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BIOSYNTHESIS OF 2-AMINO-4-METHYLHEX-4-ENOIC ACID IN *AESCULUS CALIFORNICA*: THE ROUTE FROM ISOLEUCINE AND THE ROLE OF AMINOTRANSFERASES

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Abstract—When [U-^{14}C]isoleucine was supplied to developing fruits of *Aesculus californica*, about 3 per cent of the radioactivity supplied was incorporated into 2-amino-4-methylhex-4-enoic acid (AMHA) during 6 days. If unlabelled tiglate was supplied simultaneously with ^{14}C-isoleucine, radioactivity associated with AHMA was markedly reduced, whilst that present in tiglate was strikingly increased. Fruits did not convert ^{14}C-homoisoleucine into AMHA, but some labelled homoisoleucine was formed after infiltration of ^{14}C-AMHA. These observations suggest that isoleucine may first be degraded to a tiglyl residue which then undergoes condensation with an acetate residue to yield AHMA and then finally homoisoleucine. The aminotransferase activity present in *Aesculus* seeds was investigated, because enzymes of this type logically would effect the first and last steps in the formation of AMHA from isoleucine.

INTRODUCTION

SEEDS of *Aesculus californica* (California buckeye) contain 2-amino-4-methylhex-4-enoic acid (AMHA, I) as the principal component of their free amino acid complex: this unsaturated amino acid is accompanied by smaller quantities of homoisoleucine (II), 2-amino-6-hydroxy-4-methylhex-4-enoic acid (6-hydroxy AMHA, III), γ-glutamyl AMHA and β-(methylenecyclopropyl)-β-methylalanine.[1] AMHA appears to be synthesized chiefly within the fruit, especially during the later stages of its growth.[2] These earlier experiments, performed with fruit maturing in August 1969, indicated that [U-^{14}C] isoleucine was incorporated into AMHA more effectively than any other labelled precursor tested. No radioactivity was present in AMHA after infiltration of [U-^{14}C]-leucine, [methyl-^{14}C] methionine or [3-^{14}C]serine into fruits: these latter observations discounted the possibility that synthesis of AMHA involved C_1 transfer to a terminal methyl-C of leucine.

Alternative biosynthetic pathways commencing from isoleucine can be envisaged. Pathway A (Fig. 1) implicates a series of conversions modelled on those leading from valine to leucine: the reactants in the pathway yielding AMHA would possess an additional C atom. The alternative pathway B would commence with the normal degradative steps leading from isoleucine to a tiglyl moiety; the tiglyl group then undergoes condensation with acetyl CoA to produce an unsaturated branched-chain C_7 skeleton. AMHA arises as the primary amino acid product of the latter pathway, whereas homoisoleucine is the initial product of the first biosynthetic scheme, and AMHA results only after a secondary dehydrogenation (reaction 2, see Fig. 1).

The previous study[2] had shown that no ^{14}C-label entered AMHA from [1-^{14}C]isoleucine but, although in accord with the precursor role of isoleucine in branched-chain C_7 amino

[1] L. FOWDEN and A. SMITH, *Phytochem.* **7**, 809 (1968).
[2] L. FOWDEN and M. MAZELIS, *Phytochem.* **10**, 359 (1971).

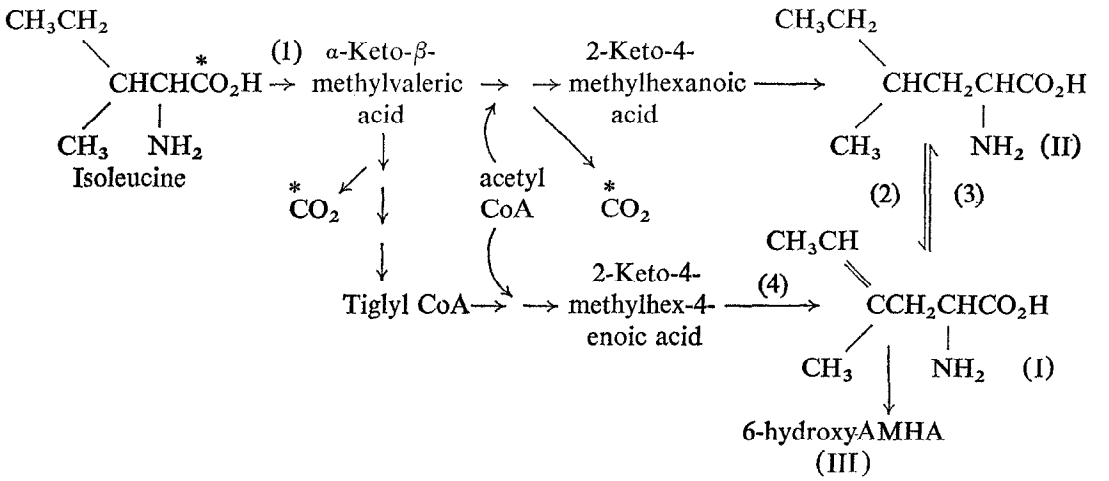

FIG. 1. POSSIBLE ALTERNATIVE ROUTES TO AMHA AND RELATED COMPOUNDS. UPPER PATHWAY A LEADS TO HOMOISOLEUCINE, LOWER PATHWAY B TO AMHA.

acid biosynthesis, this and other earlier results failed to discriminate between the two possible pathways. The present paper describes the results of new precursor feeding experiments, performed during August 1970, which strongly suggest that pathway B represents the principal route to AMHA. Some observations on the aminotransferase enzymes of buckeye seeds also are reported since reactions (1) and (4) of the scheme outlined in Fig. 1 implicate transamination reactions.

The initial step of the metabolic pathway leading to AMHA almost certainly involves loss of the amino group from isoleucine to yield α-keto-β-methylvaleric acid (reaction 1), while the final reaction yielding AMHA probably requires the amination of 2-keto-4-methylhex-4-enoic acid (reaction 4). The paper then includes some observations on the aminotransferase enzymes of buckeye seeds that catalyse transamination of isoleucine and AMHA.

Earlier work with aminotransferases has indicated that many enzymes of this type exhibit rather broad substrate specificity. This is particularly true for many preparations obtained from higher plant sources, but it is not certain whether a broad specificity is an intrinsic feature of many aminotransferases or merely an indication that enzyme fractionation has been inadequate. Enzymes catalysing the transamination of the branched-chain amino acids, valine, leucine and isoleucine, have been partially purified from a number of sources, and it is commonly concluded that all three compounds may act as substrates for a single aminotransferase. However, an early study with genetic mutants of *Escherichia coli*[3] suggested that more than one enzyme may be capable of effecting the transamination of valine and leucine, and later investigations have tended to strengthen the view that particular amino acids may form substrates for more than one species of aminotransferase.

Two possibilities of non-specific aminotransferase action may be invoked to account for transamination of AMHA. As an example of a branched-chain amino acid, AMHA may be accepted as a substrate by a leucine-isoleucine-type aminotransferase. However, AMHA behaves as a structural analogue of phenylalanine, and not of leucine or isoleucine, in relation to aminoacyl-*t*RNA synthetase enzymes; it acts as a good alternative substrate for

[3] D. RUDMAN and A. MEISTER, *J. Biol. Chem.* **200**, 591 (1953).

phenylalanyl-*t*RNA synthetase enzymes obtained from various species of *Aesculus* seed.[4] AMHA then could conceivably form a substrate for an aromatic transaminase of the type obtained from mung bean seedlings by Gamborg[5] or for a glutamate-phenylpyruvate aminotransferase such as that purified from peas by Redkina, Uspenskaya and Kretovich.[6] Finally, an aminotransferase specific for the transamination of AMHA may be elaborated by *A. californica* seed. Our fractionation of the aminotransferase complex in buckeye seed was moderately successful, and the results favoured the idea that transamination of AMHA was effected by a leucine-isoleucine-type enzyme.

RESULTS AND DISCUSSION

Labelled Precursor Feeding Experiments

The ^{14}C-labelling experiments were performed on the same *Aesculus californica* tree used previously[2] at a time when individual fruits weighed approx. 60 g, i.e. they had reached an almost mature size being intermediate between developmental stages 3 and 4 of the previous study.[2] ^{14}C-labelled compounds were supplied to the fruits via a cotton wick inserted through an incision in the pedicels. The incision was only 1–2 cm from the point of fruit attachment, and in practice the radioactive substances were absorbed quickly and translocated almost exclusively into the developing fruits. The fruits were detached 6 days later and immediately extracted with 75% ethanol.

About 3 per cent of the ^{14}C supplied as L-[U-^{14}C]isoleucine became incorporated into AMHA during the 6-day metabolic period. This figure compares favourably with the highest incorporation (0·51%) measured in similar experiments performed in the previous year, and suggests that the period of ^{14}C feeding in these new experiments coincided more exactly with the phase of maximum AMHA biosynthesis within the developing fruit. When unlabelled

TABLE 1. THE CONVERSION OF VARIOUS ^{14}C-LABELLED COMPOUNDS INTO AMHA AND RELATED COMPOUNDS PRESENT IN *A. californica* FRUITS

Labelled compound supplied†	% ^{14}C incorporated into AMHA	Homoisoleucine	6-hydroxy-AMHA	Isoleucine (protein-bound)	Tiglate
L-^{14}C-isoleucine (50 μc)	3·10	0·02	0·01	14·02	0·09
L-^{14}C-isoleucine (50 μc) + ammonium tiglate (40 mg)	0·72	0·01	t	n.d.	1·38
L-^{14}C-isoleucine (50 μc) + homosioleucine (25 mg)	2·68	0·02	0·01	n.d.	0·15
DL-AMHA (40 μc)	0·90*	0·78	10·7	0	n.d.
DL-homoisoleucine (30 μc)	0	12·8*	0	0	n.d.

Data are expressed as % of ^{14}C supplied entering the derived compounds during a 6-day metabolic period: values are means of three determinations.

* Figure represents activity remaining in compound initially supplied.

n.d. = No determination made.

t = Detectable activity, but less than 0·01% incorporation.

† = Specific activity of compound supplied were: isoleucine, 20 μc/μmole; AMHA and homoisoleucine, 6·5 μc/μmole.

[4] J. W. ANDERSON and L. FOWDEN, *Biochem. J.* **119**, 677 (1970).

[5] O. L. GAMBORG, *Can. J. Biochem.* **43**, 723 (1965).

[6] T. V. REDKINA, ZH. V. USPENSKAYA and W. L. KRETOVICH, *Biokhimiya, Engl. edit.* **34**, 247 (1969).

ammonium tiglate was supplied together with ^{14}C-isoleucine, the quantity of ^{14}C entering AMHA was reduced very markedly (Table 1). By contrast, the feeding of unlabelled homoisoleucine together with ^{14}C-isoleucine caused only a slight reduction in the amount of ^{14}C-label incorporated into AMHA. The results of these three ^{14}C-incorporation experiments provide indirect support for the operation of a pathway of type B in AMHA biosynthesis.

Table 1 further shows that the feeding of unlabelled tiglate to the buckeye fruit leads to a very considerable increase in the amount of ^{14}C-label incorporated into tiglate (or tiglyl CoA) from ^{14}C-isoleucine and, although this finding might merely reflect the role of tiglate in the normal catabolism of isoleucine, it is also in accord with its implied role as an intermediate in AMHA biosynthesis. In the related experiment involving the feeding of unlabelled homoisoleucine together with ^{14}C-isoleucine, no radioactivity accumulated in homoisoleucine, although the concentration of the amino acid within the treated buckeye fruit after the 6-day metabolic period was still much higher than in other fruits. Evidence supporting pathway A therefore was lacking in these particular feeding experiments.

The metabolic relationship between AMHA and homoisoleucine was investigated by infiltrating the ^{14}C-labelled racemic form of each amino acid into developing fruits. Subsequent combined chromatographic and radioautographic analysis showed that ^{14}C-AMHA had been converted into a number of other cationic substances. The most important of these was 6-hydroxyAMHA. However, homoisoleucine was also identified and shown to contain about 0·8 per cent of the activity supplied initially as ^{14}C-AMHA. Two unidentified radioactive products together represented 6·5 per cent of the initial ^{14}C supplied. In contrast, no conversion of ^{14}C-homoisoleucine into other cationic substances could be detected after the 6-day period. These results provide additional support for the view that AMHA, and not homoisoleucine, is the primary C_7 amino acid product.

The data in Table 1 show that after the 6-day metabolic period much of the radioactivity initially supplied had been lost from the pool of cationic substances. Even in the case of ^{14}C-homoisoleucine, where no other amino acid (cationic) substances were produced, only one-eighth of the initial radioactivity remained in homoisoleucine at extraction. No activity was detected among the amino acid constituents formed after hydrolysis of protein present in the insoluble residue remaining after extraction of fruits that had received ^{14}C-homoisoleucine or ^{14}C-AMHA. However, some 14 per cent of the ^{14}C supplied as isoleucine was present in the insoluble protein fraction: after chromatographic separation of the amino acids resulting from protein hydrolysis, all the radioactivity was shown to be present in isoleucine.

Presumably, considerable proportions of each amino acid supplied must have been degraded by catabolic mechanisms leading ultimately to the release of $^{14}CO_2$. However, the D-isomers of ^{14}C-homoisoleucine and ^{14}C-AMHA may have been converted largely into *N*-malonyl (or other *N*-acyl) derivatives, since several reports now indicate that such conversions represent an important mechanism whereby higher plants handle the unnatural stereoisomers of amino acids.[7–9]

The degree of conversion of ^{14}C-AMHA into ^{14}C-6-hydroxyAMHA was particularly interesting, for such a marked hydroxylation could not have been predicted by consideration of the relative concentrations of AMHA and its 6-hydroxy derivative present in buckeye

[7] R. L. M. SYNGE, *Ann. Rev. Pl. Physiol.* **19,** 113 (1968).
[8] J. F. THOMPSON, C. MORRIS and I. SMITH, *Ann. Rev. Pl. Physiol.* **38,** 137 (1969).
[9] N. ROSA and A. C. NEISH, *Can. J. Biochem.* **46,** 797 (1968).

fruits (AMHA concentrations are 30–50 times those of 6-hydroxyAMHA). An explanation of this finding may rest upon a localized sub-cellular distribution of the hydroxylating enzyme. Under normal circumstances, the majority of molecules of AMHA synthesized within cells of the developing buckeye fruits may be quickly translocated from a synthetic site to an adjacent storage site, presumably the vacoule: few molecules may penetrate to the site where the hydroxylase is located. However, different considerations will apply to AMHA molecules supplied exogenously, especially in relation to transport mechanisms. AMHA molecules entering the cells of buckeye fruits across the plasmalemma may come into direct contact with the hydroxylating system, which might even be bound to the plasma membrane; an enhanced formation of 6-hydroxyAMHA would ensue. These observations concerning the formation of 6-hydroxyAMHA provide a further illustration of the difficulties that may be encountered when precursor labelling techniques are applied to metabolic problems in intact plant systems, especially when the pattern of sub-cellular movement and partition of substances may strongly influence the delicate balance of metabolic systems.

Aminotransferase Studies

Extracts of buckeye seeds were treated routinely with protamine sulphate and ammonium sulphate (see Experimental) since these steps significantly enhanced the specific activities of aminotransferase preparations. Table 2 shows the activities of such preparations obtained

TABLE 2. SPECIFIC ACTIVITIES OF AMINOTRANSFERASES IN EXTRACTS OF *Aesculus* SEEDS AFTER TREATMENT WITH PROTAMINE SULPHATE AND AMMONIUM SULPHATE*

	Aminotransferase activity†			
	Leu-αKG	Isol-αKG	Phe-αKG	AMHA-αKG
Aesculus enzyme preparation	0·150 ± 0·005	0·067 ± 0·010	2·4 ± 0·005	0·156 ± 0·006

* Details of fractionation procedure are given in the Experimental.

† Specific activities are expressed as units of enzyme activity/mg protein; the standard errors are shown for each value. Reaction mixtures were as described in the Experimental. Transamination of leucine, isoleucine and AMHA was assayed by the dinitrophenylhydrazone method, whereas reactions involving phenylalanine were followed by direct measurement of phenylpyruvate absorption at 318 nm. The extinction given by phenylpyruvate in the latter assay was 3·26 times that given by an equimolar amount of α-ketoisocaproate or α-keto-β-methylvalerate in the former assay.

from *Aesculus* seed in relation to transamination of leucine, isoleucine, phenylalanine and AMHA with α-ketoglutarate. Further purification of aminotransferase activity was achieved by chromatography on Sephadex G-100. This step produced about a 2-fold increase in the specific activity of the aminotransferases from *Aesculus*. However, no separation of individual aminotransferases catalysing the reaction of leucine, isoleucine, phenylalanine and AMHA was effected, the combined enzyme peak occurring late in the protein elution profile. In a final purification step, fractions from Sephadex G-100 containing maximum aminotransferase activity were combined and the enzymes were absorbed onto DEAE cellulose. During stepwise elution with increasing concentrations of KCl in dilute tris buffer, the *Aesculus* aminotransferases were concentrated in the 0·05–0·1 M KCl fraction: again no significant separation of individual aminotransferases for leucine, isoleucine, phenylalanine and AMHA was obtained, but a further doubling of the specific activity of each enzyme was achieved.

A number of experiments were performed to determine whether mutual competition occurred when pairs of substrates, selected from leucine, isoleucine, phenylalanine and AMHA, were present in reaction mixtures. The results obtained with an aminotransferase preparation from *Aesculus*, following protamine sulphate and ammonium sulphate treatments, are given in Table 3. Evidence of competition between AMHA and leucine is clearly

TABLE 3. VALUES DETERMINED FOR TRANSAMINATION BETWEEN VARIOUS AMINO ACID SUBSTRATES, USED ALONE AND IN COMBINATION, WHEN CATALYSED BY AMINOTRANSFERASE PREPARATIONS FROM *Aesculus* SEED*

	Aminotransferase activity†	
	Aesculus preparation	
Amino acid substrate(s)	Determined values	Calculated† values
Leucine	1·08	
Isoleucine	0·22	
AMHA	1·03	
Phenylalanine	6·40	
Leucine + isoleucine	1·11	1·30
Leucine + AMHA	1·14	2·11
Leucine + phenylalanine	7·48	7·48
Isoleucine + AMHA	1·05	1·25
Isoleucine + phenylalanine	6·55	6·62
AMHA + phenylalanine	6·25	7·43

* *Aesculus* enzyme preparation was obtained after protamine sulphate and ammonium sulphate purification steps.

† Activities are expressed as units of enzyme activity under the standard assay conditions (see Experimental and Table 2): values have a standard error of about ±0·044.

‡ Calculated values are the sum of aminotransferase activities determined when amino acids were used singly.

seen, for the amount of transmination recorded in mixtures containing two substrates was considerably less than the sum of the transaminase activities determined for each amino acid separately. Competition between AMHA and isoleucine also was recorded but was less marked. No such competition was apparent when mixtures of phenylalanine with leucine or isoleucine were used. Substrate competition between AMHA and phenylalanine was somewhat equivocal on the basis of the data in Table 3.

A small batch of seed of *A. californica* was converted into an acetone powder, which was used as a source of aminotransferase activity. A sample (stored for 3 weeks at 4°) was extracted with tris buffer, and the extract was freed from low molecular weight substances by passing through a Sephadex G-25 column. The protein-containing eluate possessed aminotransferase activity for leucine, isoleucine and AMHA, which were mutually competitive as substrates, but no activity towards phenylalanine was measured. Transamination of AMHA then could not be attributed to the action of a phenylalanine aminotransferase.

The experiments described above have provided an enzymic basis for the deamination and amination reactions suggested as initial and final steps occurring in *A. californica* during the conversion of isoleucine into AMHA by pathway B. During several enzyme purification procedures, no significant separation of leucine-, isoleucine- and AMHA-

dependent aminotransferase activity was achieved, suggesting that a single enzyme catalyses amino group transfer from all 3 amino acids. Studies with aminotransferase enzymes obtained following fractionation of peanut cotyledon extracts support this view, for although AMHA is not synthesized by this species, the amino acid is effectively transaminated by enzyme preparations active with leucine and isoleucine. Slight differences in the relative rates of reaction observed with enzyme from *Aesculus* and peanut are consistent with the need for effective *in vivo* action of the *Aesculus* enzyme in relation to AMHA biosynthesis.

EXPERIMENTAL

Labelled Precursor Studies

Radioactive Compounds. Labelled L-[U-^{14}C]isoleucine was supplied by the Radiochemical Centre, Amersham; before use the specific activity was adjusted to 20 μc/μmole by addition of carrier L-isoleucine.

DL-AMHA was prepared by a micro-scale synthesis modified from the procedure of Shive and co-workers.[10] A ^{14}C-label was introduced into the C-2 position by condensing ethyl [2-^{14}C]acetamidocyanoacetate (4·4 mg; 6·52 μc/μmole; supplied by NEN Chemicals GmbH, West Germany) with tiglyl bromide: the final hydrolysis was performed with 5 N $Ba(OH)_2$ replacing the 5 N NaOH used previously.[10] AMHA was separated from small amounts of labelled contaminants by absorption upon a cationic resin, and subsequent separation of amino acids on a paper chromatogram developed in *tert*-amyl alcohol–HOAc acid–H_2O (15:1:15, by vol.; upper phase): yield 82 μc, 48% theoretical.

DL-Homoisoleucine was prepared by catalytic hydrogenation of DL-AMHA (42 μc; 6·52 μc/μmole) using Adam's PtO_2 catalyst. Homoisoleucine was separated from a small amount of unchanged AMHA by chromatography as above: yield 31 μc, 75% theoretical.

Infiltration, separation and assay procedures. Labelled compounds were supplied to individual developing fruits via cotton wicks as previously described.[2] The ^{14}C-compound was dissolved in 0·25 ml water containing streptomycin sulphate (20 μg/ml). When ammonium tiglate or DL-homoisoleucine were supplied together with ^{14}C-isoleucine, 5 mg were added to this initial 0·25 ml solution, and the remainder of each substance (see Table 1) was presented as a 20 mg/ml solution. Each precursor feeding treatment was applied to three fruits.

Six days after supplying the labelled compounds, the fruits were detached from the tree and appropriate groups of three were quickly extracted as previously.[2] Further treatment of the soluble amino acid fractions was as before.[2] Portions (100 mg) of the residues remaining after extraction of the fruits with 75% (v/v) EtOH were extracted with 7·5% (w/v) trichloroacetic acid (2 × 15 ml) at 90° for 20 min, and then with hot 75% (v/v) EtOH, hot EtOH, acetone and ether. Protein present in the residue was hydrolysed with 6 N HCl at 100° for 18 hr: the amino acid fraction was separated using small Zeokarb 225 cationic resin columns,[11] and then individual amino acids were resolved on two-dimensional paper chromatograms developed in 75% (w/w) phenol-NH_3, followed by *tert*-amyl alcohol–HOAc–H_2O as second solvent. ^{14}C activity present in amino acids was determined as previously.[2]

Radioactivity incorporated into tiglic acid (or tiglyl-CoA) was determined as follows. Aliquots of the initial fruit extracts (equivalent to 2·5 g fr. wt.) were treated with N-HCl at 100° for 30 min to hydrolyse CoA derivatives. Carrier tiglic acid (250 mg) was added, and each hydrolysate was extracted with 3 × 2·5 ml CH_2Cl_2. The combined organic layers, now containing any labelled tiglic acid plus carrier, were shaken out with 2 × 5 ml H_2O, before the tiglic acid was transferred to dilute aq. ammonia (2 × 2·5 ml). The combined ammonia layer was concentrated to about 2 ml, and then acidified to precipitate tiglic acid, which was recrystallized (×3) from hot water to yield tiglic acid of constant specific activity (determined using a Packard Tricarb 3320 scintillation counter).

Aminotransferase Studies

Enzyme fractionation. Enzyme preparations were obtained either from seed of *A. californica* (which was stored at 4° until required) or from cotyledons of peanut seedlings grown for 5 days at 30°. All extraction and fractionation steps were performed at 4°.

The plant materials were homogenized in 0·1 M tris–HCl buffer, pH 8·0; after expressing through muslin, the extract was centrifuged at 38,000 *g* for 20 min to give a clear supernatant. Aminotransferase activity present in the supernatant was occasionally determined after low molecular weight materials had been removed by passing through a column of Sephadex G-25. Normally, the supernatant was treated with 1% protamine sulphate solution (0·15 ml/ml supernatant): the precipitate was discarded and then ammonium

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[11] P. M. DUNNILL and L. FOWDEN, *Phytochem.* **4**, 933 (1965).

sulphate (560 mg/ml, 80% saturation) was added to the residual solution. After 15 min, precipitated protein was recovered by centrifuging; it was then resuspended in 0·1 M tris buffer and dialysed overnight against the same buffer to remove residual ammonium sulphate.

The next fractionation step employed a column (30 cm × 3 cm dia.) of Sephadex G-100, equilibrated against 0·1 M tris buffer, pH 8·0. Enzyme preparations obtained after ammonium sulphate treatment were applied in small volume and then the column was eluted with more 0·1 M tris buffer. Eluted fractions (5 ml) were assayed spectrophotometrically to locate protein, and aminotransferase activities were determined on each fraction as below. Fractions possessing strong aminotransferase activity were combined as appropriate and, if required, were further purified on DEAE-cellulose (Whatman column chromedia DE 52, microgranular). The DEAE-cellulose was equilibrated with 0·1 M tris buffer, pH 8·0, by repeatedly sedimenting and decanting. Enzyme (2 ml) obtained after Sephadex G-100 chromatography was stirred with DEAE-cellulose (2 g swollen weight) suspended in 0·1 M tris buffer (4 ml). After absorption, the cellulose was washed with buffer, and then eluted batchwise with buffer containing increasing concentrations of KCl, i.e. 0·05, 0·1, 0·2 and 0·5 M. The DEAE-cellulose and eluant buffer were separated on each occasion by filtration, and the eluted fractions were dialysed before assay for aminotransferase activity.

Assay of aminotransferase activity. Transamination in reaction mixtures containing α-ketoglutarate and leucine, isoleucine or AMHA (or sometimes phenylalanine) was followed by assaying the formation of the corresponding α-keto acid, colorimetrically at 440 nm, after conversion to a 2,4-dinitrophenylhydrazone derivative. The method of Ichihari and Takahashi,[12] employing liquid–liquid partition, was used to separate the dinitrophenylhydrazone of residual α-ketoglutarate from those of the mono-carboxylic α-keto acid products of the enzymic reactions. Incubation mixtures normally contained the following components, adjusted to pH 8·0: amino acid, 10 μmole; α-ketoglutarate, 10 μmole; pyridoxal phosphate, 20 μg; enzyme solution, 0·5 ml; 0·1 M tris buffer to a final vol. of 1·5 ml. Control mixtures (i.e. minus amino acid) were included in all aminotransferase assays. After incubation at 38°, reaction was stopped by addition of 20% (w/v) metaphosphoric acid (0·5 ml). Under these conditions, reaction was linear with time for at least 1 hr. A unit of aminotransferase activity was defined as that quantity of enzyme causing a change in extinction at 440 nm of 0·001 min^{-1}: under these assay conditions; 0·1 μmole of α-ketoisocaproic acid gave an extinction of 0·144.

Phenylpyruvate absorbs strongly at 318 nm in alkaline solution and its formation by transamination between α-ketoglutarate and phenylalanine can be followed by directly reading absorption at this wavelength. For these assays, the normal reaction mixture contained the following reagents at pH 8·0: phenylalanine, 20 μmoles; α-ketoglutarate, 20 μmoles; pyridoxal phosphate, 20 μg; enzyme solution, 0·5 ml; 0·1 M tris buffer to 1·5 ml final vol. After incubation at 38°, reaction was stopped by addition of ethanol. Precipitated protein was removed by centrifuging, and then 1·75 N NaOH (2 ml) was added before reading the extinction at 318 nm. This colorimetric assay was more sensitive than the one based on the formation of the dinitrophenylhydrazone of phenylpyruvate, and was much more rapid and convenient. A unit of phenylalanine aminotransferase activity was defined as the amount of enzyme giving rise to a change in extinction at 318 nm of 0·001 min^{-1}: under the conditions used, 0·1 μmole of phenylpyruvate gave an extinction of 0·47.

Protein determinations. The method of Lowry *et al.*[13] was used normally for the assay of protein present in enzyme preparations taken for measurement of aminotransferase activity. However, the less sensitive spectrophotometric method of Warburg and Christian[14] was particularly suitable for determining the profile of protein concentration during elution of the enzyme from chromatographic columns.

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