Incubation of 2-ketoglutarate dehydrogenase complex with 2-ketoisovalerate, 2-keto-4-methylvalerate, or 2-keto-3-methylvalerate leads to the appearance of a lag phase and of a progressive loss of activity in subsequent measurements of the initial rate of oxidation of 2-ketoglutarate. In the case of 2-ketoisovalerate these effects are shown to be due to the formation of an isobutyryl lipoate derivative of the enzyme, as a result of the very slow oxidation of 2-ketoisovalerate by the enzyme complex (V max = 0.15% of that for 2-ketoglutarate). Incubation of the enzyme complex with 2-keto[14C]isovalerate or 2-keto[14C]glutarate results in comparable incorporation of radioactivity, amounting to 3.5 to 5.3 nmol of isobutyryl or succinyl residues per mg of protein in the complex. Isobutyryl residues are also incorporated in the enzyme during the simultaneous oxidation of both of these substrates. During the early phase of incubation of the complex with 2-ketoisovalerate the incorporation of isobutyryl residues is much faster than the loss of enzyme activity. This observation seems to support the suggestion that each 2-ketoglutarate decarboxylase subunit of the complex may catalyze the succinylation of more than one lipoate succinyltransferase subunit.

Results are also presented showing the inactivation of pyruvate dehydrogenase complex on preincubation with 2-ketoisovalerate and of 2-ketoglutarate dehydrogenase complex with methylene-cyclopropylpyruvate, the keto acid corresponding to the toxic amino acid hypoglycin. The relevance of covalent modifications of the two keto acid dehydrogenase complexes to the pathological manifestations of maple syrup urine disease are discussed.

The branched chain keto acids, 2-ketoisovalerate, 2-keto-3-methylvalerate, and 2-keto-4-methylvalerate (keto forms of the amino acids valine, isoleucine, and leucine, respectively), are catabolized in the mitochondrion, the initial step being decarboxylation by the branched chain keto acid dehydrogenase, a multi-enzyme complex. In maple syrup urine disease, a rare genetic disorder, this enzyme is defective and, therefore, branched chain keto acids accumulate (1, 2). The plasma levels of these keto acids rise to the millimolar range (3, 4), and the level reached is a function of the remaining activity of the branched chain keto acid dehydrogenase of the patient. In the heart tissue of normal rats the combined concentration of the three branched chain keto acids is about 4 mM, while the plasma level is 40 μM (5). One would expect the concentration in the heart tissue of patients afflicted with the disease to rise in response to the increased level of branched chain keto acids found in circulating fluids.

The increased excretion of 2-ketoglutarate in the urine of untreated patients with maple syrup urine disease (6, 7), the impaired utilization of pyruvate and of 2-ketoglutarate by tissues of such patients (6), and the inhibition of O2 uptake by rat brain slices in the presence of 2-ketoisovalerate (8), suggested many years ago that the inhibition of pyruvate and 2-ketoglutarate dehydrogenase complexes by branched chain keto acids may be an important factor in the pathology of the disease (e.g. Patrick (6)). Using standard steady state kinetic methods (9) or measurements of [14C]CO2 released from carboxyl labeled substrates (10, 11), the inhibition of 2-ketoglutarate and pyruvate dehydrogenase complexes by branched chain keto acids was demonstrated, with Ki values in the millimolar range.

In the present paper it will be shown that preincubation of these enzyme complexes with branched chain keto acids leads to the acylation of lipoate residues and that extensive inactivation ensues.

EXPERIMENTAL PROCEDURES

Materials—2-Ketoglutarate dehydrogenase and pyruvate dehydrogenase complexes were isolated from beef heart mitochondria as described by Stanley and Perham (12). The activity (at V max) of most samples of 2-ketoglutarate dehydrogenase complex ranged from 15 to 18 μmol of NADH min-1 mg protein-1, consistent with the highest specific activities reported (12, 13). Preparations of 2-ketoglutarate dehydrogenase complex were 80 to 90% pure as judged by the intensity of staining with Coomassie blue on sodium dodecyl sulfate polyamide gels after electrophoresis. Some preparations, as isolated, showed a lag phase in assay, which was abolished by preincubation with 2 mM NAD+ and 0.2 mM thiamin pyrophosphate at 30 °C prior to assay. This procedure also led to a variable (up to 40%) increase in the maximum rate in subsequent assays. Where necessary, these activating agents were removed by rapid centrifugation through Sephadex G-50 (14). Removal of the activators did not lower the specific activity. Preparations of the pyruvate dehydrogenase complex were approximately 90% pure with a specific activity of ~12 μmol of NAD+ reduced min-1 mg protein-1.

1-Amino acid oxidase (twice recrystallized) was prepared by the method of Wellner and Meister (15) and general fatty acyl CoA dehydrogenase from pig kidney was a gift of Dr. C. Thorpe, University of Delaware. 2-Ket0[U-14C]glutarate and [U-14C]valine were obtained from ICN. The specific activity and purity of the former were assessed as described (16). 2-Keto[U-14C]isovalerate was prepared from [U-14C]valine (17) using purified 1-amino acid oxidase. The absence of impurities was ascertained by thin layer chromatography, and the
Fig. 1. Lag phase in the assay of the 2-ketoglutarate dehydrogenase complex following inactivation with branched chain keto acids. The complex was assayed spectrophotometrically at 30 °C in a solution containing 2 mM 2-ketoglutarate, 0.12 mM CoA, 2 mM NAD⁺, 0.2 mM thiamin pyrophosphate, 2 mM MgCl₂, 0.5 mM CaCl₂, and 2.6 mM cysteine in 50 mM Mops buffer, pH 7.6. Preincubation of the enzyme was conducted at 20 °C in 50 mM Mops, pH 7.6, containing 2 mM MgCl₂, 0.5 mM CaCl₂, and 0.2 mM thiamin pyrophosphate. Symbols: control sample (---); preincubated with 2 mM 2-ketoisovalerate for 30 min (- - -); preincubated with 2 mM 2-keto-4-methylvalerate for 30 min (● - ●).

Methods—The activity of the 2-ketoglutarate dehydrogenase complex was assayed by following the reduction of NAD⁺ at 340 nm (13), as described in the legend to Fig. 1. The same method was used for assay of the pyruvate dehydrogenase complex, except for the substitution of 2 mM pyruvate as substrate. 2-Ketoglutarate and pyruvate decarboxylase activities (also called “dehydrogenase” activities in the literature) were determined by following the reduction of 1 mM ferricyanide at 420 nm (21) by 2 mM keto acid in 50 mM potassium phosphate, pH 6.5, containing 2 mM MgCl₂, 0.5 mM CaCl₂, and 0.2 mM thiamin pyrophosphate at 30 °C. Other enzymes were assayed by published procedures, as follows: general fatty acyl-CoA dehydrogenase (22), lipoamide dehydrogenase (23), and NADH-5,5′-dithiobis-(2-nitrobenzoate) reductase (24). The latter reaction, catalyzed by lipoamide dehydrogenase, uses the enzyme-bound lipoic acid of lipoate decarboxylase (25), and NAD⁺, NADH, and Triton X-100 were from Sigma. Triton was purified as described (20).

Radioactivity covalently bound to protein was determined as follows. Concentration of 2-keto-[14C]isovalerate was determined by the procedure of Coleman and Armstrong (18), with unlabeled 2-ketoisovalerate as a standard. Hypoglycin was a gift of Dr. E. A. Keen, Kingston, Jamaica, and was converted to methylencyclopropionate (19). The sodium salts of 2-ketoisovalerate, 2-keto-3-methylvalerate, and 2-keto-4-methylvalerate, and coenzyme A, isobutyryl coenzyme A, NAD⁺, NADH, and Triton X-100 were from Sigma. Triton was purified as described (20).

Inactivation of 2-Ketoglutarate Dehydrogenase Complex by Incubation with Branched Chain Keto Acids—Preincubation of the 2-ketoglutarate dehydrogenase complex at 20 °C with 2 mM 2-ketoisovalerate or 2-keto-4-methylvalerate in the presence of thiamin pyrophosphate, Mg²⁺, and Ca²⁺ resulted in a lag phase in subsequent assays with 2-ketoglutarate as substrate (Fig. 1). This lag phase increased with the length of preincubation, and a progressive diminution of the maximal rate of 2-ketoglutarate oxidation was also observed (Fig. 2). Whereas preincubation with 2 mM 2-ketoisovalerate and 4 mM 2-keto-3-methylvalerate produced comparable decreases in the initial rate (Fig. 2A) and the maximal rate of 2-ketoglutarate oxidation (Fig. 2B), on preincubation with 2 mM 2-keto-4-methylvalerate loss of initial activity was slower (Fig. 2A), but loss of maximal activity (Fig. 2B) was more extensive.

Mechanism of the Inactivation—A sample of the complex (0.39 mg of protein ml⁻¹) was incubated for 90 min with 2 mM 2-ketoisovalerate as described in Fig. 1. This led to a 95% slower initial rate and an 87% reduction of the maximal rate of oxidation of 2-ketoglutarate in subsequent assays. Extensive dialysis against 50 mM Mops, pH 7.6, at 4 °C did not reactivate the enzyme, suggesting that a covalent modification of one of the proteins of the complex had occurred. Moreover, radioactivity became progressively incorporated into the complex when 2-keto[U-¹⁴C]isovalerate was used for inactivation.

The simplest interpretation of these observations is that the branched chain keto acids are decarboxylated to form an adduct with the thiamin pyrophosphate bound noncovalently to 2-ketoglutamate decarboxylase and that the lipoate residue of lipoate succinyltransferase is then acylated to form a thioester. This hypothesis would predict that the 2-keto acids are substrates for the complex, provided that the enzyme could...
transfer the acyl group from lipoate to CoA. The relative rate of oxidation of 2-ketoisovalerate by the complex, as measured by $V_{\text{max}}$ for the formation of NADH, was, in fact, 0.15% of the rate for 2-ketoglutarate, while $V_{\text{max}}$ for 2-keto-3-methylvalerate and 2-keto-4-methylvalerate were 0.04 and 0.06% of the rate of 2-ketoglutarate oxidation, respectively.

While it is difficult to rule out contamination of our preparations by traces of branched chain keto acid dehydrogenase complex, the polypeptides of that enzyme could not be detected following polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Furthermore, the $K_m$ value obtained for 2-ketoisovalerate, 0.33 mM, is 10-fold higher than that reported for the branched chain keto acid dehydrogenase (13 to 40 $\mu$M (29-31)). Additional evidence that 2-ketoglutarate dehydrogenase complex catalyzes the oxidation of 2-ketoisovalerate will be found in the labeling experiments presented below.

Incorporation of Radioactivity during Processing of Labeled 2-Ketoisovalerate and Its Reversal—If the inactivation of the 2-ketoglutarate dehydrogenase complex by 2-ketoisovalerate (and the analogous reactions with other keto acids) was due to the formation of isobutyryllipoate, the resulting thioester bond should be labile to performic acid (32). To test this hypothesis, the complex (1.55 mg of protein $\text{ml}^{-1}$) was incubated anaerobically for 2 h at 4°C with 0.285 mM 2-keto[U-14C]isovalerate in 100 mM Mops buffer, pH 7.0, containing 2 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.2 mM thiamin pyrophosphate. Aliquots were removed at 1 and 2 h and radioactive incorporation into the complex was determined before and after exposure to performic acid vapor, as described under "Methods." The samples not exposed to performic acid showed 3.5 and 4.6 nmol of isobutyryl residue incorporated per mg of complex at 1 and 2 h, respectively. Performic acid removed 95% of the enzyme-bound radioactivity in each experiment, showing that a thioester linkage was, indeed, involved.

Treatment of the modified enzyme with 0.5 mM CoA and 4 mM NAD$^+$ at 30°C (see under "Methods") also liberated 95% of the protein-bound radioactivity, with an accompanying increase in the specific activity of the 2-ketoglutarate dehydrogenase complex from 10% of that of untreated samples before addition of CoA and NAD$^+$ to 80% after 20 min of incubation. In the experiments of Fig. 3, enzyme inactivated with 2 mM 2-ketoisovalerate was incubated with NAD$^+$, CoA, and thiamin pyrophosphate, with restoration of full activity. Control experiments showed that of these additions, only CoA is effective in reversing the inactivation. The transfer of isobutyryl residues from lipoate to CoA with relief of inhibition, confirms that 2-ketoisovalerate is a substrate for the overall reaction of the complex. Incubation with CoA also abolishes the lag phase in assays. The lag phase produced by branched chain keto acids (Fig. 1) is thus distinct from that seen in occasional samples of untreated enzyme, which is unaffected by CoA, but is abolished by preincubation with NAD$^+$ and thiamin pyrophosphate.

In order to determine the stoichiometry of incorporation of isobutyryl residues into the enzyme complex, the latter was incubated anaerobically with 0.3 mM 2-keto[U-14C]isovalerate in 100 mM Mops, pH 7.0, containing 2 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.2 mM thiamin pyrophosphate at 4°C. The extent of incorporation was in the range of 3.5 to 5.3 nmol of isobutyryl residue per mg of protein in the complex in several experiments. Increasing the concentration of 2-ketoisovalerate to 0.5 mM or the temperature to 25°C increased the rate but not the extent of incorporation. The incorporation of succinyl residues from 2-keto[U-14C]glutamate in analogous studies was the same as that of isobutyryl residues for each preparation, although it occurred far more rapidly. Label originating from either substrate was over 95% released on exposure to performic acid vapor for 48 h. Hence, 2-ketoglutarate and 2-ketoisovalerate seem to form thioester bonds with the same residues in the enzyme complex.

Calculation of the stoichiometry of incorporation per lipoic acid residue in the 2-ketoglutarate dehydrogenase complex is difficult, because no reliable values have been reported for the lipoate content of the enzyme from mammalian sources. The only analyses reported in the literature (33) used alkaline hydrolysis and a microbiological assay, which are not conducive to great accuracy. Using the data given above, and assuming that the subunit stoichiometry of the beef heart 2-ketoglutarate dehydrogenase complex is decarboxylase:lipoate succinyltransferase:lipoamide dehydrogenase = 12:24:12 and the molecular weight 2,700,000 as reported (33), the stoichiometry of incorporation of isobutyryl (or succinyl) residues would be 0.4 to 0.6 mol per mol of lipoate succinyltransferase. If the mammalian enzyme complex, like the enzyme from *Escherichia coli* (34-36), contains 1 mol of lipoate per mol of lipoate succinyltransferase, as seems likely, the incorporation would be 0.4 to 0.6 mol of residue per mol of lipoic acid residue. We have, as yet, no explanation for the apparently substoichiometric labeling of the lipoate residues.

Other Evidence for the Proposed Mechanisms—If the mechanism of inactivation of the 2-ketoglutarate dehydrogenase complex by branched chain keto acids involves the formation of lipoate thioesters which can only slowly transfer their acyl moieties to CoA, it should be possible to inactivate the enzyme with isobutyryl-CoA and NADH, by reversal of the lipoate succinyltransferase and lipoamide dehydrogenase reactions. Fig. 4 shows that this is indeed the case.

If acylation of the lipoate residues of lipoate succinyltransferase were the cause of inactivation, only those reactions of the enzyme complex which involve lipoic acid should be inhibited. It was indeed found that the complete reaction of the complex, as measured by the reduction of NADH, and the reduction of 5,5'-dithiobis-(2-nitrobenzoate) by NADH, reac-
formation of isobutyryllipoate and loss of 2-ketoglutarate dehydrolase was incubated with 50 µM unlabeled 2-ketoisovalerate in 100 mM Mops, pH 7.6, containing 2 mM MgCl₂, 0.5 mM CaCl₂, 2.6 mM cysteine, and 0.2 mM thiamin pyrophosphate with 1 mM isobutyryl-CoA and 0.2 mM NADH present (C). Control samples were similarly incubated but without isobutyryl-CoA (C), without NADH (E), or without either of these present (C). The initial rate of NADH reduction was measured on aliquots and is expressed as the percent of the rate at the start of the incubation.

Relation of the Loss of Activity to the Formation of Isobutyryllipoate—To determine the relationship between the formation of isobutyryllipoate and loss of 2-ketoglutarate dehydrogenase complex activity, the enzyme (0.088 mg ml⁻¹) was incubated with 50 µM 2-keto[¹⁴C]isovalerate in 100 mM Mops, pH 7.0, containing 2 mM MgCl₂, 0.5 mM CaCl₂, 4 mM NAD⁺, and 0.2 mM thiamin pyrophosphate at 15°C. At intervals, samples were removed, precipitated with cold 10% (w/v) trichloroacetic acid, and counted. Another sample of the enzyme was incubated with 50 µM unlabeled 2-ketoisovalerate under the same conditions, and at intervals aliquots were reacted in a stopped flow apparatus at 15°C in a 1:1 ratio with an assay mixture to give final concentrations of 5 mM 2-ketoglutarate, 0.12 mM CoA, 2 mM NAD⁺, and 0.2 mM thiamin pyrophosphate in 100 mM Mops, pH 7.0, containing 2 mM MgCl₂, 0.5 mM CaCl₂, and 2.6 mM cysteine. (The 25 µM 2-ketoisovalerate carried over into the assay did not affect the initial rate significantly.) No reactivation was noted within the first 4 s following mixing. Rates of NAD⁺ reduction were routinely measured at 0.25 to 0.5 s after mixing at 15°C. In order to assure that no loss of the bound moiety occurred under the conditions of the stopped flow assay within 0.5 s, a sample of the enzyme complex (0.086 mg protein/ml) was incubated with 2-keto[¹⁴C]isovalerate for 30 min under the conditions given above, and a sample was then rapidly mixed with an equal volume of 10 mM 2-ketoglutarate, containing all the other components used in the stopped flow assay. Aliquots precipitated with trichloroacetic acid and counted, as above, showed 2.8 ± 1.5% (S.E.) of the radioactivity released within 20 s after mixing. Hence, it is valid to compare the kinetics of the labeling of the enzyme by isobutyryl residues with the rate of inactivation, as in Fig. 5. It may be noted that the loss of activity shows a lag not seen in the incorporation of isobutyryl residues. By plotting these parameters against each other (Fig. 6), it is evident that labeling of 20 and 40% of the available binding sites results in only 5 and 11% of inactivation, respectively. By the time 1 mol of isobutyryl residue was incorporated per mol of site, no reduction of NAD⁺ could be detected within 10 s in stopped flow experiments, although over longer periods of assay (1 min) some reactivation was noted. Contamination by pyruvate dehydrogenase complex (present at about 2% of the concentration of 2-ketoglutarate dehydrogenase complex) was far too small to account for the discrepancy between the initial loss of activity and incorporation of label.

Incorporation of Isobutyryl Residues in the Presence of 2-Ketoglutarate and CoA—The data presented so far suggest that the lipoate residues of the 2-ketoglutarate dehydrogenase complex are acetylated by these branched chain keto acids and that the acyl residues formed are only slowly transferred to CoA. However, within the mitochondrion the natural substrate, 2-ketoglutarate, will compete with the branched chain keto acids and the presence of CoA will permit a degree of
decacylation of the lipoate.

During initial rate assays in the presence of inhibitor (the reaction being started with enzyme), the rate of reduction of NAD+ decreases much more in the course of the assay than in the control without inhibitor, particularly at high concentrations of the inhibitor. This is probably due to the rapid formation of isobutyryllipoate leading to quasi-irreversible inhibition. Under these conditions the steady state assumptions will be invalid. The failure to appreciate that these keto acids inhibit by covalent modification, as well as by competing at the 2-ketoglutarate-binding site, may explain some of the discrepancies in the literature.

Nevertheless, during the first few seconds after the mixing of the reactants the absorbance change is sufficiently linear to suggest that the quasi-irreversible loss of activity is not yet a significant factor. From such initial rates the occurrence of negative cooperativity was readily confirmed in the presence or absence of inhibitor (Fig. 7). Moreover, in the range of 0-20 mM 2-ketoisovalerate, the $K_{i}$ for 2-ketoglutarate increased from 0.3 to 1.2 mM, whereas $V_{max}$ remained unchanged (Table II). Thus, 2-ketoisovalerate appears to be a competitive inhibitor with respect to 2-ketoglutarate. 2-Keto-3-methylvalerate and 2-keto-4-methylvalerate were found to be considerably weaker inhibitors than 2-ketoisovalerate, yielding at 0 to 5 mM inhibitor a slight decrease in $V_{max}$ and only a marginal increase in $S_{max}$. Although the presence of negative cooper-

![Figure 7](http://www.jbc.org/)

**TABLE I**

Incorporation of isobutyryl residues into 2-ketoglutarate dehydrogenase complex during the simultaneous oxidation of 2-ketoglutarate and 2-ketoisovalerate

| Concentration of 2-ketoglutarate dehydrogenase complex | Incorporation of isobutyryl residues | Concentration of inhibitor |
|--------------------------------------------------------|-------------------------------------|---------------------------|
| 0.25 mg protein/ml                                      | 0.81 ± 0.06                         | 0.25 mM                    |
| 0.26                                                     | 0.27 ± 0.056                        | 0.26                       |
| 0.39                                                     | 0.16 ± 0.029                        | 0.39                       |
| 0.45                                                     | 0.58 ± 0.06                         | 0.45                       |
| 0.26                                                     | 0.19 ± 0.025                        | 0.26                       |
| 0.36                                                     | 0.14 ± 0.008                        | 0.36                       |
| 0.50                                                     | 0.68 ± 0.11                         | 0.50                       |
| 0.26                                                     | 0.24 ± 0.068                        | 0.26                       |
| 0.39                                                     | 0.12 ± 0.026                        | 0.39                       |

* Since the CoA concentration was 0.5 mM, the reaction was complete when 0.5 mM NAD+ had been reduced.

The kinetic parameters were estimated from Eadie-Hofstee plots (Fig. 7) of the initial rate of 2-ketoglutarate oxidation.

![Figure 8](http://www.jbc.org/)

**TABLE II**

Kinetic parameters for the inhibition of 2-ketoglutarate dehydrogenase by 2-ketoisovalerate

The kinetic parameters were estimated from Eadie-Hofstee plots (Fig. 7) of the initial rate of 2-ketoglutarate oxidation.

| Concentration of 2-ketoisovalerate | $S_{max}^{0.5}$ for 2-ketoglutarate | $V_{max}$ |
|-----------------------------------|-----------------------------------|-----------|
| 0                                 | 0.27±0.32                         | 17.3±0.2  |
| 2                                 | 0.38                              | 16.7±0.5  |
| 5                                 | 0.57                              | 17.1      |
| 10                                | 0.75                              | 18.3      |
| 20                                | 1.20                              | 17.5      |
tivity precludes the conventional calculation of $K_i$ values, in our hands the inhibition of the purified enzyme complex from beef heart by these two keto acids is much less than has been reported by others (9-11).

In the other experiments, isobutyryl-CoA, the product of oxidative decarboxylation of 2-ketoisovalerate, was found to be a competitive inhibitor with respect to CoA, with an apparent $K_i$ of 0.55 mM for the range of concentrations of CoA over which substrate inhibition cannot be detected (37).

**Effect of Branched Chain Keto Acids on Pyruvate Dehydrogenase Complex**—Incubation of the pyruvate dehydrogenase complex from beef heart with 2 mM 2-ketoisovalerate prior to assay leads to extensive loss of activity in the overall reaction, whether initial rates of pyruvate oxidation or the maximal rate attained are measured (Fig. 8). The characteristics of the inactivation were found to be very similar to those observed with the 2-ketoglutarate dehydrogenase complex. Thus, the loss of activity was not reversed by dialysis and affected only those activities of the pyruvate dehydrogenase complex in which the lipoic acid component of lipoate acetyltransferase participates (i.e. the overall reduction of NAD$^+$ by pyruvate and the NADH-dependent reduction of 5,5'-dithiobis(2-nitrobenzoate)), whereas the lipoamide dehydrogenase reaction and the reduction of Fe(CN)$_3$$^-$$^-$ by pyruvate, catalyzed by the decarboxylase, were unaffected.

Incubation of the complex with 2 mM 2-keto-4-methylvalerate or 4 mM DL-2-keto-3-methylvalerate under the conditions of the experiment in Fig. 8 produced no inactivation. As expected, these keto acids were also not perceptibly oxidized by the pyruvate dehydrogenase complex, while 2-ketoisovalerate was oxidized, as determined by NADH production, but only at 0.05% of the maximal rate of pyruvate oxidation even at 20 mM.

2-Ketoisovalerate was a competitive inhibitor with respect to pyruvate in initial rate studies (i.e. without preincubation) with an apparent $K_i$ of 1.3 mM. Although this is close to the value reported by Johnson and Connelly (10) (1.4 mM), but lower than that reported by Kanzaki et al. (9) (3.4 mM), the similarity may be fortuitous, as in the experiments quoted (10) $K_i$ values were derived from assays of 20-min duration, during which time covalent modification was likely to have occurred.

**Inactivation of the 2-Ketoglutarate Dehydrogenase Complex by Methylene cyclopropylpyruvate**—Methylene cyclopropylpyruvate, the keto form of hypoglycin, contains a cyclopropyl ring that is analogous to the branched side chain of the keto acids considered above. This compound had no effect on initial rates of the 2-ketoglutarte dehydrogenase complex at a concentration of 2 mM, but major and progressive inactivation was produced on preincubation with 1 mM methylene cyclopropylpyruvate prior to assay (Fig. 9). As in the case of branched chain keto acids (Fig. 1), the partially inactivated enzyme shows a lag phase in assay. The lag phase was abolished and the activity regenerated by incubation of the inactivated enzyme complex for 5 min at 30 °C with the assay mixture (minus 2-ketoglutarate), showing that the adduct formed with the lipoate moiety can be slowly transferred to CoA. In a typical experiment after 2-h incubation with 1 mM methylene cyclopropylpyruvate the activity (initial rates) fell from 8.6 to 0.89 μmol of NADH produced per min per mg, but 5-min incubation with the assay mixture raised the activity to 7.6 μmol of NADH per min per mg.

The inactivation could also be reversed and the lag phase in assays abolished by 0.45 mM hydroxylamine, which presumablycleaves the thioester in the inactivated enzyme, forming lipoic acid and methylene cyclopropylacetylhydroxamate.

That inactivation by this keto acid results from esterification of the lipoic acid residues of lipoate succinyltransferase, as has been concluded for the action of branched chain keto acids, is supported by the facts that only lipoate-dependent

**Fig. 8. Inactivation of the pyruvate dehydrogenase complex on incubation with 2-ketoisovalerate.** The complex (0.3 mg of protein per ml) was incubated with 2 mM 2-ketoisovalerate in 50 mM Mops, pH 7.6, containing 2 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 0.2 mM thiamin pyrophosphate at 20 °C, and aliquots were assayed at intervals by following the reduction of NAD$^+$. The activity is expressed as the per cent of the rate given by untreated samples, ||initial rate; □, maximal rate attained in the assay, ■, activity of a control sample incubated without 2-ketoisovalerate. Note that in the latter the initial and maximal rates are the same.

**Fig. 9. Inactivation of the 2-ketoglutarte dehydrogenase complex by methylene cyclopropylpyruvate.** The complex at 0.38 mg of protein per ml was incubated with 2-ketoglutarate dehydrogenase complex (0.38 mg of protein per ml) at 20 °C in the presence of 5 μM general fatty acyl-CoA dehydrogenase, 1.2 mM CoA, 0.2 μM thiamin pyrophosphate, 2 mM NAD$^+$, 100 mM Mops, pH 7.6, 2 mM MgCl$_2$, 0.5 mM CaCl$_2$, and 2.6 mM cysteine. The ordinate represents the per cent of the activity of the general fatty acyl-CoA dehydrogenase remaining. Note that prior to assay of the latter activity, cysteine and unreacted CoA were removed with 5 μM 5,5'-dithiobis(2-nitrobenzoate). This treatment had only a trivial effect on fatty acyl-CoA dehydrogenase activity. ○, complete mixture; □, 2-ketoglutarate dehydrogenase complex omitted; △, methylene cyclopropylpyruvate omitted from incubation mixture.
reactions were affected by the inactivation, whereas the reduction of Fe(CN)$_6^{3-}$ by 2-ketoglutarate and the reactions of lipoamide dehydrogenase were not.

The oxidation of methylenecyclopropylpyruvate to methylenecycloproplacetetyl-CoA was too slow to be conveniently detected by NADH formation in the complete assay system (less 2-ketoglutarate). It could be readily followed, however, by measuring the suicide inhibition of the general fatty acyl-CoA dehydrogenase by methylenecyclopropylacetetyl-CoA (38) (Fig. 10). Incubation of the pyruvate dehydrogenase complex with 1 mM methylenecyclopropylpyruvate led to no detectable inactivation, in agreement with the results of Kean and Pogson (39).

**DISCUSSION**

It is clear that the 2-ketoglutarate dehydrogenase complex from beef heart has a broader specificity for keto acids than has been previously reported for this enzyme from mammalian tissues, although a broad specificity has been noted in studies of the reverse reaction, i.e. the NADH-dependent acylation of lipoate residues (40). 2-Ketoglutarate dehydrogenase complex catalyzes the oxidative decarboxylation of 2-ketoisovalerate, 2-keto-3-methylvalerate, 2-keto-4-methylvalerate, and of methylenecyclopropylpyruvate. The failure to detect the oxidation of 2-ketoisovalerate by the complex from pig heart (9, 30) may be due to the slow rate, which would be easily overlooked unless a sufficiently high concentration of enzyme were used. However, Kanzaki et al. (9) did note that the enzyme catalyzed the reduction of Fe(CN)$_6^{3-}$, but not that of NAD$^+$, by 2-ketoisovalerate. We found that pyruvate dehydrogenase complex also catalyzes the oxidative decarboxylation of 2-ketoisovalerate, but the high activity toward 2-keto-4-methylvalerate previously reported (9) was not detected.

An important conclusion from this study is that the catalytic activity of the 2-ketoglutarate dehydrogenase complex toward keto acids is determined not only by the substrate site of the decarboxylase moiety, but also by the catalytic site of lipoate succinyltransferase for the transfer of acyl residues from lipoate to CoA. Thus, the rate of this latter reaction is dependent on the nature of the acyl group bound to the lipoate.

The evidence presented in this paper indicates that the loss of activity of the two complexes caused by branched chain keto acids is determined not equally by the substrate site of the decarboxylase moiety, but also by the catalytic site of lipoate succinyltransferase. The catalytic events have been modified, provided that the catalytic events on to CoA. This model predicts that the loss of activity should be proportional to the fraction of lipoic acid modified, as was found for the inactivation of the 2-ketoglutarate dehydrogenase complex of E. coli by N-ethylmaleimide in the presence of 2-ketoglutarate (34). 2) Each 2-ketoglutarate dehydrogenase subunit catalyzes the succinylation of a specific lipoate residue and the succinyl residue is then directly transferred to CoA. This model predicts that loss of activity be initially less extensive than incorporation of acyl residues derived from the inhibitor. 3) Each 2-ketoglutarate dehydrogenase subunit catalyzes the succinylation of a specific lipoic acid residue, but the succinyl group is obligatorily transferred to a second lipoic acid moiety prior to being passed on to CoA. This model requires that at any time inactivation be more extensive than incorporation of acyl residues derived from the inhibitor. 4) Each 2-ketoglutarate dehydrogenase subunit catalyzes the succinylation of a specific lipoic acid residue band to separate lipoate succinyltransferase chains. This model predicts that loss of activity be initially less extensive than the incorporation of isobutyryl residues, as found in Fig. 6, because the enzyme complex remains catalytically competent even though some of its lipoate moieties have been modified, provided that the catalytic events involving lipoate succinyltransferase are not rate-limiting in the oxidation of 2-ketoglutarate. Stepp et al. (42) previously reported that loss of activity of both the pyruvate and 2-ketoglutarate dehydrogenase complexes of E. coli was slower than the removal of lipoic acid residues and lipoamidase or of the lipoate domains by trypsin digestion. Similar results for the two complexes from E. coli were interpreted by Danson et al. (43) and Berman et al. (44) in terms of the "lipoic acid takeover mechanism" summarized above (mechanism 3).

The strong dependence of the extent of formation of iso- butyrylipoate in the presence of substrates on enzyme concentration (Table I) has already been pointed out. A possible explanation is as follows. 2-Ketoglutarate and 2-ketoisovalerate compete for the first two reactions of the complex yielding thiosteres with the lipoate residues. Succinyl groups are then transferred to CoA more rapidly than isobutyryl groups, freeing the lipoate residues to which succinyl groups were bound. This permits further competition between 2-ketoglutarate and 2-ketoisovalerate and gradual accumulation of isobutyrylipoate. Since the lower its concentration the more times the enzyme must turn over to reduce the same amount of NAD$^+$, this mechanism predicts that the extent of inactivation would be inversely related to the enzyme concentration, as noted.

The observation that at low levels of incorporation the labeling of the complex by isobutyryl residues is more extensive than is the loss of activity (Figs. 5 and 6) requires an explanation. According to the accepted model (41), in the 2-ketoglutarate dehydrogenase complex of E. coli, succinyl residues originating from 2-ketoglutarate form thioester linkages with the lipoate residues of lipoate succinyltransferase, which serve as swinging arms between the three enzymes of the complex. These lipoic acids are thought to transfer succinyl residues to other lipoic acid moieties in the core of the complex from E. coli (35). The general features of the model are usually thought to apply also to the mammalian enzyme complex.

One may visualize three possible mechanisms for the cinculation of lipoate residues. 1) Each 2-ketoglutarate decarboxylase subunit catalyzes the succinylation of a specific lipoate residue and the succinyl residue is then directly transferred to CoA. This model predicts that the loss of activity should be proportional to the fraction of lipoic acid modified, as was found for the inactivation of the 2-ketoglutarate dehydrogenase complex of E. coli by N-ethylmaleimide in the presence of 2-ketoglutarate (34). 2) Each 2-ketoglutarate dehydrogenase subunit catalyzes the succinylation of a specific lipoic acid residue, but the succinyl group is obligatorily transferred to a second lipoic acid moiety prior to being passed on to CoA. This model requires that at any time inactivation be more extensive than incorporation of acyl residues derived from the inhibitor. 3) Each 2-ketoglutarate decarboxylase subunit catalyzes the succinylation of a specific lipoic acid residue band to separate lipoate succinyltransferase chains. This model predicts that loss of activity be initially less extensive than the incorporation of isobutyryl residues, as found in Fig. 6, because the enzyme complex remains catalytically competent even though some of its lipoate moieties have been modified, provided that the catalytic events involving lipoate succinyltransferase are not rate-limiting in the oxidation of 2-ketoglutarate. Stepp et al. (42) previously reported that loss of activity of both the pyruvate and 2-ketoglutarate dehydrogenase complexes from E. coli was slower than the removal of lipoic acid residues and lipoamidase or of the lipoate domains by trypsin digestion. Similar results for the two complexes from E. coli were interpreted by Danson et al. (43) and Berman et al. (44) in terms of the "lipoic acid takeover mechanism" summarized above (mechanism 3).
if two lipote residues interact with each 2-ketoglutarate dehydrogenase subunit.

It remains to discuss the relevance of the finding that branched chain keto acids inactivate the 2-ketoglutarate and pyruvate dehydrogenase complexes to the pathology of maple syrup urine disease. Elevated plasma levels of branched chain keto acids are known to occur in uncontrolled diabetes, during elevated caloabolism of protein, and especially in maple syrup urine disease (47, 48). These high levels are known to be toxic, even when the catabolism of these keto acids is normal, but the effect is aggravated in patients with maple syrup urine disease, who have a defective or deficient branched chain keto acid dehydrogenase.

The deleterious effects of branched chain keto acids on metabolism have been clearly demonstrated in studies in Williamson’s laboratory with isolated hepatocytes from normal rats (49–51). Incubation with 1 mM 2-ketoisovalerate severely inhibited fluxes through the 2-ketoglutarate and pyruvate dehydrogenase complexes. Thus, flux between oxaloacetate and succinyl-CoA decreased by 97%, with a dramatic decline in the succinyl-CoA content and a rise in the concentration of isovaleryl-CoA, propionyl-CoA, methylmalonyl-CoA, and in the NADH/NAD+ ratio, while flux through the pyruvate dehydrogenase complex diminished by 60% under these conditions. These marked effects cannot be explained by competitive inhibition of the two keto acid dehydrogenase complexes by 2-ketoisovalerate, but would not be unexpected in view of the covalent modification of the complexes as described in this paper. From our data, at 2 mM 2-ketoisovalerate and in the presence of saturating NAD+ and CoA and 0.4 mM 2-ketoglutarate (within the range of mitochondrial concentrations estimated by LaNoue et al. (52)), the inhibition of the 2-ketoglutarate dehydrogenase complex is only 8% in initial rate studies. The effect of 1 mM 2-ketoisovalerate on initial rates of the pyruvate dehydrogenase complex reaction at the expected prevailing concentration of pyruvate would be similarly negligible. Whether the increased concentrations of methylmalonyl-CoA and propionyl-CoA contribute significantly to the severe decline in flux through the 2-ketoglutarate dehydrogenase complex in the presence of 1 mM 2-ketoisovalerate (49, 50) cannot be evaluated, since the \( K_i \) values of the enzyme for these compounds are not known. However, since the \( K_i \) for isobutyryl-CoA, with respect to CoA, from our data is 0.55 mM, whereas the \( K_i \) for succinyl-CoA, with respect to CoA, is 6.9 \( \mu M \) (53) under comparable conditions, it is clear that the competitive inhibition by the increased level of isobutyryl-CoA would be more than compensated for by the drop in the level of succinyl-CoA. Fruktolysis due to the increased NADH/NAD+ ratio (54) could contribute to the decrease in flux through the 2-ketoglutarate dehydrogenase complex. The severity of the decrease, however, suggests that the drop is largely caused by another factor, probably the incorporation of covalently bound isobutyryl residues into the enzyme. In contrast, inactivation of the pyruvate dehydrogenase complex by covalent modification may be less important, since the decrease in flux through that enzyme complex is also unlikely to contribute significantly to the development of hypoglycemia. Since the oxidation of methylencyclopropyruvate by this enzyme complex is also very slow, but the action of branched chain keto acid dehydrogenase on the compound would be expected to be more rapid in view of its similarity to branched chain keto acids, the 2-ketoglutarate dehydrogenase complex is also unlikely to be the predominant system for the metabolic conversion of methylencyclopropyruvate to methylencyclopropylacetoyl-CoA.

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