated amounts of 1-^{14}C-KIC (3600 cpm per μmole) and inhibitor. All other experimental conditions, reaction mixture components and assay methods were as stated for Figure 31. (v_i, nanomoles CO_2 produced per minute per mg of protein).

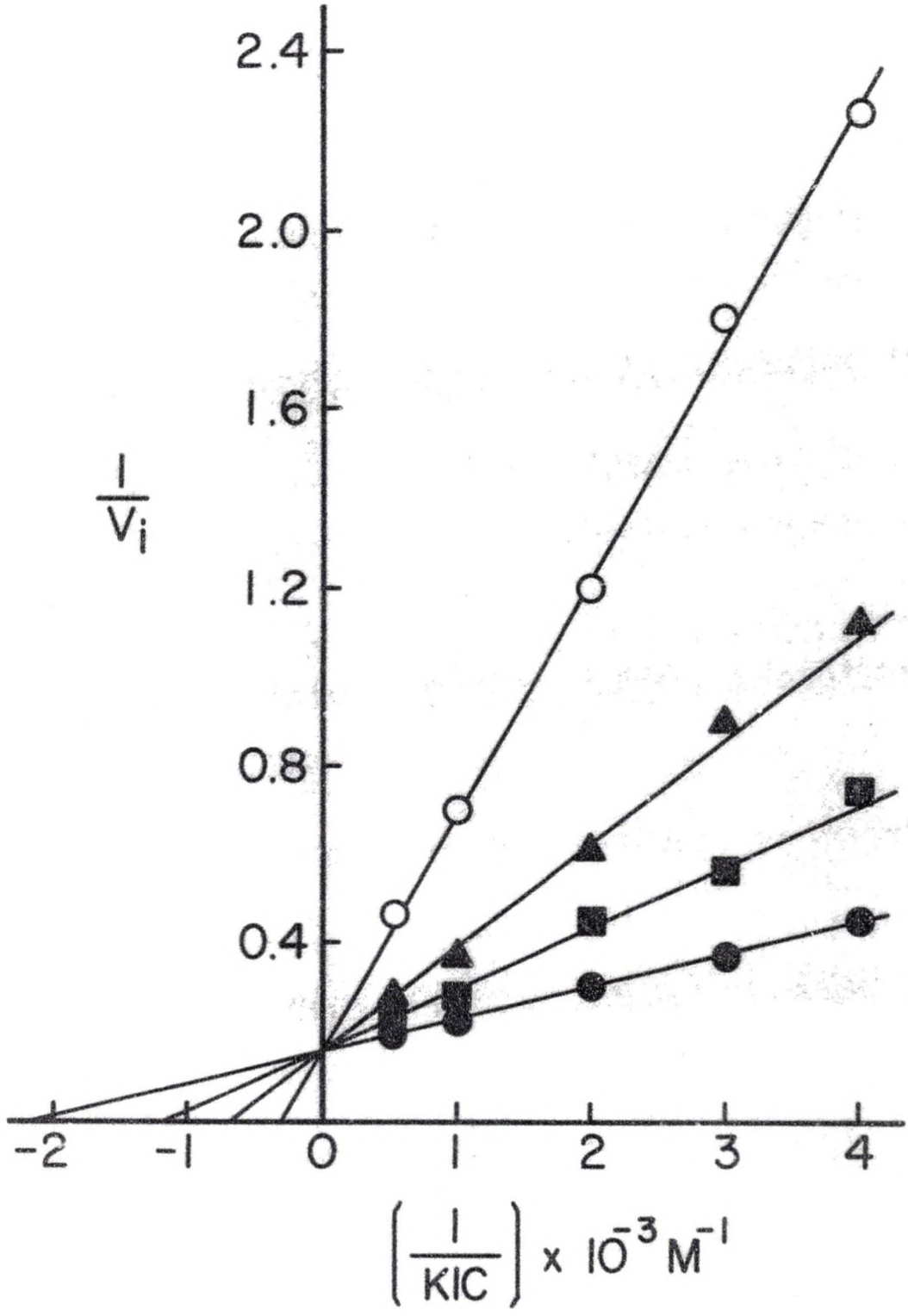

2.4
2.0
1.6
1.2
0.8
0.4
1/Vi
-2
-1
0
1
2
3
4
(1/KIC) x 10⁻³ M⁻¹

Fig. 33. -- Lineweaver-Burk plots of the inhibition of α-ketoisovalerate dehydrogenase activity by α-ketoisocaproate

Lineweaver-Burk plots with 1-^{12}C-KIC as inhibitor and 1-^{14}C-KIV as varied substrate. (●——●) no inhibitor, (■——■) 2.5×10^{-4} M KIC, (▲——▲) 5.0×10^{-4} M KIC, (○——○) 7.5×10^{-4} M KIC. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria and the indicated amounts of 1-^{14}C-KIV (3500 cpm per μmole) and inhibitor. All other experimental conditions, reaction mixture components and assay methods were as stated for Figure 31. (v_i, nanomoles of CO_2 produced per minute per mg of protein).

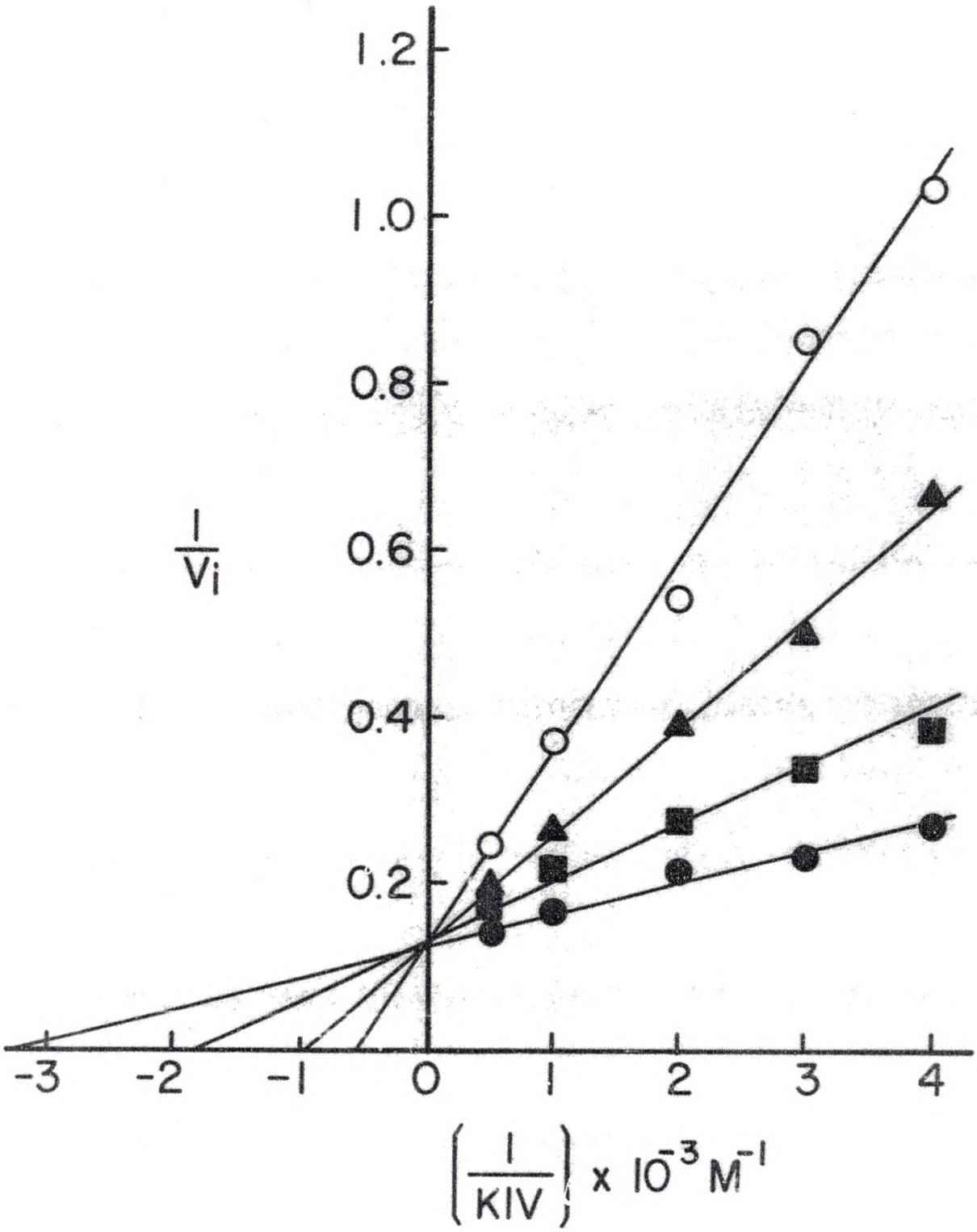
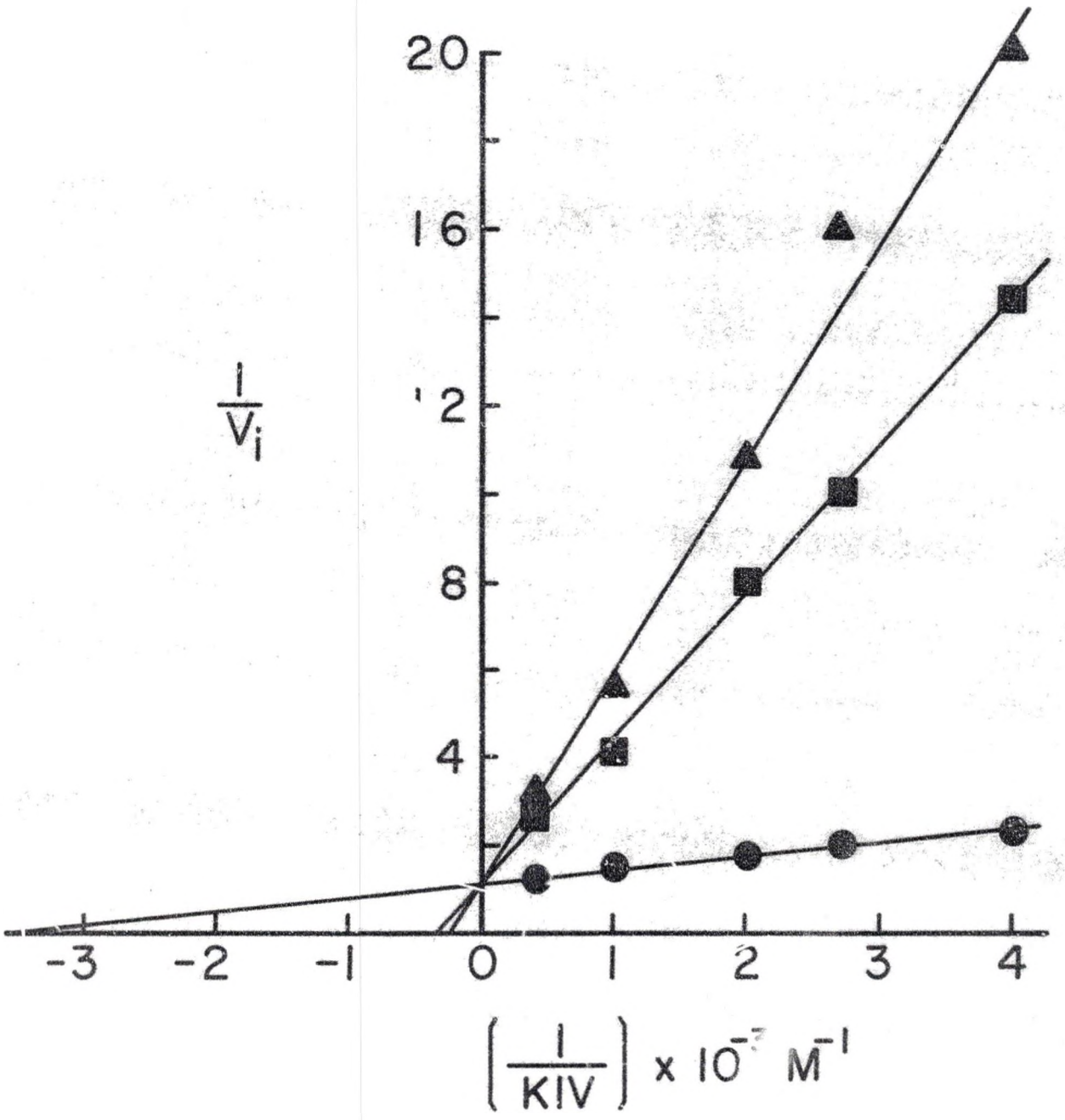

1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-3
-2
-1
0
1
2
3
4
(1/KIV) x 10⁻³ M⁻¹

Fig. 34. -- Lineweaver-Burk plots of the inhibition of α-ketoisovalerate dehydrogenase activity by α-keto-β-methylvalerate

Lineweaver-Burk plots with 1-^{12}C-KMV as inhibitor and 1-^{14}C-KIV as varied substrate. (●——●) no inhibitor, (■——■) 5×10^{-4}M KMV, (▲——▲) 1.0×10^{-3}M KMV. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria and the indicated amounts of 1-^{14}C-KIV (3500 cpm per μmole) and inhibitor. All other experimental conditions, reaction mixture components and assay methods were as described in Figure 31.


20
16
12
8
4
$\frac{1}{V_i}$
-3
-2
-1
0
1
2
3
4
$\left(\frac{1}{KIV}\right) \times 10^{-7}\ M^{-1}$

decarboxylation of KIV is inhibited competitively by either KMV or KIC in this system. Production of $^{14}CO_2$ from carboxyl-labeled KMV is also subject to competitive inhibition by KIC or KIV as seen in the Lineweaver-Burk plots of Figures 35 and 36. Thus, each of the branched-chain α-keto acids is able to compete for the oxidative process in the mitochondrial system.

The rate of in vitro oxidative decarboxylation of pyruvate by bovine liver mitochondria was decreased significantly in the presence of the branched-chain keto acids. Figure 37 indicates, in double reciprocal form, that KIC acts as a competitive inhibitor of pyruvate oxidation. The Dixon plots (84) of Figure 38 disclose a similar competitive effect for KIV upon the utilization of pyruvate by bovine liver mitochondria. The Dixon plots of Figure 39 illustrate the competitive inhibition of pyruvate oxidative decarboxylation by KMV.

The catabolism of α-ketoglutarate was inhibited by KIC (Figure 40), KMV (Figure 41), and KIV (Figure 42). The apparent mixed type of inhibition was noted with each of the branched-chain α-keto acids. These findings agree with those previously obtained for the purified α-ketoglutarate dehydrogenase complex from pig heart mitochondria (38).

The influence of α-ketocaproate (KC), a straight-chain analogue of KIC and KMV, on the production of $^{14}CO_2$ from carboxyl-labeled KIC or KIV was also examined. It was assumed that KC would act as a competitive inhibitor of the oxidation of these two branched-chain keto acids, and could thus be used as an agent for

Fig. 35. -- Lineweaver-Burk plots of the inhibition of α-keto-β-methylvalerate dehydrogenase activity by α-ketoisocaproate

Lineweaver-Burk plots with 1-^{12}C-KIC as inhibitor and carboxyl-labeled KMV as varied substrate. (●——●) no inhibitor, (■——■) 2.5×10^{-4} M KIC, (▲——▲) 7.5×10^{-4} M KIC. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria and the indicated amounts of labeled KMV (2050 cpm per μmole) and inhibitor. All other experimental conditions, reaction media and assay methods were as stated for Figure 31. (v_i, nanomoles CO_2 produced per minute per mg of protein).

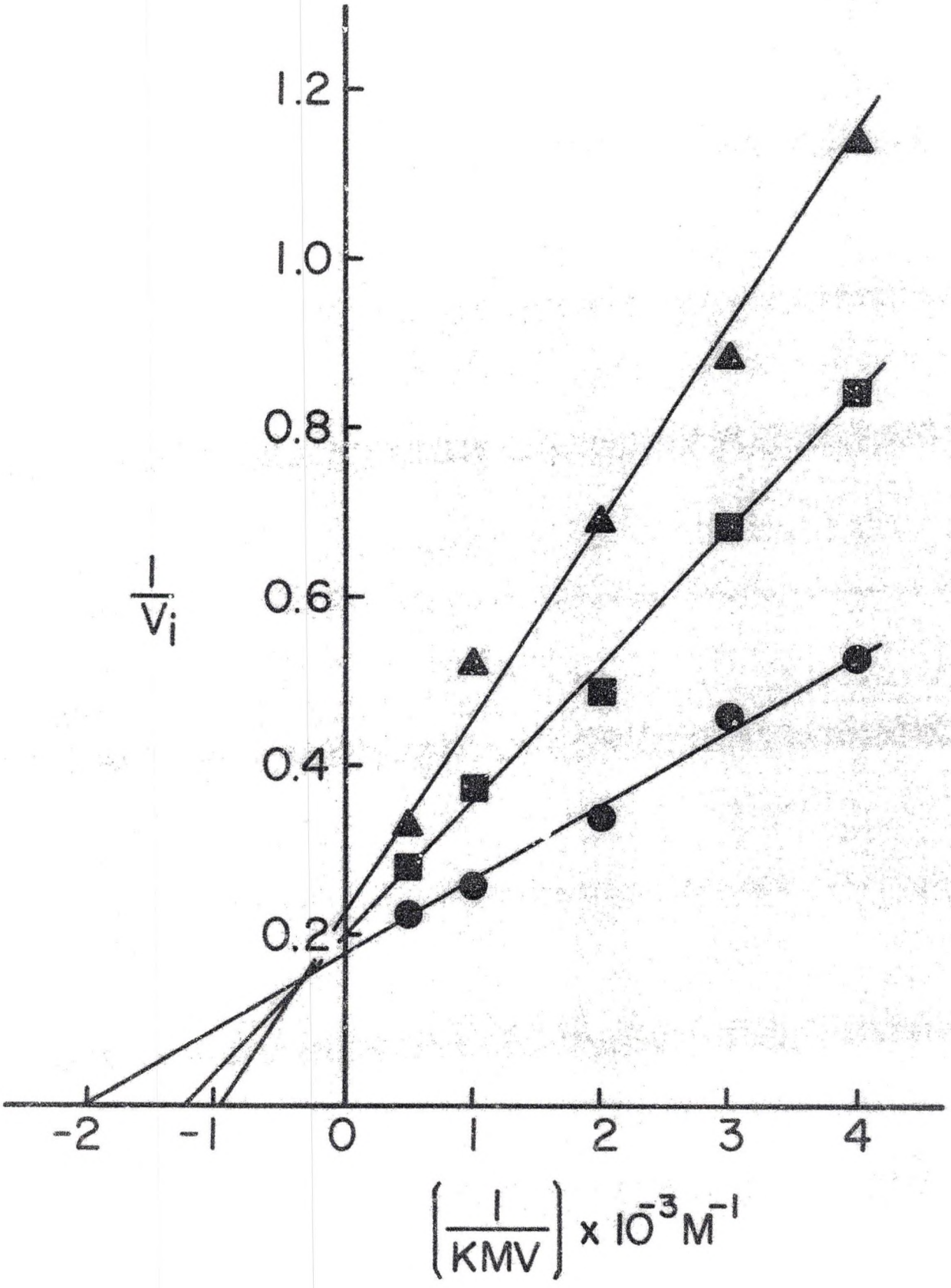

1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-2
-1
0
1
2
3
4
(1/KMV) x 10⁻³M⁻¹

Fig. 36. -- Lineweaver-Burk plots of the inhibition of α-keto-β-methylvalerate dehydrogenase activity by α-ketoisocaproate

Lineweaver-Burk plots with 1-^{12}C-KIC as inhibitor and carboxyl-labeled KMV as varied substrate. (●——●) no inhibitor, (■——■) 2.5 x 10^{-4}M KIC, (▲——▲) 5.0 x 10^{-4}M KIC, (○——○) 7.5 x 10^{-4}M KIC. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria, the indicated amount of labeled KMV (2050 cpm per μmole) and inhibitor. All other experimental conditions, reaction media and assay methods were as stated for Figure 31. (v_i, nanomoles CO_2 produced per minute per mg of protein).

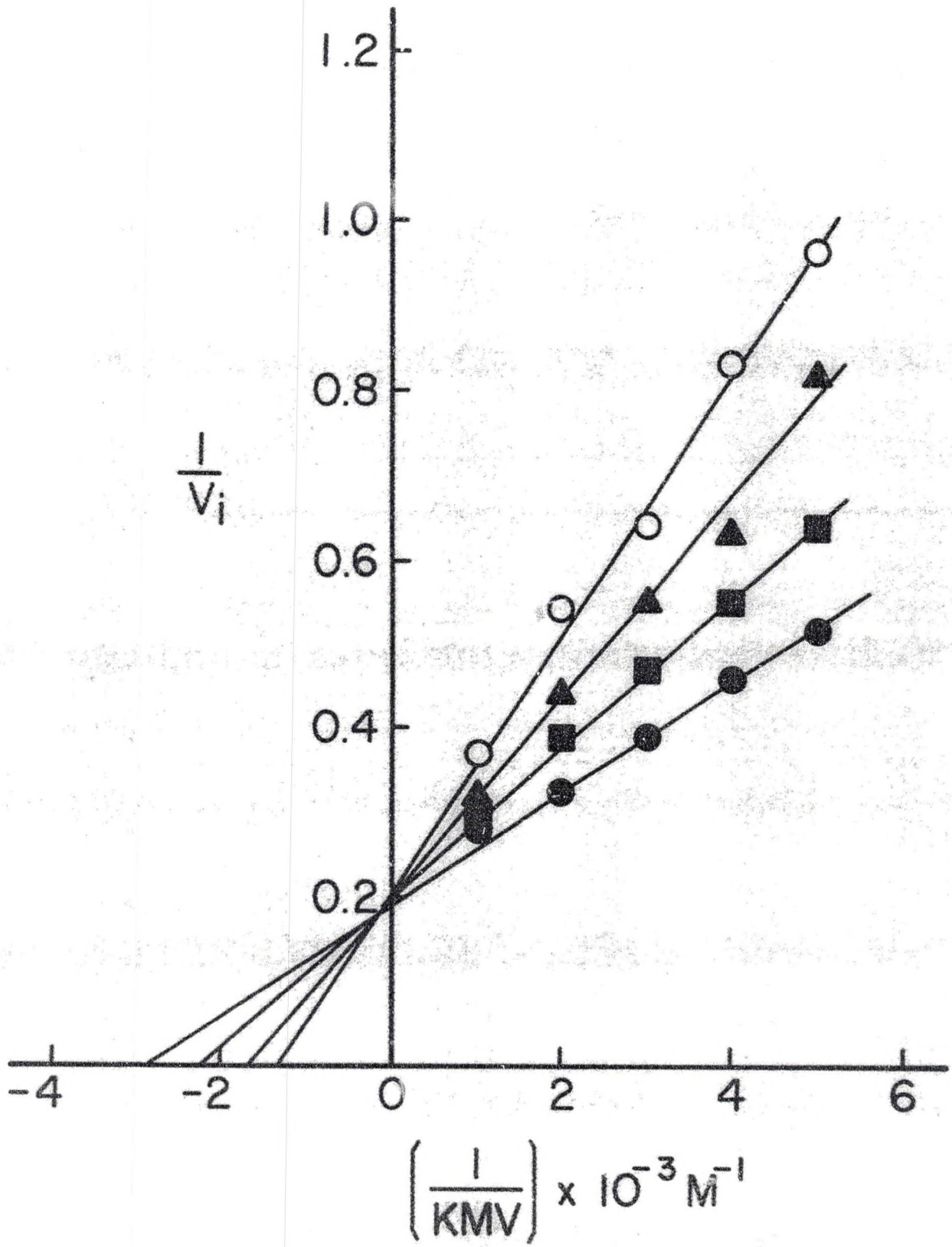

1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-4
-2
0
2
4
6
(1/KMV) x 10^-3 M^-1

Fig. 37. -- Lineweaver-Burk plots of the inhibition of pyruvate dehydrogenase activity by α-ketoisocaproate

Lineweaver-Burk plots with 1-^{12}C-KIC as inhibitor and 1-^{14}C-pyruvate as varied substrate. (●——●) no inhibitor, (■——■) 2.5 x 10^{-4}M 1-^{12}C-KIC, (▲——▲) 7.5 x 10^{-4}M 1-^{12}C-KIC, (○——○) 1.0 x 10^{-3}M 1-^{12}C-KIC, (□——□) 2.0 x 10^{-4}M 1-^{12}C-KIC. Pyruvate dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles), mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.05 ml of a washed preparation of bovine liver mitochondria (1.3 mg protein) and the indicated amounts of 1-^{14}C-pyruvate and inhibitor. The reactions were allowed to proceed for 10 min at 30°C. (v_i, nanomoles CO_2 produced per minute per mg protein).

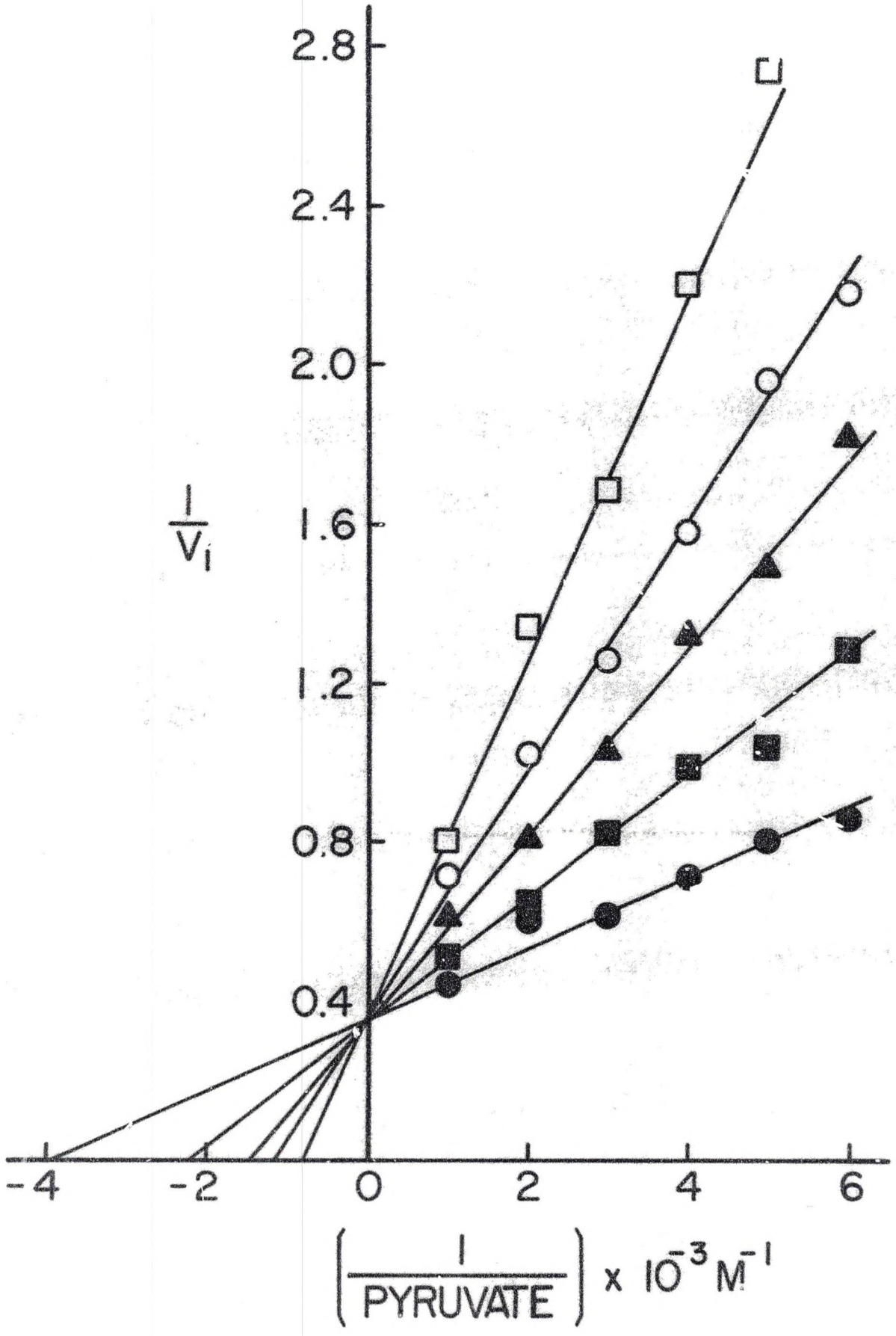

2.8
2.4
2.0
1.6
1.2
0.8
0.4
1/Vi
-4
-2
0
2
4
6
(1/PYRUVATE) x 10⁻³ M⁻¹

Fig. 38. -- Dixon plot of the inhibition of pyruvate dehydrogenase activity by α-ketoisovalerate

Dixon plot with 1-^{12}C-KIV as varied inhibitor and 1-^{14}C-pyruvate as substrate. (●——●) 1.0 x 10^{-4}M 1-^{14}C-pyr (■——■) 3.0 x 10^{-4}M 1-^{14}C-pyr. Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria, the indicated amounts of 1-^{14}C-pyr (58,500 cpm per μmole) and quantities of inhibitor as noted in the figure. All other experimental conditions, reaction media and assay methods were as stated for Figure 37. (v_i, nanomoles of CO_2 produced per minute per mg of protein).

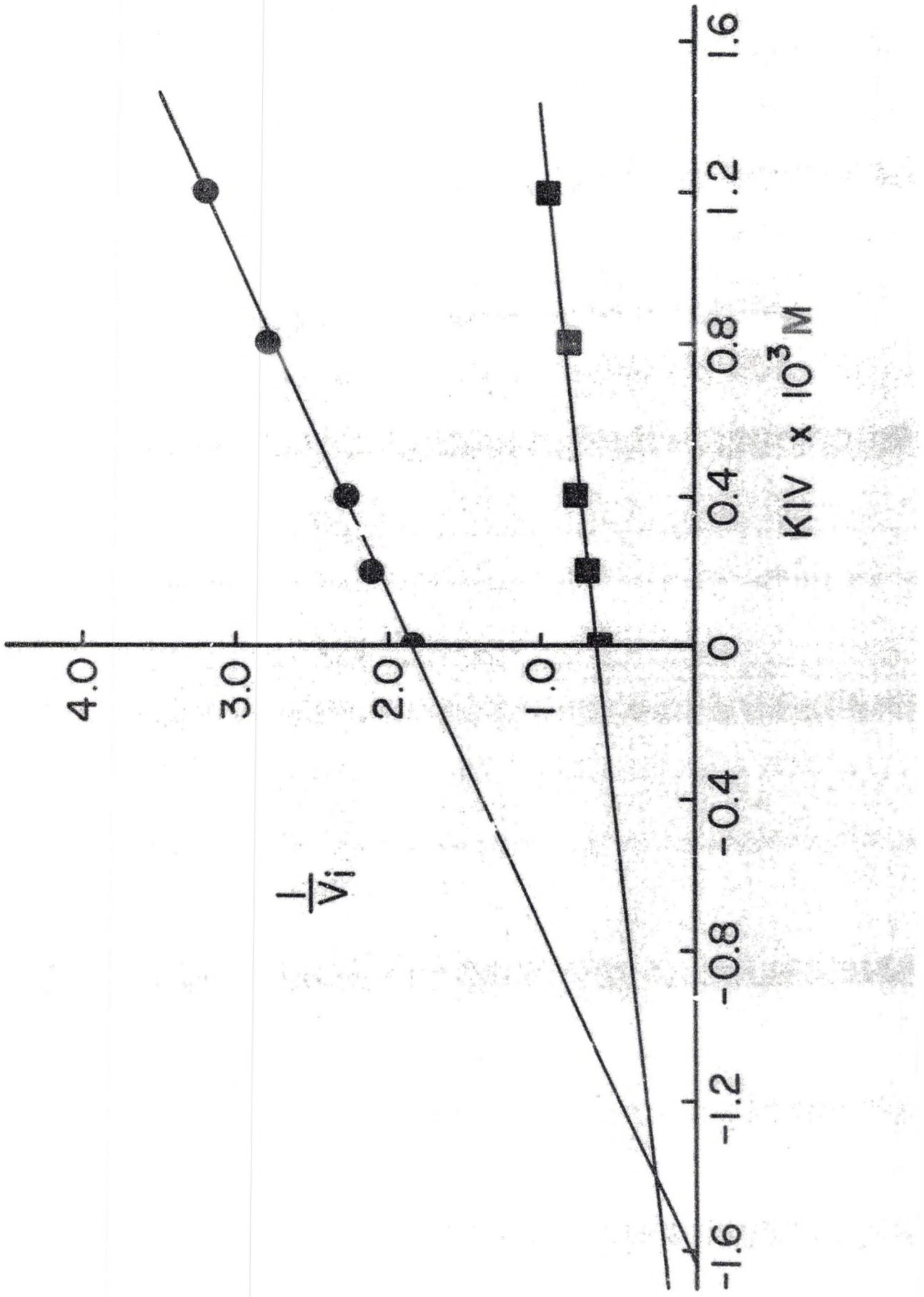

4.0
3.0
2.0
1.0
0
-0.4
-0.8
-1.2
-1.6
0.4
0.8
1.2
1.6
$\frac{1}{V_i}$
KIV x 10^3 M

Fig. 39. -- Dixon plot of the inhibition of pyruvate dehydrogenase activity by α-keto-β-methylvalerate

Dixon plot with 1-^{12}C-KMV as varied inhibitor and 1-^{14}C-pyruvate as substrate. (●——●) 5 x 10^{-5}M 1-^{14}C-pyr, (■——■) 1.5 x 10^{-4}M 1-^{14}C-pyr. Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria with the indicated amounts of 1-^{14}C-pyr (58,500 cpm per μmole) and inhibitor. All other experimental conditions, reaction media and assay methods were as stated for Figure 37. (v_i, nanomoles of CO_2 produced per minute per mg protein).

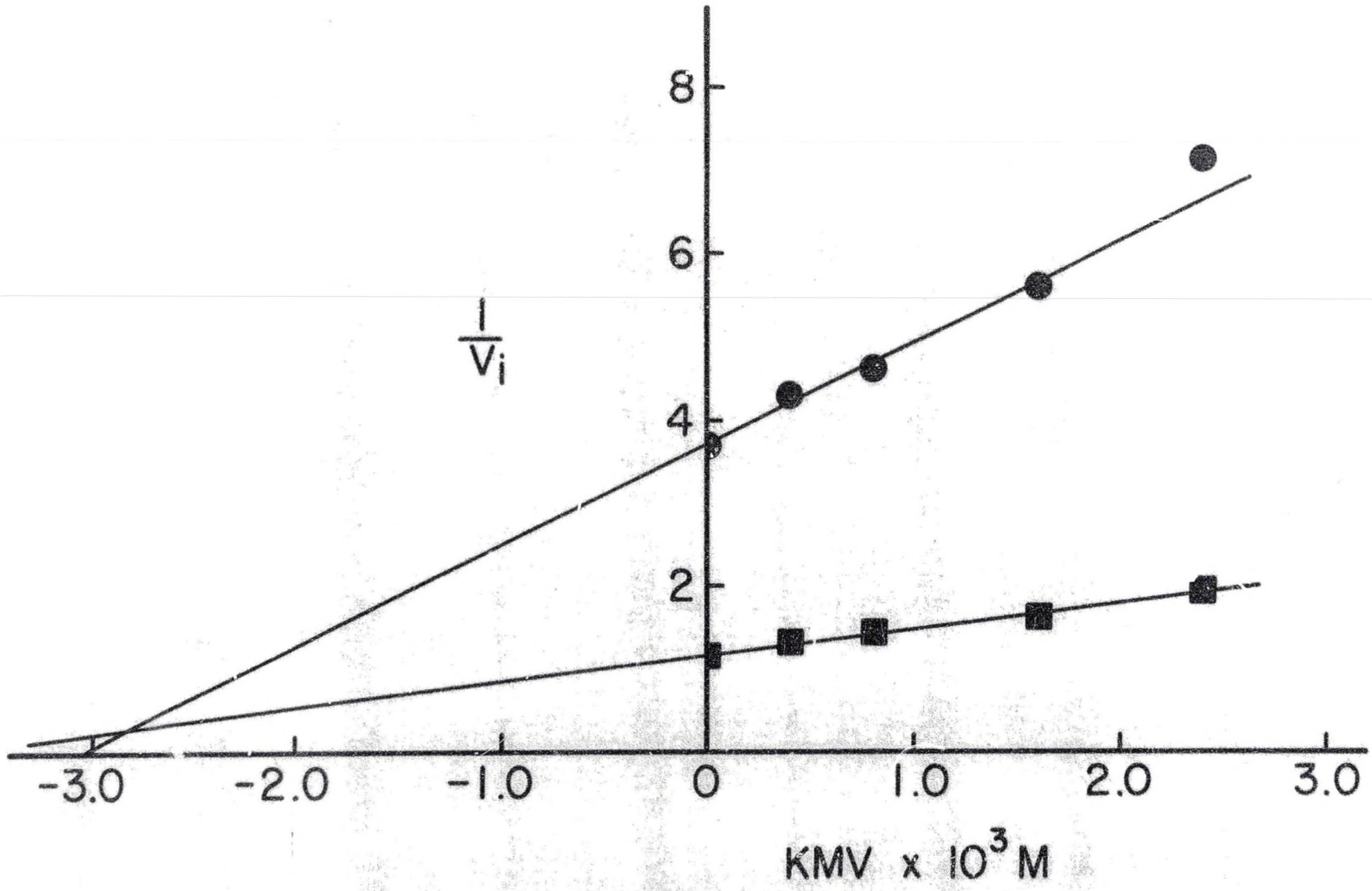

8
6
4
2
$\frac{1}{V_i}$
-3.0
-2.0
-1.0
0
1.0
2.0
3.0
KMV x 10^3 M

Fig. 40. -- Lineweaver-Burk plots of the inhibition of α-ketoglutarate dehydrogenase activity by α-ketoisocaproate

Lineweaver-Burk plots with 1-^{12}C-KIC as inhibitor and 1-^{14}C-α-ketoglutarate as the varied substrate. (●——●) no inhibitor, (■——■) 5 x 10^{-4}M KIC, (▲——▲) 1.5 x 10^{-3}M KIC. α-Ketoglutarate dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section (v_i, nanomoles CO_2 produced per minute per mg protein). Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles), mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.05 ml of a washed preparation of bovine liver mitochondria (1.3 mg protein), and the indicated amounts of 1-^{14}C-α-ketoglutarate (50,000 cpm per μmole) and inhibitor. All reactions were allowed to proceed for 10 minutes at 30°C.

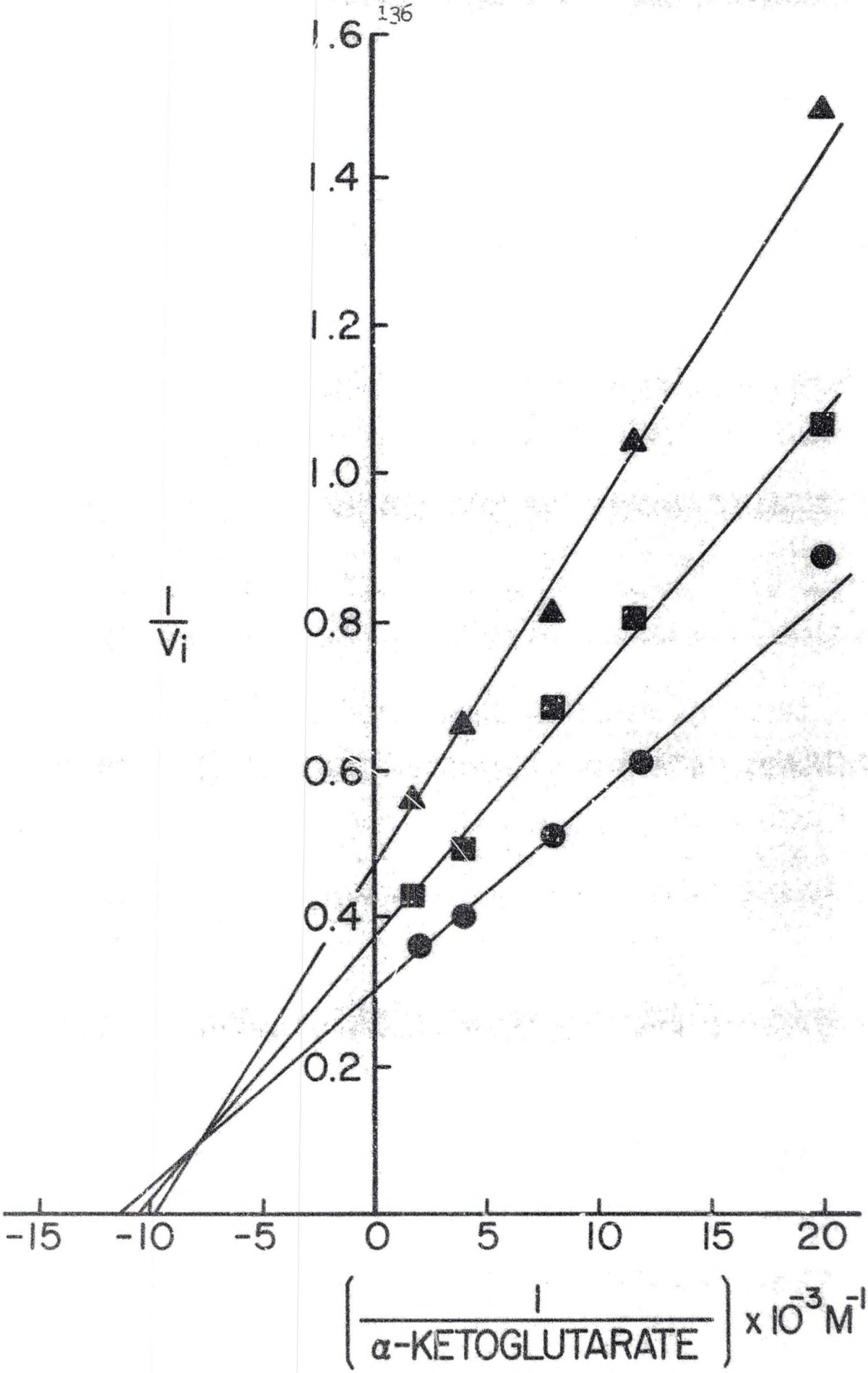

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-15
-10
-5
0
5
10
15
20
(1/α-KETOGLUTARATE) x 10⁻³ M⁻¹

Fig. 41. -- Lineweaver-Burk plots of the inhibition of α-ketoglutarate dehydrogenase activity by α-keto-β-methylvalerate

Lineweaver-Burk plots with 1-^{12}C-KMV as inhibitor and 1-^{14}C-KG as the varied substrate. (●——●) no inhibitor, (■——■) 5.0×10^{-4}M KMV, (▲——▲) 1.5×10^{-3}M KMV. Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria with the indicated amounts of 1-^{14}C-KG (50,000 cpm per μmole), and inhibitor. All other experimental conditions, reaction media and assay methods were as stated for Figure 40. (v_i, nanomoles CO_2 produced per minute per mg of protein).

CORRECTION

THIS DOCUMENT

HAS BEEN REPHOTOGRAPHED

TO ASSURE LEGIBILITY

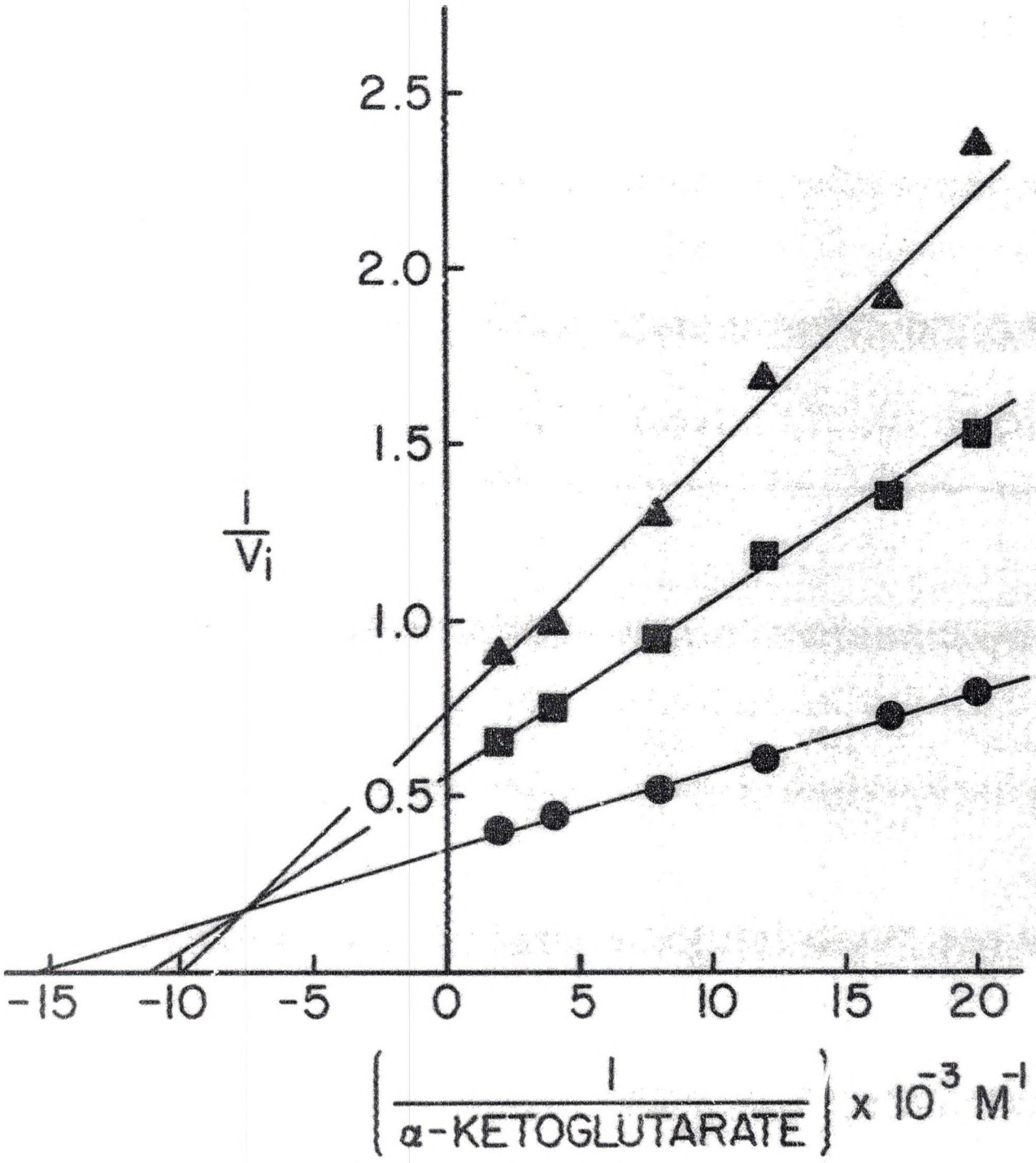
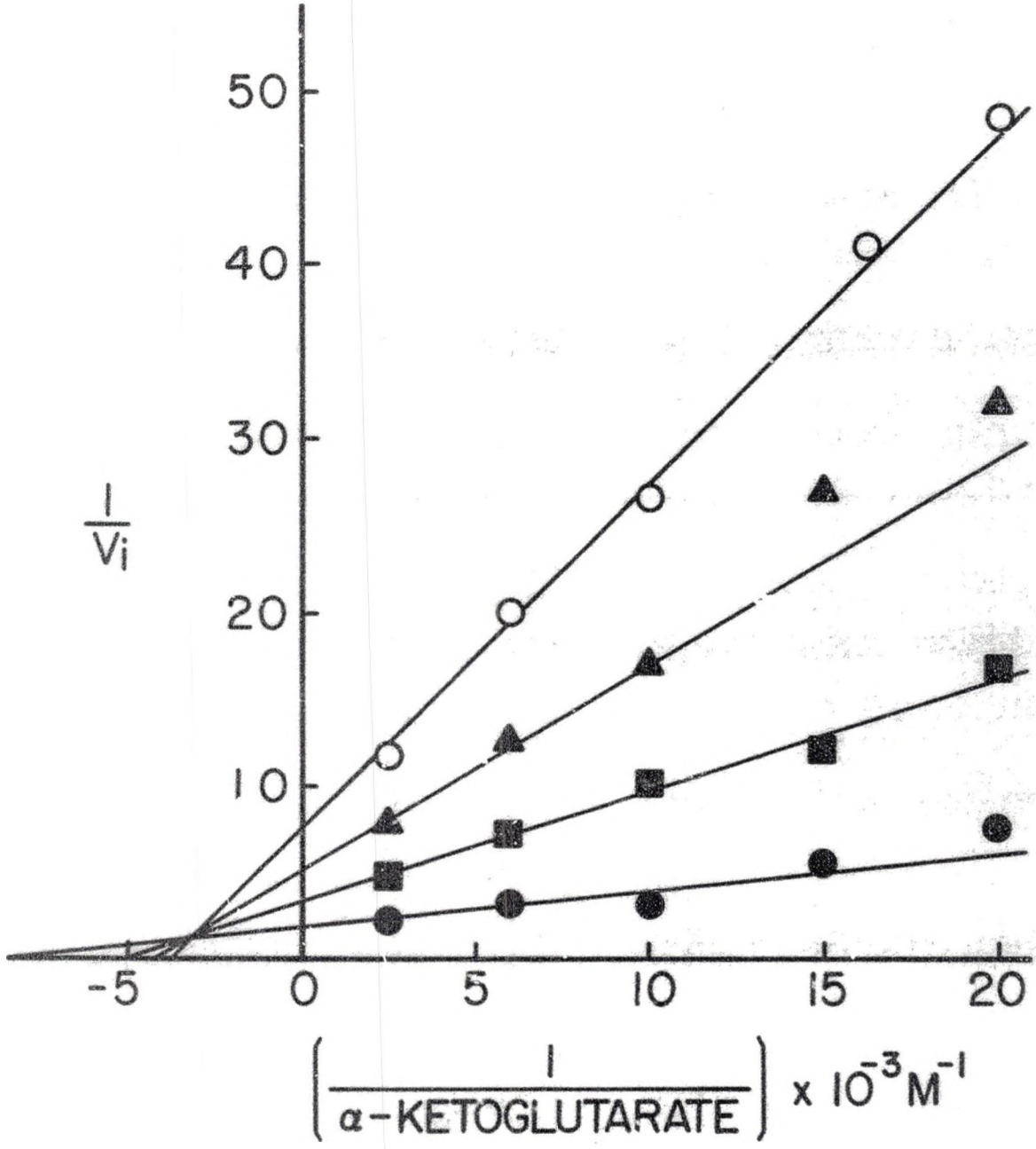

2.5
2.0
1.5
1.0
0.5
1/Vi
-15
-10
-5
0
5
10
15
20
1/α-KETOGLUTARATE
x 10⁻³ M⁻¹

Fig. 42. -- Lineaver-Burk plots of the inhibition of α-
etoglutarate dehydrogenase activity by α-ketoisovalerate

ineweaver-Burk plots with 1-^{12}C-KIV as inhibitor and 1-^{14}C-KG as
aried substrate. (●——●) no inhibitor, (■——■)
.0 x 10^{-4}M KIV, (▲——▲) 1.0 x 10^{-3}M KIV, (○——○)
.0 x 10^{-3}M KIV. Each reaction contained 0.05 ml of a washed
reparation of bovine liver mitochondria with the indicated amounts
f 1-^{14}C-KG (50,000 cpm per μmole) and inhibitor. All other
xperimental conditions, reaction media and assay methods were as
tated for Figure 40. (v_i, nanomoles CO_2 produced per minute per
g of protein).


50
40
30
20
10
1/Vi
-5
0
5
10
15
20
(1/α-KETOGLUTARATE) x 10⁻³M⁻¹

further investigation of the number of enzymes involved in the oxidation of KIC and KIV, since if only one enzyme is concerned, a competitive inhibitor should give the same value of K_i when tested with either substrate. While KC did in fact inhibit the dehydrogenase activity with either KIC (Figure 43) or KIV (Figure 44), the inhibition appeared to be of a mixed nature. Though the K_i values were calculated (2.4×10^{-4}M and 4.8×10^{-4}M), their value as a diagnostic tool is definitely lessened by the suggestion that KC may be interacting with the enzyme(s) at other than the catalytic site since the inhibition observed appeared to be of a mixed type. Oxidation of KIV is also inhibited by its straight-chain analogue, α-ketovalerate (KV), as evidenced by the data shown in the Dixon plots of Figure 45. The observation that the values of K_i for the straight-chain keto acids KC and KV are somewhat lower (see Table 9) than are the K_m values for the branched-chain analogues KIC and KIV may indicate preference of the enzyme for the unbranched compounds. This findings is of only incidental interest at present, since the physiological function or occurrence of KC and KV have not been investigated in mammalian tissues. On the other hand, α-keto-butyrate (KB), an α-keto acid of considerable importance in the catabolic pathways of methionine and threonine, has been reported to accumulate in the plasma of branched-chain ketoaciduric patients (16). Recent investigation has shown that KB is oxidized to propionyl-CoA by the pyruvate dehydrogenase complex (38) at a substantial rate. The oxidation of KIC (Figure 46)

TABLE 9

K_i VALUES OF VARIOUS α-KETO ACIDS ON BOVINE LIVER MITOCHONDRIA α-KETO ACID DEHYDROGENASE ACTIVITIES[a]

Inhibitor	K_i (M)				
	α-keto-β-methyl-valerate dehydrogenase	α-ketoisocaproate dehydrogenase	α-keto-isovalerate dehydrogenase	pyruvate dehydrogenase	α-ketoglutarate dehydrogenase
1-^{12}C-KMV------		8.6×10^{-4}	2.5×10^{-4}	2.3×10^{-3}	1.4×10^{-3}
1-^{12}C-KIC------	6.9×10^{-4}		1.3×10^{-4}	5.4×10^{-4}	2.4×10^{-3}
1-^{12}C-KIV------	2.2×10^{-4}	1.95×10^{-4}		1.4×10^{-3}	8.2×10^{-4}
1-^{12}C-KV-------			0.5×10^{-4}		
1-^{12}C-KC-------		2.2×10^{-4}	4.8×10^{-4}		
1-^{12}C-KB-------		1.1×10^{-3}		4.1×10^{-4}	

[a] α-Keto acid dehydrogenase activities were measured by quantitation of $^{14}CO_2$ produced from carboxyl-labeled α-keto acids.

Reaction conditions and assay methods are described in the legends to Figures 31-47 and in the Methods. Each K_i value tabulated represents the average of at least three determinations.

Fig. 43. -- Lineweaver-Burk plots of the inhibition of α-ketoisocaproate dehydrogenase activity by α-ketocaproate (KC)

Lineweaver-Burk plots with 1-^{12}C-α-KC as inhibitor and 1-^{14}C-KIC as the varied substrate. (●——●) no inhibitor, (■——■) 5.0×10^{-4} M 1-^{12}C-KC. α-Ketoisocaproate dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles), mannitol, 150; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.1 ml of a washed preparation of bovine liver mitochondria (2.6 mg protein), and the indicated amounts of 1-^{14}C-KIC and inhibitor. All reactions were allowed to proceed for 20 minutes at 30°C. (v_i, nanomoles CO_2 produced per minute per mg protein).

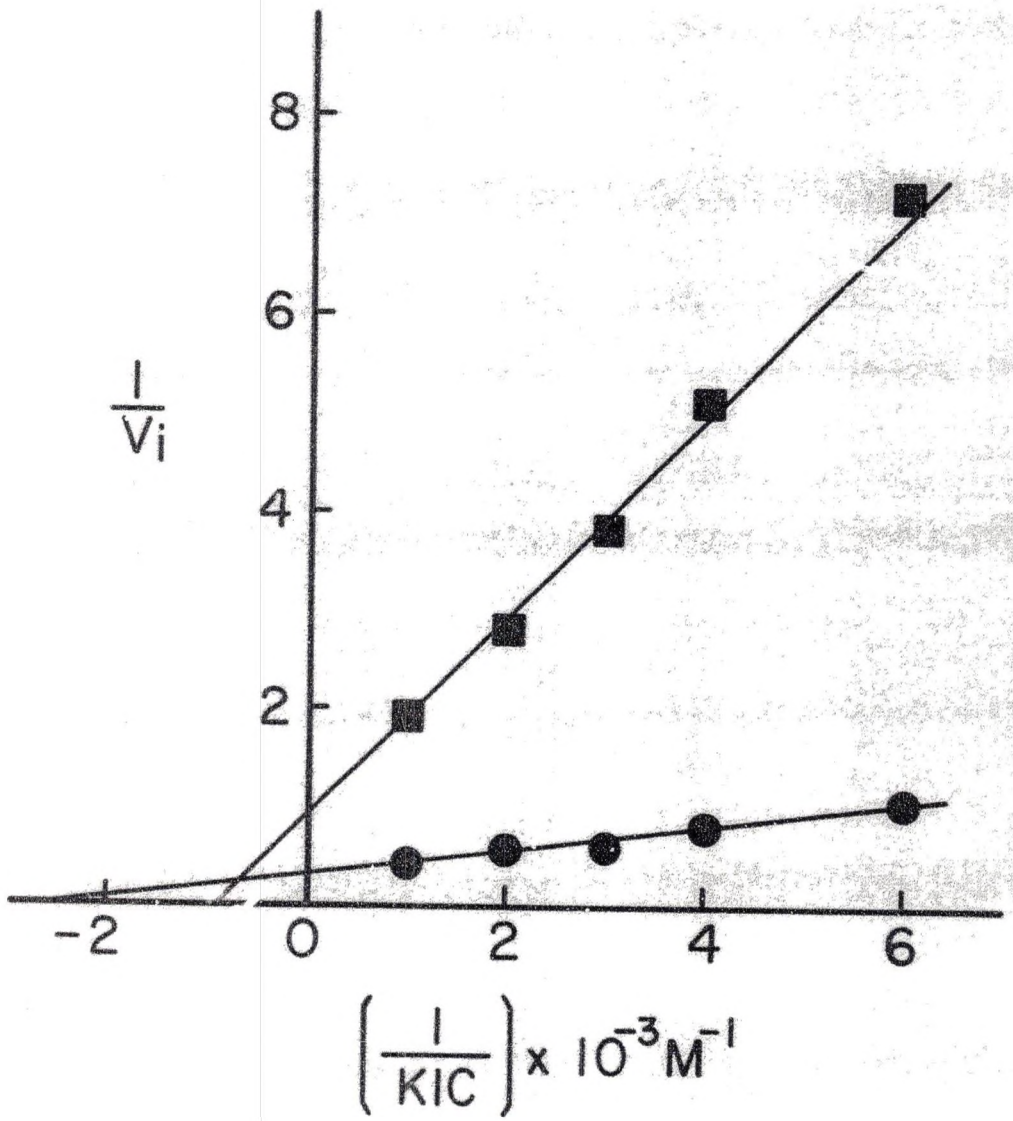

8
6
4
2
1/Vi
-2
0
2
4
6
(1/KIC) x 10⁻³M⁻¹

Fig. 44. -- Lineweaver-Burk plots of the inhibition of α-
:toisovalerate dehydrogenase activity by α-ketocaproate

.neweaver-Burk plots with 1-^{12}C-α-ketocaproate (α-KC) as inhibitor
id 1-^{14}C-α-KIV as varied substrate. (●——●) no inhibitor,
■——■) 1.0 x 10^{-4} M α-KC. α-Ketoisovalerate dehydrogenase
:tivity was measured by the $^{14}CO_2$ assay as described in the
:thods section (v_i, nanomoles CO_2 produced per minute per mg of
'otein). Each reaction mixture contained in a final volume of
0 ml, the following (in micromoles), mannitol, 150; potassium
osphate buffer, pH 7.2, 33; β-NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0;
Cl_2, 1.0; Na_2CO_3, 1.0; 0.1 ml of a washed preparation of bovine
ver mitochondria (2.6 mg protein), and the indicated amounts of
^{14}C-KIV and inhibitor. All reactions were allowed to proceed for
minutes at 30°C.

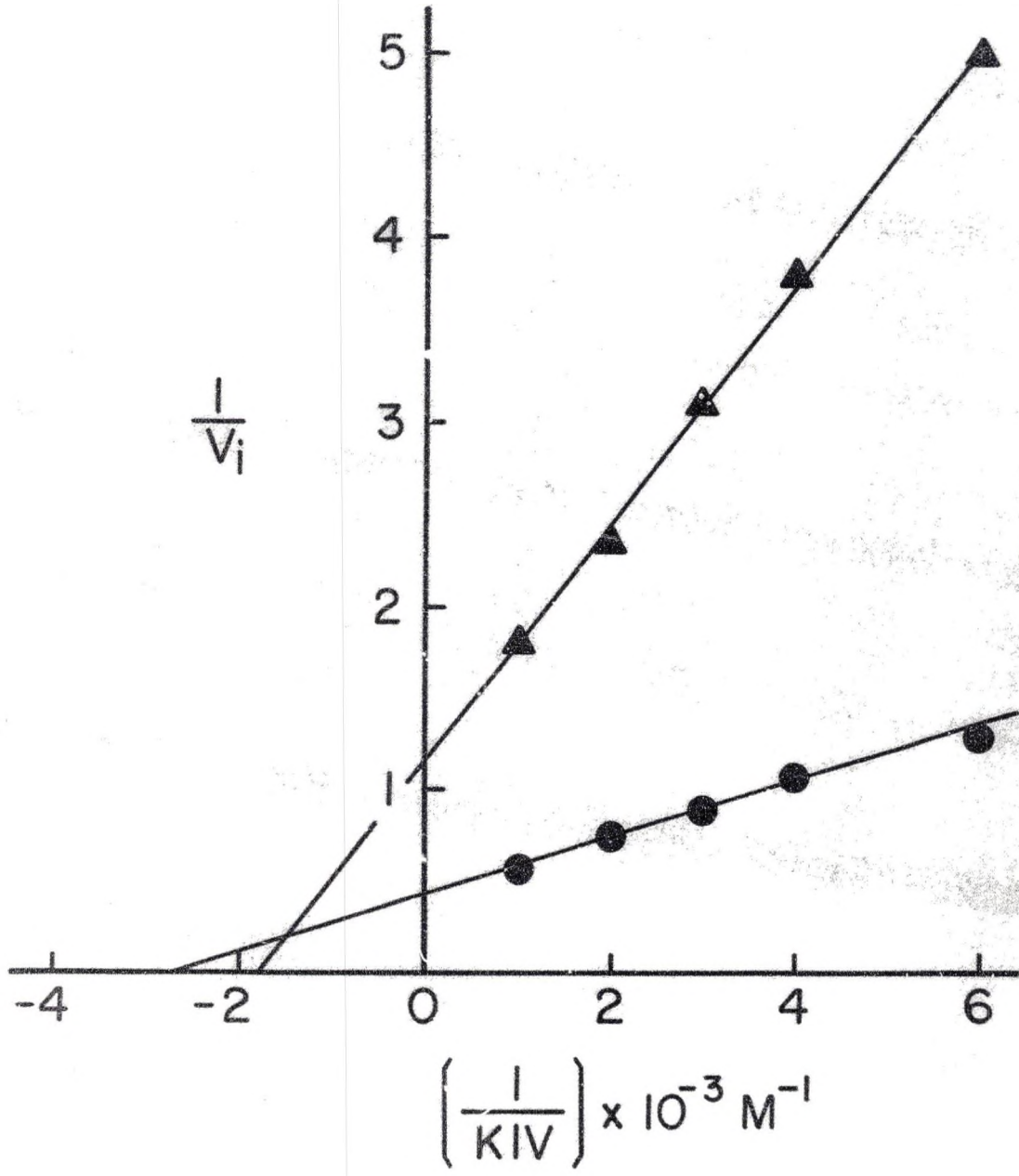

5
4
3
2
1
$\frac{1}{V_i}$
-4
-2
0
2
4
6
$\left(\frac{1}{KIV}\right) \times 10^{-3}\ M^{-1}$

Fig. 45. -- Dixon plot of the inhibition of α-ketoisovalerate dehydrogenase activity by α-ketovalerate (KV)

Dixon plot with 1-^{12}C-KV as the varied inhibitor and 1-^{14}C-α-KIV as the keto acid substrate. (●——●) 2.5 x 10^{-4} M KIV-1-^{14}C, (■——■) 5.0 x 10^{-4} M KIV-1-^{14}C, 1-^{14}C-KIV. α-Ketoisovalerate dehydrogenase activity was measured by the $^{14}CO_2$ assay as described in the Methods section. Each reaction mixture contained in a final volume of 1.0 ml, the following (in micromoles); mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; 0.1 ml of a washed preparation of bovine liver mitochondria, and the indicated amounts of 1-^{14}C-KIV and inhibitor. The reactions were allowed to proceed for 10 minutes at 30°C. (v_i, nanomoles CO_2 per minute per mg protein).

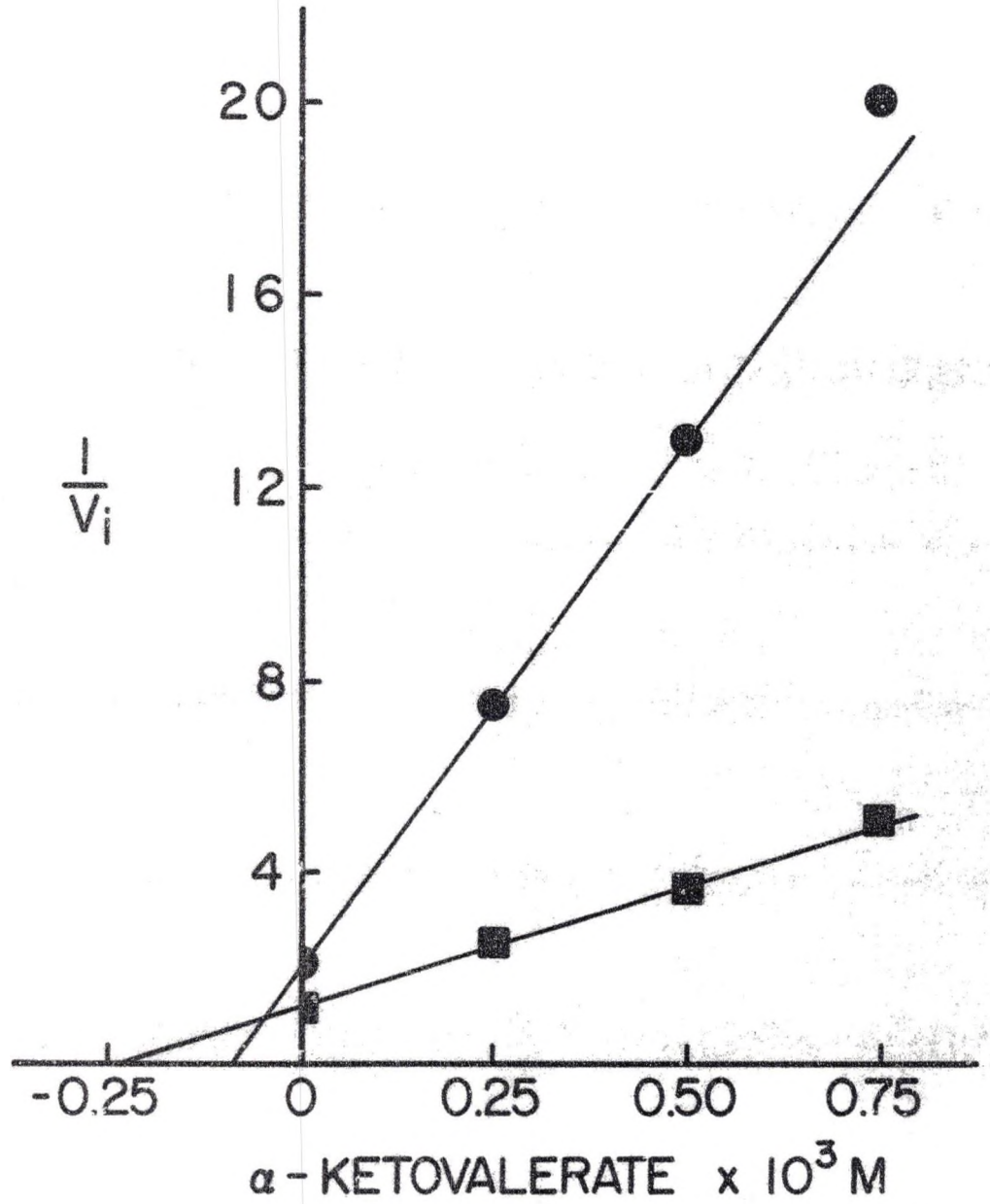
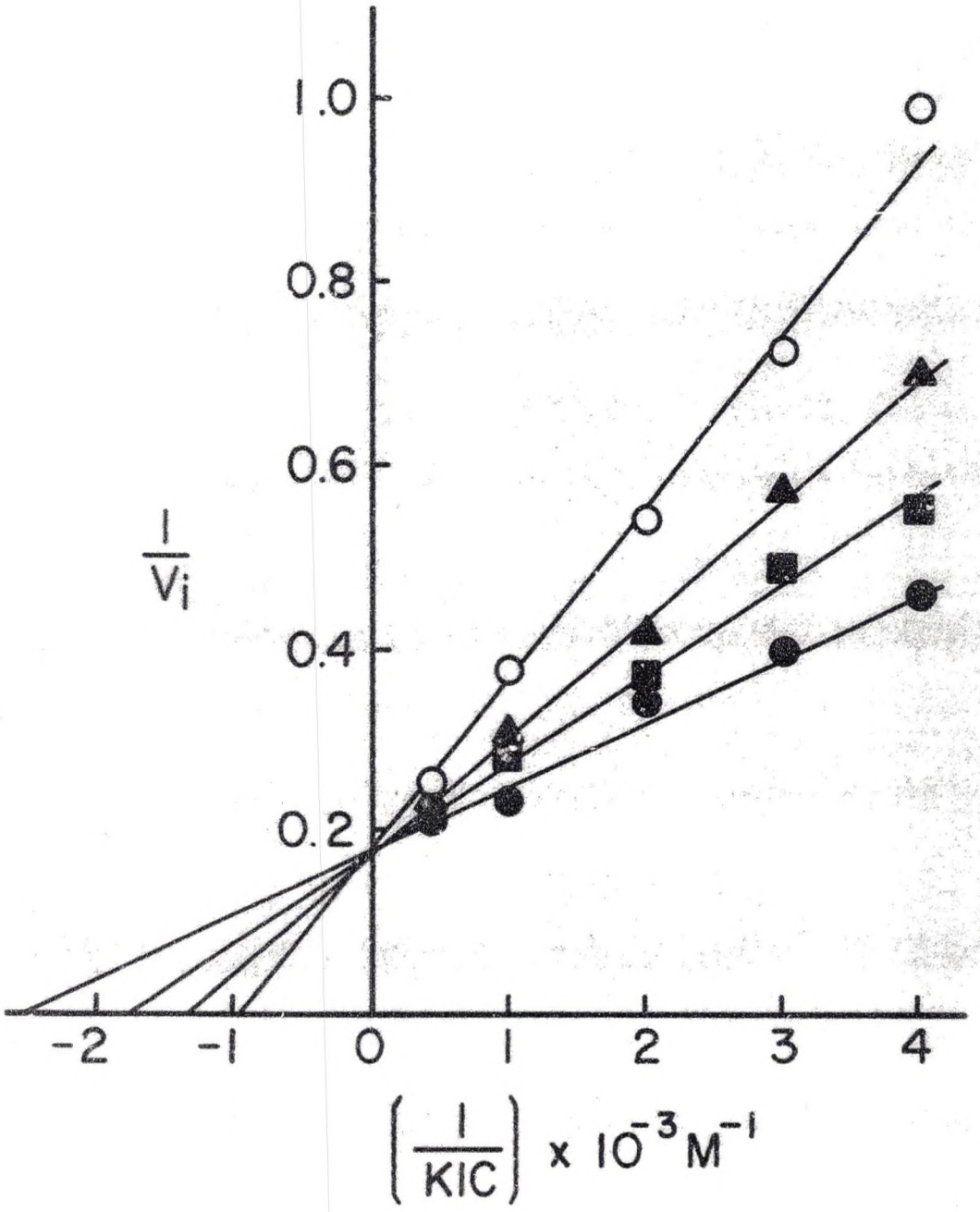

20
16
12
8
4
1/Vi
-0.25
0
0.25
0.50
0.75
α-KETOVALERATE x 10³ M

Fig. 46. -- Lineweaver-Burk plots of the inhibition of α-ketoisocaproate dehydrogenase activity by α-ketobutyrate (KB)

Lineweaver-Burk plots with 1-^{12}C-KB as inhibitor and 1-^{14}C-KIC as varied substrate. (●——●) no inhibitor, (■——■) 0.25 mM KB, (▲——▲) 0.75 mM KB, (○——○) 1.50 mM KB. Each reaction mixture contained 0.1 ml of a washed preparation of bovine liver mitochondria (2.4 mg of protein) with the indicated amounts of 1-^{14}C-KIC (3600 cpm per μmole) and inhibitor. All other experimental conditions, reaction media, and assay methods were as stated for Figure 43. (v_i, nanomoles CO_2 produced per minute per mg protein).

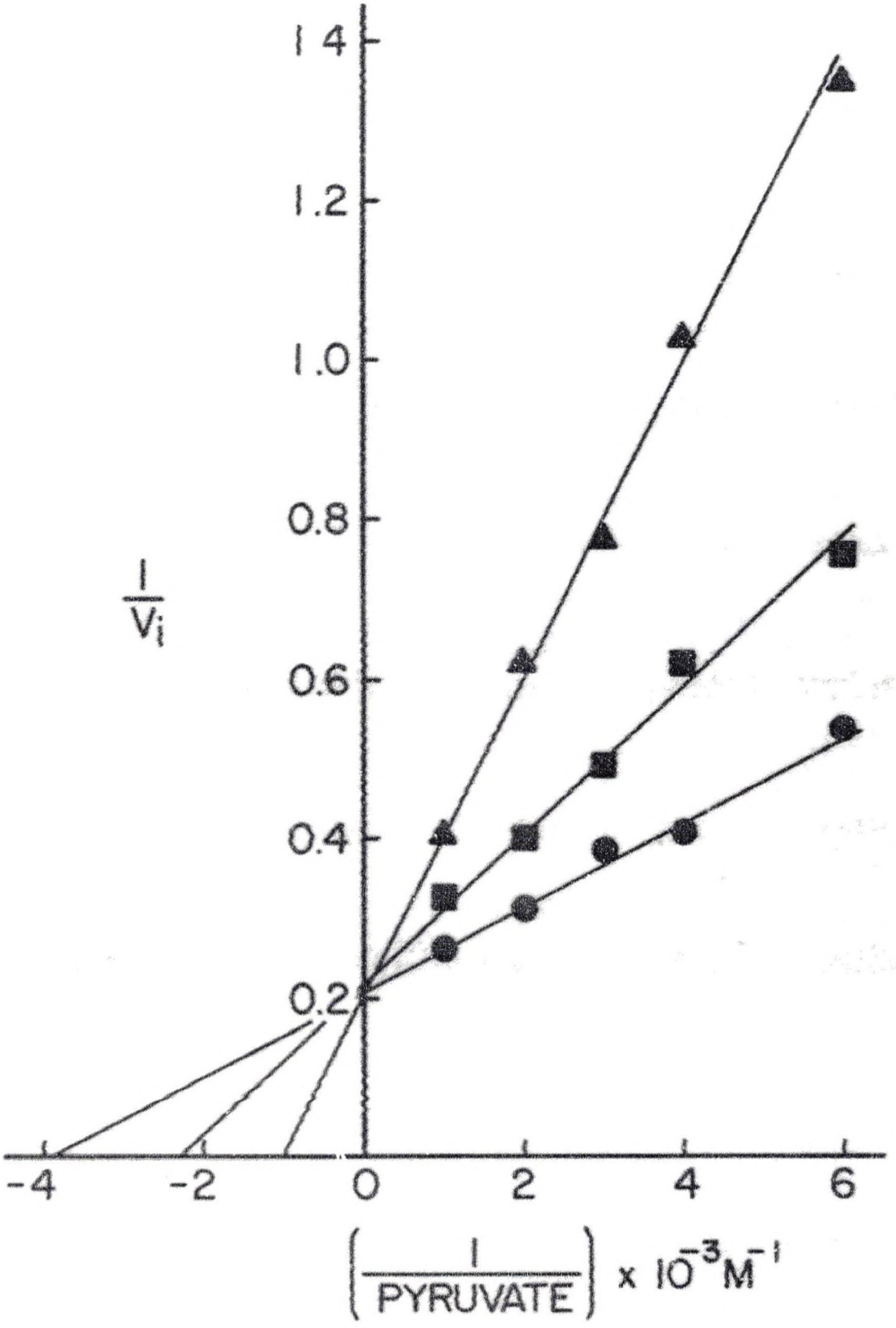

1.0
0.8
0.6
0.4
0.2
1/Vi
-2
-1
0
1
2
3
4
(1/KIC) x 10⁻³ M⁻¹

and of pyruvate (Figure 47) was affected deleteriously by low levels of KB *in vitro*.

The results from the inhibition studies illustrated by Figures 31 to 47 and summarized in Table 9 indicate that the metabolism of these keto acids is markedly influenced by the relative levels of the various α-keto acids in the normal liver cell (see Appendix II for normal levels of some amino acids and keto acids). From these data it is evident that one consequence of increased KIC concentrations will be the effective and detrimental influence upon the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase. Further implications of the mutual interactions among the α-keto acids KMV, KIC and KIV and the effects of these keto acids on the catabolism of α-ketoglutarate and pyruvate with reference to the normal metabolism of the liver and with respect to the aberrant situation associated with the diseased (branched-chain ketoaciduria) state will be considered in the "Discussion".

Fig. 47. -- Lineweaver-Burk plots of the inhibition of pyruvate dehydrogenase activity by α-ketobutyrate

Lineweaver-Burk plots with 1-^{12}C-KB as inhibitor and 1-^{14}C-pyruvate (52,600 cpm per μmole) as the varied substrate. (●——●) no inhibitor, (■——■) 0.25 mM KB, (▲——▲) 0.75 mM KB. Each reaction mixture contained 0.05 ml of a washed preparation of bovine liver mitochondria (1.2 mg of protein) with the indicated amounts of 1-^{14}C-pyr (58,500 cpm per μmole) and inhibitor. Reactions were allowed to proceed for 10 minutes at 30°C. All other experimental conditions, reaction media and assay methods were as stated for Figure 43. (v_i, nanomoles CO_2 produced per minute per mg of protein).


1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/Vi
-4
-2
0
2
4
6
(1/PYRUVATE) x 10⁻³M⁻¹

DISCUSSION

A comprehensive delineation of the intricate regulatory mechanisms which modify the catabolism of the branched-chain amino acids is only recently emerging from several investigations of the catabolic enzymes. The major thrust of initial studies of the metabolism of the branched-chain amino acids centered upon the characterization of the overall degradative pathways of leucine, isoleucine and valine (1); elucidation of the site of the catabolism of these compounds in the genetic disease branched-chain ketoaciduria (21,23,24) and development of therapeutic dietary treatment designed to control the symptoms of the disease (20,32). Recently however, there has been evidenced a growing awareness of the complexity and multiplicity of the regulatory mechanisms which may function to maintain a fine balance among the cellular levels of the branched-chain amino acids and keto acids in the normal mammalian system.

A thorough description of the regulation of the metabolism of these compounds must integrate such known facts as the variation in distribution among tissues of the transaminases and α-keto acid dehydrogenases, differences in the adaptive responses of these enzymes to variation in diet or availability of substrates, and the number and specificities of these enzymes. Furthermore, of considerable importance to such a discussion is the consideration of the mode and extent of any mutual interferences among these

reactions. The influence of permeability barriers between substrates and enzymes imposed by physical compartmentation of constituent enzymes involved with the catabolic sequence may also contribute to the overall control apparatus.

The metabolism of the amino groups of leucine, isoleucine and valine appears to be regulated by several factors, including the availability of amino group acceptor (16) and induction of amino-transferase enzymes (11,85). That the further catabolism of these amino acids is subject to genetic control is evident from the studies of Wohlheuter and Harper (11), who have shown a parallel increase in the branched-chain α-keto acid dehydrogenase activities in response to increased dietary intake of protein. During the course of their investigations of this phenomenon, they noted a second modulation of the dehydrogenase activities, not affected by known inducing agents, which was tentatively identified as a pattern of diurnal variation.

A substantial elevation of the plasma levels of the branched-chain amino acids occurs in animals fed a diet high in protein. For example, Anderson et al. (86) have reported leucine concentrations of approximately 10^{-4}M in the plasma of rats fed a controlled diet containing five per cent casein hydrolysate. However, within one day after initiation of a force-fed diet which contained 25 per cent protein, the levels of leucine increased nearly five-fold. Leucine, isoleucine and valine concentrations remained elevated for two to three days if the 25 per cent casein diet was continued, then decreased gradually to a steady state slightly higher than was

observed initially. Meister (14) has shown that the relative concentrations of free branched-chain amino acids and their respective α-keto acids in mammalian liver are nearly equivalent, based on the equilibrium constants for their interconversion. This would indicate that under conditions of abnormally increased leucine, isoleucine and valine levels the concentrations of KIC, KMV and KIV would be expected to increase concurrently.

In view of the importance which must be attached to the degradative pathways for the essential amino acids leucine, isoleucine and valine, a discussion of these pathways and a consideration of potential regulatory mechanisms is important to the understanding of how the metabolism of these compounds might be controlled. Recognition and quantitation of the characteristics of the individual enzymatic steps and of the interactions among these metabolites in the normal course of metabolism also may result in a clearer understanding of the aberrant events associated with branched-chain ketoaciduria.

The oxidative decarboxylation of the branched-chain α-keto acids, the second step in the catabolism of the corresponding amino acids, may be speculated to be a logical candidate as a site of metabolic regulation, owning to the complex nature of the multienzyme units which catalyze this conversion, and to the essentially irreversible diversion of carbon away from the mainstream of amino acid and protein metabolism.

It has been variously postulated that one, two or even three separate enzymes may be responsible for the oxidative decarboxylation

of KIC, KMV and KIV in mammals. The combined substrate experiments performed in these studies imply the existence of a single dehydrogenase, as do the similar responses, in vitro, to perturbing influences such as subjection to mild heating, pH variation, and arsenite treatment. Mutual inhibition among KIC, KMV and KIV (Table 8) approximate that which will be expected for competitive interaction at a single binding site. However, the values for K_m and K_i which were determined for the branched-chain keto acids are sufficiently similar so that the observed pattern could be attributed to the influence of multiple dehydrogenases for KMV, KIC and KIV. Nevertheless, the weight of evidence from this work appears to favor the former hypothesis. Ultimately the acceptance of a one enzyme model requires the demonstration of a single homogeneous enzyme complex having activity with all three keto acids.

The assumption of a single branched-chain α-keto acid dehydrogenase from mitochondria should not be construed as contradictory to the demonstration, by Connelly et al. (27,80), of a soluble KIC-KMV dehydrogenase from bovine liver cell preparations. This enzyme was partially purified, and by a variety of criteria, was considered to be a single enzyme. The subcellular distribution studies reported here indicate that in bovine liver, the dehydrogenase activity with KIC, KMV and KIV is predominantly associated with the mitochondria, with roughly ten per cent of the total decarboxylase activity with either KMV or KIC occurring in the "soluble" (i.e. not sedimented at 80,000 x g for one hour) fraction. Very little KIV decarboxylase activity was detected in

the soluble phase. In contrast to the notable dependence of the mitochondrial enzyme activities upon exogenous NAD^+ and CoA, the soluble activities exhibited some requirement for NAD^+, but no observable need for added CoA. These results merit discussion in view of the observed absence of branched-chain keto acid dehydrogenase activity in branched-chain α-keto aciduria (BCKA). If two types of branched-chain keto acid dehydrogenase exist, one localized in the mitochondria and another in the soluble phase of the cell they are evidently closely related at the genetic level. Loss of these enzymatic capabilities, through an inherited genetic lesion, would then result in total inability (in classic branched-chain ketoaciduria) to catabolize these keto acids.

It is generally accepted that the oxidative decarboxylation of pyruvate and α-ketoglutarate, in mammalian tissues, is catalyzed by separate dehydrogenases complexes (38), each possessing a relatively high degree of substrate specificity. α-Ketoglutarate dehydrogenase in nearly completely specific for α-ketoglutarate with a minor ability to decarboxylate α-ketoadipate. On the other hand, the pyruvate dehydrogenase of pig heart mitochondria is capable of oxidizing either pyruvate or α-ketobutyrate at substantial rates (38). Studies reported here, i.e. differential response of these activities to mild heating, and nearly additive reaction rates in the mixed substrate experiments (Table 7) support the conclusion of separate α-ketoglutarate and pyruvate dehydrogenase complexes in bovine liver mitochondria.

Close scrutiny of the data shown in Appendix II will reveal that protein intake (discussed above) results in accumulation of KIC, KMV

and KIV in concentrations which approach, and in some instances exceed, the calculated inhibition constants for these keto acids (see Table 9). For example the concentration of KIC may reach 0.5 mM, which is significantly greater than its inhibition constant against KIV dehydrogenase activity. These mutual inhibitory interactions among the branched-chain α-keto acids would thus enable the organism to maintain cellular homeostasis by permitting the preferential oxidation of a particular keto acid present in excess amounts. This phenomenon could attain a significant physiological role in the event of a disparity in the concentration of these metabolites in the tissues. In dietary intake of protein from certain sources, for example, large amounts of milk protein, which has a high leucine content (32), the situation described above would be expected to occur quite commonly.

Possibly the most important consequence of elevated branched-chain amino acid and α-keto acid concentrations in the cells is the potential modulating effect of these acids upon the utilization of carbohydrate. As can be seen in Table 9, each of the branched-chain α-keto acids is able to inhibit the oxidation of pyruvate and α-ketoglutarate. The apparent inhibition constant (K_i) for KIC against the effective pyruvate dehydrogenase activity was determined to be approximately 0.5 mM. When the level of KIC in plasma and liver increases subsequent to high dietary consumption of protein, this K_i is sufficiently low so as to allow a marked reduction in the rate of oxidation of pyruvate to acetyl CoA.

Kinetic constants, K_m, were determined conventionally by application of the relationship shown in equation 12. Furthermore, since the inhibition of pyruvate oxidation by KIC is of the competitive type, equation 13 was applicable for the calculation of the inhibitor constants, K_i. Use of these constants then allowed for calculation of the per cent inhibition of the dehydrogenase activity with equation 14, as proposed by Nordlie (87), at any suitable levels of substrate and inhibitor (v_i as used in equations 13 and 14 refers to the initial reaction velocity in the presence of inhibitor).

$$v = \frac{V_{max}\ /\overline{pyruvate}/}{K_m + /\overline{pyruvate}/} \qquad (12)$$

$$v_i = \frac{V_{max}\ /\overline{pyruvate}/}{\left(1 + \frac{/\overline{KIC}/}{K_i}\right)K_m + /\overline{pyruvate}/} \qquad (13)$$

$$\text{Per cent inhibition} = \frac{(v - v_i) \times 100}{v} \qquad (14)$$

Pyruvate concentrations in rat liver are reported to range from 0.034 mM for 24 hour fasted animals to approximately 0.2 mM in the fed rat (88). Assuming that these results can be extrapolated to other mammals, the following situations would obtain. In mammalian liver, KIC concentrations of about 0.1 mM (lower end of the normal range, see Appendix II) would decrease the pyruvate dehydrogenase activity only slightly (five to ten per cent) in the fed animal. However, if the level of KIC reaches a somewhat elevated concentration, such as is known to occur after consumption

of increased amounts of protein, inhibition is considerably greater (about 35 per cent). Under these conditions, this represents nearly a four-fold potentiation in the inhibition of pyruvate oxidation. By comparison, KMV concentrations in the normal range of 0.08 mM to 0.2 mM (see Appendix II) would result in merely a two to five per cent inhibition of pyruvate dehydrogenase activity in the fed animal.

In contrast, the oxidation of α-ketoglutarate is less likely to be influenced by these conditions, since the reported levels of plasma and liver branched-chain α-keto acids remain somewhat below the apparent K_i values for KIC, KMV and KIV against the α-ketoglutarate dehydrogenase activity. By way of illustration, continued administration of a 25 per cent casein hydrolysate to diet-stabilized rats caused the concentration of α-ketoisocaproate to rise significantly, to a value of approximately 0.4 mM. This concentration is considerably lower than the estimated K_i (2.4 mM) for KIC.

A second, indirect effect of elevated KIC, KMV and KIV lies in the fact that the catabolism of these compounds leads ultimately to succinyl-CoA and acetyl-CoA, each of which has been reported to inhibit the enzymatic functions of α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (89). The combined effects of these direct and indirect inhibitions by branched-chain α-keto acids on the metabolism of pyruvate and α-ketoglutarate could thus constitute a significant physiological regulatory phenomenon. In this way, the branched-chain α-keto acids may be able to promote their own catabolism, since oxidation of excess branched-chain compounds would be favored, until normal cellular concentrations are reattained, at which time the inhibitory effects would become slight.

Other potential points of inter-regulation of branched-chain α-keto acid catabolism are suggested by the effects of added Mg^{+2} and ATP. Linn, Pettit and Reed (90) have recently reported that mammalian pyruvate dehydrogenase is inactivated by ATP in the presence of a specific kinase enzyme. A phosphatase, in the presence of Mg^{+2}, reactivates the pyruvate dehydrogenase complex. While speculation that a similar sequence of events may function to regulate the catabolism of the branched-chain α-keto acids is intriguing, the ultimate proof of such a mechanism will depend upon eventual isolation of the dehydrogenase complex. The dramatic stimulation of branched-chain α-keto acid dehydrogenase activity of whole mitochondria by Ca^{+2} and inorganic phosphate is not understood. A similar effect of Ca^{+2} on this activity was noted by Wohlheuter and Harper (11) in rat liver mitochondria. They also utilized rather high concentrations of phosphate buffer (150 mM), but apparently did not investigate the effects of this agent on the branched-chain α-keto acid dehydrogenase activities. Inorganic phosphate (91) and Ca^{+2} (92) are known to be potent mitochondrial swelling agents at the concentrations utilized in the assay medium upon which these studies are largely based. The possibility that changes in the structural state of mitochondria give rise to the observed enzymatic changes can not be disregarded. The relative intramitochondrial concentrations of Mg^{+2}, Ca^{+2}, ATP and P_i may provide a finely intermeshed regulatory system to supplement the major regulatory aspects mentioned earlier in this discussion.

Interposition of a physical barrier such as a biological membrane between a metabolite and an enzyme functioning in the conversion of that metabolite may introduce an additional regulatory aspect. The selective permeability of the inner membrane of mammalian mitochondria could constitute such a barrier. If the metabolite is formed outside of this membrane, and is to be catabolized inside of the membrane, that substrate must either diffuse through or be transported across the membrane barrier. The enzyme localization studies presented here, which indicate that the branched-chain α-keto acid dehydrogenase oxidation site is on the outer face of the inner membrane, would apparently abolish the consideration of permeation of substrate or translocase action as a factor controlling the oxidation of the branched-chain α-keto acids. In contrast, the mixed type of inhibition of α-ketoglutarate dehydrogenase activity, which was noted above, could in theory reflect dual inhibitory effects of the branched-chain α-keto acids at the α-ketoglutarate translocase (53) site, and at a catalytic site on the enzyme within the mitochondrial inner membrane. Although the localization studies predict that pyruvate dehydrogenase faces the matrix side of the inner membrane, earlier studies have demonstrated that pyruvate can transverse the inner membrane with little difficulty (93).

Among the outstanding biochemical characteristics of the disease branched-chain ketoaciduria are (a) abnormal accumulation of leucine, isoleucine and valine in the brain, liver, plasma and spinal fluid; (b) a corresponding increase in the branched-chain

α-keto acid (KIC, KMV, KIV) concentrations in these same tissues; and (c) extraordinarily large amounts of these amino and α-keto acids in the urine of the diseased patients (17). The distinctive maple syrup odor of the urine and other bodily secretions has been attributed to the accumulation of a polymeric form of α-hydroxybutyrate derived from α-ketobutyrate (94,95), or to the presence of α-keto-β-methylvaleric acid (KMV) (19). Menkes (17) reported that the excretion of pyruvate and α-ketoglutarate in the urine of persons with branched-chain ketoaciduria was normal. In a more recent communication, however, Woody (96) indicates that α-ketoglutarate levels of the urine increase significantly in those patients showing advanced neurological symptoms of the disease. Increased excretion of this compound could be a consequence of the effective inhibition of α-ketoglutarate oxidation by the branched-chain α-keto acids or alternatively by an inhibitory effect of these keto acids on reabsorption of KG in the renal tubules, as was suggested by Lysiak et al. (97).

Inhibition of the activity of pyruvate dehydrogenase by KIC has been investigated semi-quantitatively in rat liver (37,39) and brain (37). Kanzaki et al. (38) have shown that KMV and KIV inhibit the activity of the purified pyruvate dehydrogenase from pig heart. They did not establish the inhibitory capacity of KIC upon pyruvate dehydrogenase activity because they considered this keto acid as a substrate. Their results, which describe an apparent Michaelis constant value of 4.3×10^{-2} M for KIC, indicate that this branched-chain α-keto acid is an extremely poor substrate for the

pyruvate dehydrogenase complex. Normal levels of KIC in the liver cell are two to three orders of magnitude below the K_m that they have specified. In general, the results obtained here dealing with the effects of the branched-chain α-keto acids on bovine liver pyruvate dehydrogenase correspond closely to those determined for the purified complex. As was noted earlier, the branched-chain α-keto acids are also capable of inhibiting the oxidative decarboxylation of α-ketoglutarate. Based upon the apparent inhibition constants (Table 9), each of which was found to be near 10^{-3}M, and the reported concentrations reported for the branched-chain α-keto acids in the branched-chain ketoaciduria individuals (see Appendix II), a significant depression of α-ketoglutarate dehydrogenase activity would be likely to result. That the inhibitory effects on α-ketoglutarate dehydrogenase are evident only when tissue levels of KIC, KMV and KIV are highest is consistent with the previous observation that α-ketoglutarate accumulates in the plasma and is excreted in abnormal amounts by the kidney during the semi-comatase, ataxic state of the terminal stages of the disease.

Although the exact cause of the neurological pathology commonly associated with branched-chain ketoaciduria is not known, a correlation between the neurologic symptoms and elevated leucine and KIC has been established (19). The results of the mutual inhibition studies which were summarized in Table 9 disclose that KIC is the most potent inhibitor of pyruvate oxidation among the three branched-chain keto acids. Concentrations of KIC have been reported to exceed 2.5 mM in plasma, and 0.8 mM in brain tissue of

BCKA patients (18,19,37). It is evident that the metabolism of pyruvate dehydrogenase would be severely impaired by these concentrations of KIC, which range from two to five times the calculated value for the K_i (0.54 mM) of this branched-chain keto acid. The effects of KMV and KIV on the oxidative decarboxylation of pyruvate would be minimal by comparison to those of KIC, in view of their considerably higher inhibition constants on pyruvate dehydrogenase.

The observed accumulation of α-ketobutyrate in tissues of persons afflicted with branched-chain ketoaciduria may also result from inhibition of the pyruvate dehydrogenase complex, if in fact this enzyme is the primary decarboxylase of α-ketobutyrate.

The decreased production of acetyl CoA from pyruvate, which is a natural result of the obstruction of pyruvate dehydrogenase activity, has at least two major consequences. The first, and probably the more important of these effects, is the limitation placed upon the amount of acetyl-CoA entering the tricarboxylic acid cyle from carbohydrate metabolism. This phenomenon, coupled with the simultaneous inhibition of α-ketoglutarate dehydrogenase activity, would greatly diminish the total energy production by the affected tissues. Secondly, reduced availability of acetyl-CoA would have a depressing effect upon lipid synthesis, necessary for the deposition of myelin in the developing brain. Decreased myelination in certain primitive areas of the brain has been proposed as a contributory factor to the mental deficiencies of untreated branched-chain ketoaciduria (94). Elevated levels of KIC are also

theorized to affect the synthesis of the cerebronic acids which are essential for myelin formation (34). It should be noted that clinical studies indicate that the lack of myelination which does occur is probably not in itself sufficient to account for the acute neurological syndrome (19,34).

Finally, two other effects of elevated branched-chain α-keto and amino acids have been noted. Concentrations of these amino acids in the range of 10^{-3}M are known to inhibit the activity of ornithine-keto acid transaminase (OKT), an enzyme which has a role in regulating the activity of the Krebs urea cycle (98). Impaired function of this pathway would be expected to have an undesirable influence upon the nitrogen metabolism of the organism. Excessive leucine (2 mM) and alloisoleucine are known to induce hypoglycemia and to inhibit gluconeogenesis from lactate (40,41,42). The site of this inhibition has not been elucidated.

In general, then, a variety of potentially deleterious consequences of the reduced ability of the organism to metabolize α-ketoglutarate and pyruvate may be expected to underlie the symptoms and pathology of branched-chain ketoaciduria. The catabolism of carbohydrate by the Krebs tricarboxylic acid cycle, to which pyruvate dehydrogenase is an important point of entry and of which α-ketoglutarate dehydrogenase is a part, is responsible for production of a significant portion of the reduced pyridine nucleotide (NADH) necessary for the subsequent formation of high energy phosphate bonds. Interference with these processes through the effective inhibition of pyruvate and α-ketoglutarate oxidation

would seriously affect the ability of the organism to maintain the desired cellular homeostasis, and would decrease the anabolic capacity of the affected tissues.

Thus, in the normal, and certainly more so in the abnormal (excess branched-chain amino and α-keto acids) mammal, fluctuations in the levels of branched-chain α-keto acids could contribute significantly to the regulation of the levels of these metabolites, to the status of the rates of pyruvate and α-ketoglutarate metabolism, and to the resulting metabolic consequences.

APPENDICES

APPENDIX I

Appendix I describes the preparation of some reagent solutions.

A. Preparation of 2 % (w/v) Digitonin Solution.

Add five ml of warm 0.25 M sucrose to 0.1 g of powdered digitonin. Mix briefly. Sonicate for one to two minutes in an ultrasonic bath. The resulting clear solution remains clear for 30 to 60 min at 30°C.

B. Triton-Urea Reagent (4%).

Saturate 500 ml of distilled-deionized water with solid urea at room temperature. Add 4 % (by volume) Triton X-100 detergent.

C. Biuret Reagent.

Dissolve 6.0 g of sodium-potassium tartrate in 500 ml of water. Add 1.5 g of $CuSO_4 \cdot 5H_2O$. Combine with 300 ml of 10 % sodium hydroxide solution. Dilute to one liter with water.

D. Folin-Ciocalteau Reagents (for Lowry protein determinations).

Reagent A: Combined 20 g of Na_2CO_3, 4 g of solid NaOH, and 200 mg of sodium potassium tartrate. Dilute to one liter with distilled-deionized water.

Reagent B: Combine five grams of $CuSO_4 \cdot 5H_2O$ with one liter of water.

Reagent C: Combine 50 parts Reagent A with one part Reagent B. Prepare fresh daily.

APPENDIX II

Concentration ranges for some amino acids and α-keto acids in normal mammalian tissues and in human tissues from individuals with branched-chain ketoaciduria.

TABLE 10

SOME AMINO ACID AND α-KETO ACID CONCENTRATIONS IN NORMAL AND BRANCHED-CHAIN KETOACIDURIC TISSUE FLUIDS

Amino Acid	Normal Concentration Range	BCKA[a] Concentration Range	α-Keto Acid	Approximate[b] Normal Range	Approximate BCKA Range	Tissue	References
	mM	mM		mM	mM		
Leucine	0.1 - 0.5	-	α-ketoiso-caproate	0.1 - 0.5	-	rat liver	(86)(99)
Leucine	0.1 - 0.4	4-6	α-ketoiso-caproate	0.1 - 0.4	2-4	human plasma	(19)
Leucine	0.01	0.43	α-Ketoiso-caproate	0.01	0.43	human spinal fluid	(19)
Isoleucine	0.08-0.2	-	α-keto-β-methylvalerate	0.08-0.2	-	rat liver	(99)
Isoleucine	0.07-0.25	1.0-1.5	α-keto-β-methylvalerate	0.07-0.25	1.0-1.5	human plasma	(19)
Isoleucine	0.004	0.22	α-keto-β-methylvalerate	0.004	0.22	human spinal fluid	(19)
Valine	0.1 - 0.4	-	α-ketoiso-valerate	0.1 - 0.4	-	rat liver	(99)

TABLE 10 -- Continued

Amino Acid	Normal Concentration Range	BCKA[a] Concentration Range	α-Keto Acid	Approximate[b] Normal Range	Approximate BCKA Range	Tissues	References
Valine	0.24	1.85	α-ketoisovalerate	0.25	1.85	human plasma	(19)
Valine	0.014	0.32	α-ketoisovalerate	0.014	0.32	human spinal fluid	(19)
			pyruvate	0.2	-	rat liver (fed)	(88)
			pyruvate	0.03	-	rat liver (24 hr. fast)	(88)

[a] BCKA is the abbreviation for branched-chain ketoaciduria.

[b] Based on a K_{eq} of approximately one for the transamination reaction (14).

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