The sizes of the protein subunits are not known for bromegrass mosaic and broad bean mottle virus, although preliminary experiments in both cases suggest that they will consist of roughly 190 amino acid residues. Thus the coding ratio would be about 17.

Summary.—Broad bean mottle virus has been obtained as an electrophoretically and ultracentrifugally homogeneous preparation. The molecular weight of the virus was found to be 5.20×10^6, based on a sedimentation coefficient of $(84.8-0.47c)S$ and a diffusion coefficient of 1.38×10^{-7} cm^2 per second and a partial specific volume of 0.717 ml per gm. The virus contains about 1.1×10^6 molecular weight units of ribonucleic acid.

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AN EFFECT OF L-LEUCINE AND OTHER ESSENTIAL AMINO ACIDS ON THE STRUCTURE AND ACTIVITY OF GLUTAMIC DEHYDROGENASE

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Glutamic dehydrogenase (GDH) catalyzes the interconversion of α-ketoglutarate (α-KG), a Krebs cycle component, and L-glutamate. With the crystalline protein from beef liver, it has been shown that the activity of the enzyme, which is composed of subunits, depends on its state of aggregation.[1] Our recent finding that several steroid hormones promote the reversible dissociation of the enzyme into subunits[2] suggested that control of the physical state of this protein, and perhaps of others, may be a means by which enzymic reactions are regulated. This idea

seems especially attractive since we have also found[3] that the subunits are enzymically active, but have a different substrate specificity from that of the aggregate.

Experiments described in the present communication suggest that essential amino acids could also regulate glutamate metabolism, since certain essential amino acids can affect the structure (and function) of GDH.

Materials and Methods.—Steroid hormones, diethylstilbestrol, nucleotides, and a suspension of crystalline beef liver glutamic dehydrogenase in Na_2SO_4 were obtained from the Sigma Chemical Company. The amino acids were purchased from Nutritional Biochemical Corporation, and phenanthridine was produced by Aldrich Chemical Company, Inc.

Mitochondria, separated from rat liver according to the technique of Hogeboom,[4] were suspended in 0.025 *M* phosphate buffer pH 7.5, containing 0.75 *M* KCl. These suspensions, 1 ml of which was equivalent to 125 mg of the original tissue, were frozen and thawed four times before use.

Glutamic dehydrogenase activity was assayed spectrophotometrically by following oxidation or reduction of the pyridine nucleotide.[2] Sedimentation experiments were performed in the Spinco model E analytical ultracentrifuge as a rotor speed of 59,780 essentially as described earlier.[2]

Results.—Glutamate oxidation by either DPN or TPN, catalyzed by crystalline glutamic dehydrogenase prepared from beef liver, was stimulated by L-leucine (Fig. 1, curves A and B). During the course of these experiments, four different

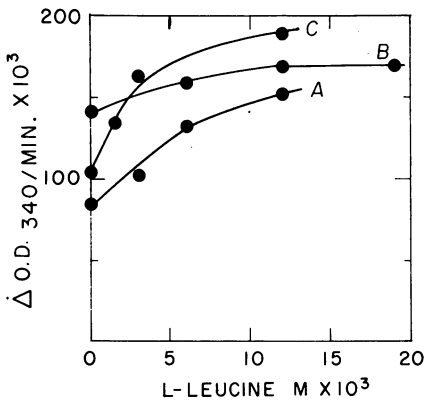

FIG. 1.—Effects of L-leucine on glutamate oxidation. *A*, *B*: 2.5 ml reaction mixture contained 0.01 *M* tris buffer pH 8.0, EDTA 1×10^{-4} *M*, glutamate 0.1 *M*, DPN 2×10^{-4} *M*, 0.01 mg of crystalline beef liver glutamic dehydrogenase (see text) and L-leucine as shown; *B*: 2.5 ml. reaction mixture contained 0.01 *M* tris buffer pH 8.0, EDTA 1×10^{-4} *M*, α-ketoglutarate 5×10^{-3} *M*, NH_4Cl 0.1 *M*, DPNH 5×10^{-5} *M*, mitochondrial suspension equivalent to 1.25 mg of rat liver, and L-leucine as shown.

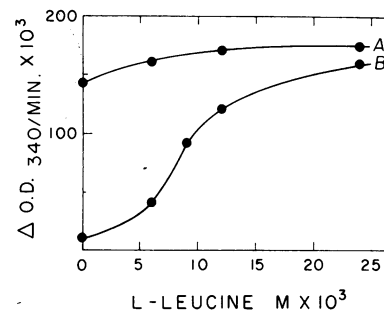

FIG. 2.—Effect of L-leucine in inhibition of glutamic dehydrogenase by diethylstilbestrol. Experiment as in Figure 1 *A*, *B* except for presence of ethanol 0.4% in each, and diethylstilbestrol, 8×10^{-6} *M* in *B*.

preparations of the crystalline enzyme were examined, and the extent of stimulation by 1.2×10^{-2} *M* L-leucine varied from 20 to 100 per cent depending on the preparation. This monocarboxylic amino acid also stimulated GDH activity in mitochondrial preparations from rat liver (Fig. 1, curve C). In the latter case 1.5×10^{-3} *M* L-leucine caused a 30 per cent stimulation of glutamate oxidation, and 1.2

$\times 10^{-2}$ M doubled the rate. As expected, no oxidation of L-leucine could be observed with the amount of enzyme used in the present experiments. This is consistent with the observation of Struck and Sizer[5] that although GDH catalyzes the oxidation of L-leucine, this occurs at a rate only 1.7 per cent that of L-glutamate. It should be emphasized that leucine stimulates the oxidation of glutamate rather than vice versa. This is attested to by the finding that leucine stimulates equally well the reduction of α-KG.

D-leucine had virtually no effect on the GDH reaction and, moreover, when used in equimolar concentrations with the L-isomer, did not reduce the stimulation produced by the latter. Neither the α-keto analog of leucine, α-ketoisocaproate, nor isocaproate itself influenced the rate of the enzymic reaction. Leucinamide, leucylleucine, and leucylglycine were also ineffective. Methionine, isoleucine, and the unnatural amino acid norvaline, though considerably less active, were also able to stimulate the GDH reaction. Further effects of these amino acids will be discussed below. Glycine, alanine, valine, norleucine, lysine, arginine, ornithine, serine, threonine, phenylalanine, tyrosine, tryptophan, α-aminobutyrate and histidine had no influence on the GDH reaction.

Mechanism of amino acid stimulation of GDH: Diethylstilbestrol, a steroid analog, inhibits the GDH reaction by dissociating the enzyme molecule into subunits.[2] As shown in Figure 2, 2.5×10^{-2} M L-leucine completely overcame the 91 per cent inhibition of the crystalline enzyme resulting from 8×10^{-6} M diethylstilbestrol. Again L-methionine, L-isoleucine, and L-norvaline could overcome the steroid inhibition but were somewhat less effective than L-leucine. Table 1 shows the concentrations of these compounds required to restore 50 per cent of the enzyme activity after inhibition by 8×10^{-6} M DES.

TABLE 1

ABILITY OF AMINO ACIDS TO RESTORE GDH ACTIVITY AFTER INHIBITION WITH DIETHYLSTILBESTROL

Amino acid	Conc. to restore 50% of GDH activity after inhibition with 8×10^{-6} M diethylstilbestrol
L-leucine	8×10^{-3} M
L-methionine	1.6×10^{-2} M
L-isoleucine	3.6×10^{-2} M
DL-norvaline	6×10^{-2} M

Experiment conducted as in Figure 2 except for additions as noted.

Phenanthridine, like DES,[2] inhibits the GDH reaction by disrupting the enzyme molecule.[6] L-leucine was also able to overcome the inhibition produced by this compound (Table 2).

Frieden[1] has made the observation that relatively high concentrations of DPNH can disaggregate the GDH molecule and thus inhibit the enzymic reaction. The inhibitory effect of the reduced pyridine nucleotide could also be prevented by L-leucine. Curve A in Figure 3 shows the inhibition of α-ketoglutarate reduction at higher concentrations of DPNH. As shown by curve B, the presence of 1.2×10^{-2} M L-leucine prevented this effect of DPNH.

Since L-leucine could antagonize the inhibitory action of a number of compounds known to dissociate the GDH molecule into subunits, it seemed likely that this

amino acid could prevent such reagents from dissociating the enzyme. Accordingly, the effects of L-leucine on the DES-induced disruption of the enzyme were studied in the ultracentrifuge. Figure 4 (curve A) illustrates the alteration of GDH in

TABLE 2

EFFECT OF PHENANTHRIDINE AND L-LEUCINE ON GDH ACTIVITY

Additive	ΔO.D.$_{340}$/min $\times 10^{-3}$
None	156
Phenanthridine 2×10^{-4} *M*	10
Phenanthridine 2×10^{-4} *M* + L-leucine 1.2×10^{-2} *M*	180

Experiment performed as in Figure 1A, B except for presence of 2×10^{-4} *M* phenanthridine and 1% ethanol.

the presence of 2×10^{-4} *M* DES, as shown earlier.[2] Curve B shows the same experiment with the addition of 2.4×10^{-2} *M* L-leucine. It is apparent that the amino acid can, indeed, suppress the disaggregation of the GDH molecule caused by DES.

It appears from these experiments that L-leucine has an effect on the enzyme very similar to that reported for DPN and ADP.[7] These nucleotides have been shown to favor the aggregation of the enzyme, and therefore to antagonize the action of DPNH[1] and the steroids.[2] It was therefore of interest to determine whether these nucleotides and L-leucine had the same site of action. Suffice it to say that when maximal stimulation by ADP of glutamate oxidation was obtained, L-leucine produced no additional effect. However, when a similar degree of activation was produced with DPN, L-leucine could cause as much stimulation as in the absence of the excess DPN.

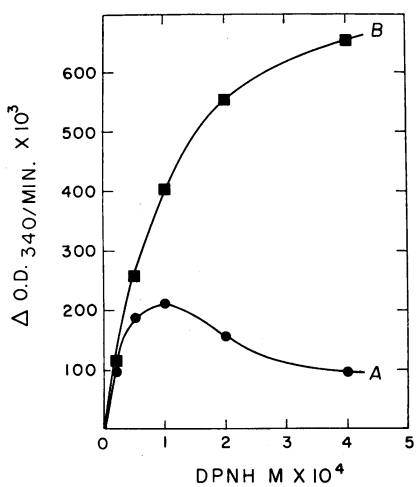

FIG. 3.—Effect of L-leucine and DPNH on the reductive amination of α-ketoglutarate. 2.5 ml reaction mixture contained 0.01 *M* tris buffer pH 8, EDTA 1×10^{-4} *M*, α-ketoglutarate 5×10^{-3} *M*, NH_4Cl 0.1 *M*, 0.0025 mg of crystalline beef liver glutamic dehydrogenase, DPNH as shown, and in curve B: L-leucine 9×10^{-3} *M*.

Because of the structural similarity between L-leucine and glutamate and because L-leucine has also been shown to be a substrate for the enzyme,[5] it might be supposed that the leucine and glutamate binding sites are the same. The fact that L-leucine stimulated rather than inhibited the oxidation of glutamate showed that this is not the case. The further observation that the stimulation of the GDH reaction by L-leucine was independent of the glutamate concentration (Fig. 5) also indicated that leucine and glutamate do not have a common binding site.

In our previous studies on the alanine dehydrogenase reaction[3] catalyzed by GDH, some evidence was obtained that the alanine site is different from the glutamate site. The effect of alanine on the leucine stimulation was therefore examined. Rela-

tively high concentrations of alanine (0.12 M) could not prevent the L-leucine activation (Table 3), which makes it appear that the sites for leucine and alanine are also different.

Discussion.—The results of these investigations indicate that the L-isomers of leucine, methionine, norvaline, and isoleucine have a pronounced effect on the struc-

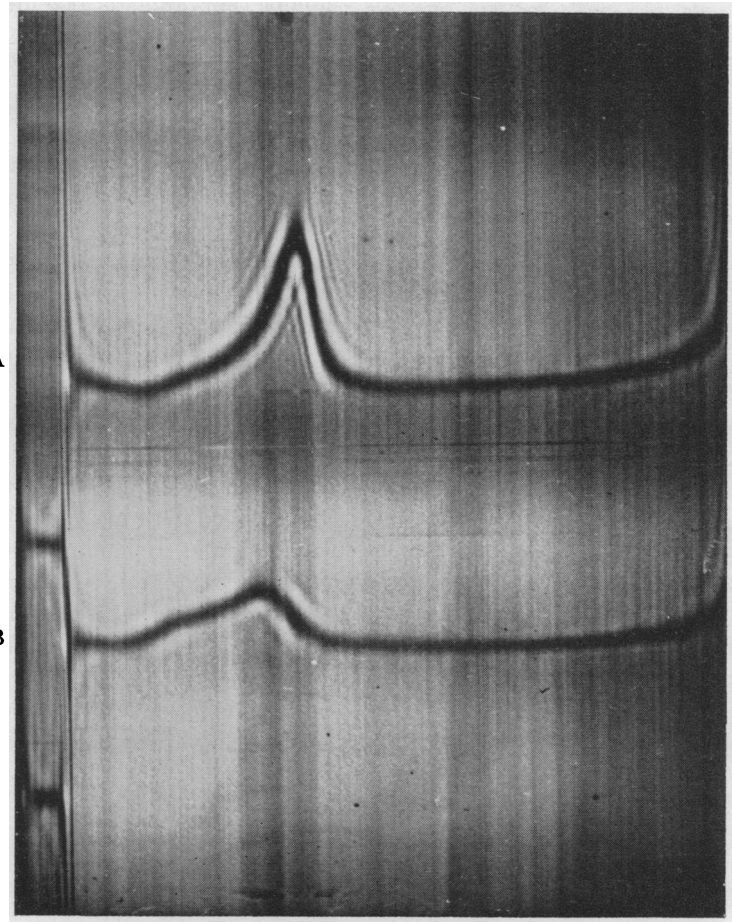

FIG. 4.—Effect of diethylstilbestrol and L-leucine on sedimentation behavior of glutamic dehydrogenase. Reaction mixture contained 0.05 M tris pH 8.0, EDTA 5×10^{-4} M, crystalline beef liver glutamic dehydrogenase 5 mg/ml, propylene glycol 2%, diethylstilbestrol 2×10^{-4} M, and in B: L-leucine 2.4×10^{-2} M. Sedimentation is from left to right for 16 minutes at a rotor speed of 59,780 rpm.

ture of crystalline glutamic dehydrogenase. These amino acids apparently favor aggregation of the subunits of the enzyme, thereby antagonizing the effects of compounds which cause its disruption. Functionally, these structural alterations are evidenced by the ability of the amino acids to stimulate the enzymic reaction and to overcome the inhibition produced by DES, DPNH, etc.

The data show that there are specific structural requirements for the amino acids which activate GDH. Since neither the fatty acid nor the α-keto acid corresponding to leucine were active, an α-amino group is necessary, and the ineffectiveness

of the D-antipode indicates a requirement for the L-configuration of the α-carbon.

All of the stimulatory amino acids had a straight chain of five atoms, and neither a longer molecule (norleucine) nor a shorter one (α aminobutyrate) affected the enzyme. The greater sensitivity of GDH to methionine than to its carbon analog, norvaline, is also interesting to note.

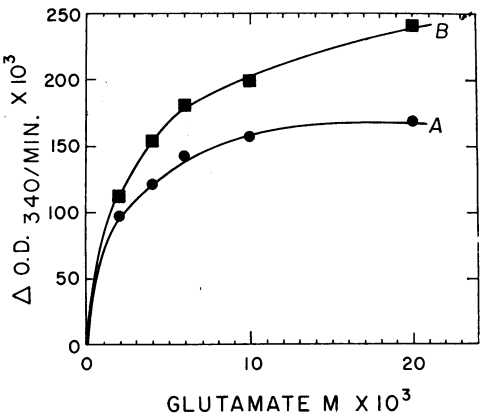

FIG. 5.—Effect of glutamate concentration and L-leucine on the glutamic dehydrogenase reaction. Experiment was done as in Figure 1 *A*, *B*, except for glutamate concentration as shown, and the presence of L-leucine 1.2×10^{-2} *M* in *B*.

Finally, attention should be directed to the influence of the branched chain in leucine, which confers upon that amino acid a 6- to 8-fold greater activity than its nonmethylated analog, norvaline.

It is, at this time, impossible to give a satisfactory chemical explanation for the action of these amino acids on the enzyme molecule, since there is no detailed information about the forces which bind the subunits together. It seems, in general, that molecules such as the steroids, phenanthridine, and the reduced pyridine nucleotides, which cause disaggregation of the enzyme, have in common a planar, nonpolar area. This suggests that adjacent polypeptide chains may interact, to some extent, by way of aromatic residues and that these interactions can be disrupted by the enzyme inhibitors. Since the molecule has been reported to dissociate on dilution,[8] it is apparent that these binding forces are not very strong.

The mechanisms by which DPN, ADP, and leucine favor protein association are also obscure, but might be due to neutralization of repulsive charges on adjacent chains. It may be relevant that when activated by ADP, GDH could no longer be stimulated by leucine which suggests that the nucleoside diphosphate and the amino acid have a common mode of action.

TABLE 3

EFFECTS OF L-LEUCINE AND L-ALANINE ON THE GLUTAMIC DEHYDROGENASE REACTION

Additive	ΔO.D.$_{340}$/min × 10^{-3}	
	No leucine	L-leucine 1.2×10^{-2} *M*
None	116	184
L-alanine 4×10^{-2} *M*	120	192

Conditions were identical to those for Figure 1A, B except for addition of L-leucine and L-alanine as shown.

Aside from the chemical implications of these findings, biologically it is of interest that none of the three natural amino acids which can influence GDH structure, leucine, methione, and isoleucine, can be synthesized to any extent by mammals. Thus, the dietary intake of these amino acids could regulate the metabolism of other amino acids by influencing the state of aggregation of GDH.

Current ideas about the control of enzyme action, at least in microorganisms,

have centered around the concept of negative feed-back[9] in which a metabolic end-product can inhibit the first definitive step in its own biosynthesis. Functionally, the effect of leucine, methionine, and isoleucine on glutamate metabolism is clearly different, since these amino acids are not biosynthetically related to glutamate, but can still affect its metabolism. Furthermore, rather than producing inhibition, they stimulate glutamate metabolism.

In more general terms, the influence of leucine, described here, and of the steroid hormones[2,3] on the structure of glutamic dehydrogenase indicate that metabolic control could be mediated through changes in enzyme structure.

The leucine site which causes stimulation of the enzyme is apparently distinct from those which bind the substrates or catalyze the chemical transformations. Similarly, Frieden has proposed an activating site on GDH for DPN and ADP,[7] and an inhibitory site for DPNH.[1] GDH, therefore, carries with it not only an active center, but quite specific areas devoted to control of the enzymic reaction. It may be that the large size of this enzyme molecule, and perhaps of others, is required to accommodate not only catalytic loci but specific control sites as well.

Summary—1. L-leucine, L-methione, L-isoleucine, and L-norvaline stimulate the glutamic dehydrogenase reaction catalyzed by either a crude rat liver preparation or a crystalline enzyme from beef liver. D-leucine was ineffective.

2. The inhibition of the enzyme by DPNH, diethylstilbestrol, and other compounds can be overcome by these L-amino acids.

3. L-leucine prevents the disaggregation of the enzyme caused by diethylstilbestrol.

4. The effect of L-leucine on the kinetics of the enzymic reaction can be explained by its influence on the aggregation state of the protein.

5. These findings suggest a mechanism by which these essential amino acids can regulate general amino acid metabolism.

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