lactone and phenyl *N*-acetylglucosaminide and of the corresponding galactosamine derivatives were measured. For each substance the value obtained with *p*-nitrophenyl *N*-acetylglucosaminide as substrate was almost the same as that obtained with *p*-nitrophenyl *N*-acetylgalactosaminide.

6. The competition of *p*-nitrophenyl *N*-acetylglucosaminide and *p*-nitrophenyl *N*-acetylgalactosaminide for one enzyme site was confirmed by an experiment with mixtures of these substrates.

We acknowledge the kind gifts of 2-acetamido-2-deoxygalactonolactone from Dr G. A. Levvy and of freeze-dried ram-testis extract from Benger Ltd. This work was assisted by grants from the Research Fund of the University of London and the Nuffield Foundation.

REFERENCES

Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1952). *Biochem. J.* **51**, 379.
Borooah, J., Leaback, D. H. & Walker, P. G. (1961). *Biochem. J.* **78**, 106.
Clark, W. M. & Lubs, H. A. (1916). *J. biol. Chem.* **25**, 479.
Conchie, J. & Levvy, G. A. (1957). *Biochem. J.* **65**, 389.
Dixon, M. & Webb, E. C. (1958). *Enzymes*. London: Longmans, Green and Co. Ltd.
Findlay, J., Levvy, G. A. & Marsh, C. A. (1958). *Biochem. J.* **69**, 467.
Foster, R. J. & Niemann, C. (1951). *J. Amer. chem. Soc.* **73**, 1552.
Heyworth, R., Borooah, J. & Leaback, D. H. (1957). *Biochem. J.* **67**, 21P.
Heyworth, R., Leaback, D. H. & Walker, P. G. (1959). *J. chem. Soc.* p. 4121.
Heyworth, R. & Walker, P. G. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 1–48, p. 7.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Leaback, D. H. & Walker, P. G. (1957). *J. chem. Soc.* p. 4754.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Linker, A., Meyer, K. & Weissmann, B. (1955). *J. biol. Chem.* **213**, 237.
Marsh, C. A. & Levvy, G. A. (1958). *Biochem. J.* **68**, 610.
Pugh, D., Leaback, D. H. & Walker, P. G. (1957). *Biochem. J.* **65**, 464.
Roseman, S. & Ludowieg, J. (1954). *J. Amer. chem. Soc.* **76**, 301.
Thorn, M. B. (1949). *Nature, Lond.*, **164**, 27.
Tsuboi, K. K. & Hudson, P. B. (1955). *Arch. Biochem. Biophys.* **55**, 191.
Veibel, S. (1950). In *The Enzymes*, vol. 1, p. 624. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
Woollen, J. W., Heyworth, R. & Walker, P. G. (1959). *Biochem. J.* **73**, 40P.

Biochem. J. (1961) **78**, 116

Mechanism of Transaminase Action

BY T. OSHIMA
Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Tokyo, Japan

AND N. TAMIYA
School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan

(*Received* 29 *April* 1960)

It is known that during the course of transaminase action the α-hydrogen atom of an amino acid exchanges with a hydrogen atom of the medium water (Konikova, Dobbert & Braunstein, 1947; Konikova, Kritzmann & Teiss, 1942). We have found that during the reaction an exchange also occurs with the hydrogen atoms at the β position of amino acid. This paper presents the experimental evidence for such an exchange and discusses the significance of the phenomenon for the mechanism of the transaminase reaction. A preliminary report of this work has already appeared (Oshima & Tamiya, 1959).

MATERIALS AND METHODS

Enzyme. An alanine–glutamate transaminase preparation was obtained from pig-heart homogenate as described by Green, Leloir & Nocito (1945). After the third ammonium sulphate precipitation and dialysis, the preparation was freeze-dried and stored at −20°.

Calcium pyridoxal phosphate. Pyridoxal phosphate was prepared from pyridoxamine dihydrochloride according to the method of Wilson & Harris (1951).

α-*Deutero*-DL-*alanine* (DL-[2-^{2}H]*alanine*). α-Deuterium-substituted DL-alanine [$CH_3 \cdot CD(NH_2) \cdot CO_2H$] was prepared by the electrolytic reduction of α-*iso*nitrosopropionic acid [$CH_3 \cdot C(:N \cdot OH) \cdot CO_2H$] in deuterium oxide. The method

of its preparation and the assignment of its infrared-absorption peaks have been described elsewhere (Suzuki *et al.* 1959). Mass-spectrometric analysis showed that the preparation contained 1·0 atom of deuterium per molecule.

Mass-spectrometric analysis. The deuterium content of the compounds was analysed by a modification (Tamiya, 1960) of the method described by Graff & Rittenberg (1952), with a mass spectrometer, model M-60 (Process and Instrument, Brooklyn, New York, U.S.A.).

Infrared-absorption spectra. The infrared-absorption spectra of alanine preparations were recorded by the potassium bromide disk method (thickness, 1 mm.) with a sodium chloride prism in a Koken infrared spectrometer, model DS-301 (Japan Spectroscopic Manufacturing Co. Ltd., Itabashiku, Tokyo, Japan). L-Alanine and α-oxoglutaric acid were obtained from the Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.

EXPERIMENTAL AND RESULTS

Preparation of αβ-tetradeutero-L-alanine (L-[2-^{2}H:3-^{2}H$_3$]alanine)

L-Alanine (42 mg.), the enzyme preparation (20 mg.), pyridoxal phosphate (0·05 mg.) and α-oxoglutaric acid (0·5 mg.) were dissolved in 99·8 % deuterium oxide (5 ml.), containing 0·08 M-phosphate buffer, pH 7·0, made of disodium hydrogen phosphate and potassium dihydrogen phosphate. The final pH of the mixture was adjusted with concentrated sodium hydroxide in 99·8 % deuterium oxide to give pH 7·6 (estimated with bromothymol blue). One drop of toluene was added as preservative.

After incubation for 38 hr. at 37° the reaction mixture was boiled and filtered. The amino acid in the filtrate was adsorbed on a column (1·8 cm.^{2} × 7 cm.) of Amberlite IR-112, H type. The column was washed with distilled water and the amino acid eluted with aq. 0·15 N-ammonia solution. The eluate was evaporated and dried under reduced pressure at about 50°; the residue was dissolved again in water (10 ml.) and passed through another column (1·8 cm.^{2} × 3 cm.) of Amberlite IR-45, acetate type, to remove acidic impurities. The solution was then evaporated to dryness under reduced pressure. The alanine thus obtained was dissolved in a minimum amount of water, recrystallized by the addition of ethanol and dried under reduced pressure at 100°. The recovery of alanine was 33 mg., which, as analysed by mass spectrometry, was found to contain deuterium in an amount of 53·7 atoms % of its total hydrogen, or 3·76 atoms of deuterium per molecule. It is evident that these four deuterium atoms were attached to the α- and β-carbon atoms of the alanine, because the other three hydrogen atoms must have readily exchanged with normal hydrogen during the course of isolation.

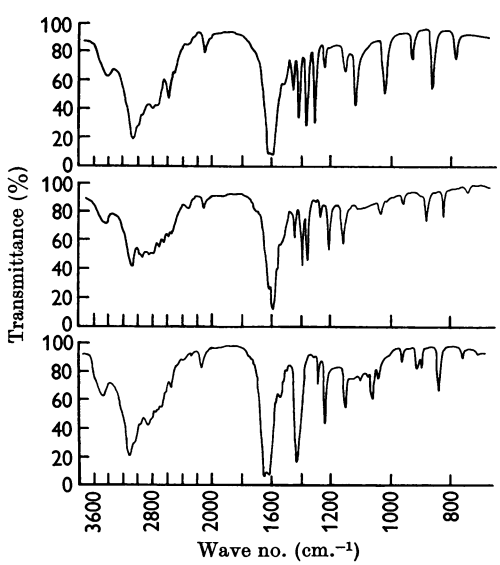


Fig. 1. Infrared-absorption spectra of non-deuterium-substituted L-alanine, α-deutero-DL-alanine and enzyme–D_2O-treated L-alanine.

Fig. 1 shows the infrared-absorption spectra of L-alanine, chemically prepared α-deutero-DL-alanine and enzymically prepared αβ-tetradeutero-L-alanine. It is known that non-deuterium-substituted D-, L- and DL-alanine give the same infrared-absorption spectra. The absorption peak at 1308 cm.$^{-1}$, which is to be assigned to the α-CH deformation (Fukushima, Onishi, Shimanouchi & Mizushima, 1959; Suzuki *et al.* 1959), disappeared in both chemically prepared α-deutero-DL-alanine and the enzyme-treated L-alanine.

A new absorption of α-CD deformation appeared at about 960 cm.$^{-1}$, which corresponded to approx. $1/\sqrt{2}$ of the wave number of the original α-CH absorption. The three absorption peaks at 1451 cm.$^{-1}$, 1356 cm.$^{-1}$ and 1018 cm.$^{-1}$, which were to be assigned to degenerating deformation, symmetric deformation and rocking of the $-CH_3$ group of alanine respectively (Fukushima *et al.* 1959; Suzuki *et al.* 1959), remained unchanged with α-deutero-DL-alanine and they completely disappeared with enzymically prepared αβ-tetradeutero-L-alanine.

The L-alanine, which was recovered from the deuterium oxide medium containing boiled enzyme or no enzyme, was revealed by infrared analysis not to have deuterium incorporated into its molecule.

Recovery of L-alanine from the αβ-tetradeutero-L-alanine

The enzymically prepared αβ-tetradeutero-L-alanine was treated again with transaminase in water to recover L-alanine. αβ-Tetradeutero-L-alanine (10 mg.), the enzyme preparation (5 mg.), α-oxoglutaric acid (0·5 mg.) and pyridoxal phosphate (0·05 mg.) were dissolved in 0·08 M-phosphate

buffer (4 ml.), pH 7·6, made up with water, disodium hydrogen phosphate and potassium dihydrogen phosphate. After addition of one drop of toluene, the mixture was incubated for 10 hr. at 37° and L-alanine (7 mg.) was recovered from the reaction mixture by a method similar to that described above. The recovered alanine showed an infrared-absorption spectrum reproduced in Fig. 2, which indicates the recovery of alanine containing no deuterium.

Comparison of the rates of exchange at α and β positions

L-Alanine (20 mg.) was treated with 99·8% deuterium oxide–phosphate buffer (4 ml.; 0·08 M, pH 7·0) in the presence of transaminase (7 mg.), α-oxoglutaric acid (0·5 mg.) and pyridoxal phosphate (0·05 mg.). The pH of the mixture was adjusted to 7·6 with concentrated sodium hydroxide in 99·8% deuterium oxide. After 10 and 25 min. incubation at 37°, 2 ml. samples of the reaction mixture were withdrawn and the alanine was isolated and purified as described above. The decrease in the absorbance of the infrared peaks at 1308 cm.$^{-1}$ (α-CH deformation) and 1451 cm.$^{-1}$ ($-CH_3$ degenerating deformation) was compared with that at 1410 cm.$^{-1}$ ($-CO_2^-$ symmetric stretching), which remained unchanged. The absorption at 1451 cm.$^{-1}$ would disappear when only one hydrogen atom in the methyl group was replaced. The results are summarized in Table 1. The values obtained with chemically synthesized α-deutero-DL-alanine and with L-alanine, which had been recovered from the reaction mixture containing boiled enzyme or no enzyme, are also given in the Table. It can be seen that the rate of exchange was about the same at the β position as at the α position. The chemically prepared α-deutero-DL-alanine seemed to contain some of its deuterium at the β position; the amount of β-incorporation increased when the reduction temperature was raised.

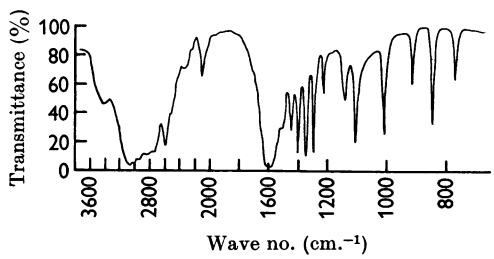


Fig. 2. Infrared-absorption spectrum of recovered non-deuterium-substituted L-alanine.

Table 1. *Incorporation of deuterium into α and β positions of alanine*

L-Alanine (20 mg.) was treated with 99·8% deuterium oxide–phosphate buffer (4 ml.), pH 7·6, in the presence of transaminase (7 mg.), α-oxoglutarate (0·5 mg.) and pyridoxal phosphate (0·05 mg.). After 10 and 25 min. incubation samples (2 ml.) were withdrawn and the alanine was isolated and purified as described in the text. α-Deutero-DL-alanine was prepared by electrolytic reduction of α-*iso*-nitrosopropionic acid in 99·8% deuterium oxide (Suzuki *et al.* 1959).

	Decrease in absorbance (%)	
Alanine preparations	1308 cm.$^{-1}$ (α – CH)	1451 cm.$^{-1}$ ($-CH_3$)
10 min. incubation	13	14
25 min. incubation	20	29
16 hr. with boiled enzyme (100°, 3 min.)	2	0
16 hr. without enzyme	0	0
Chemically synthesized α-deutero-DL-alanine	> 90	7

DISCUSSION

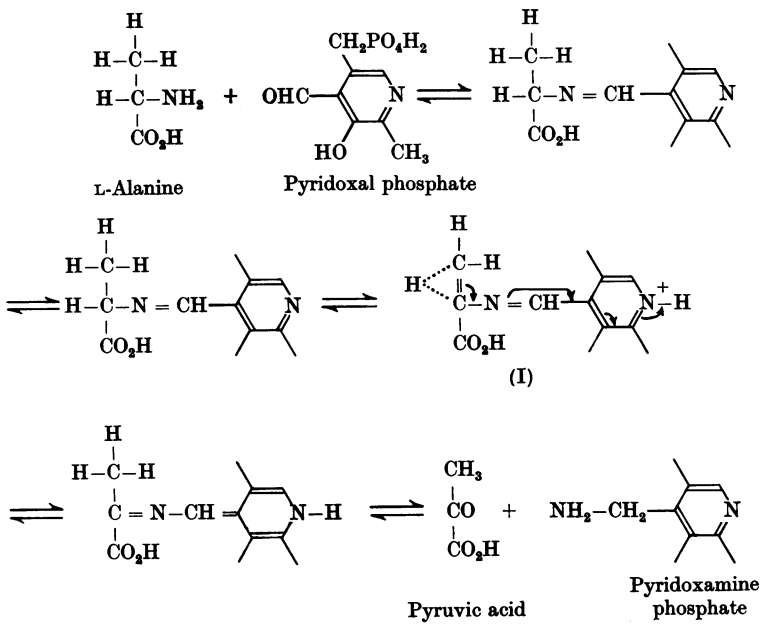
The above-described experiments show that when the transaminase reaction occurred in a medium of heavy water, the hydrogen atoms attached to the β-carbon atom of L-alanine exchanged with the deuterium in the medium. The fact that the rate of exchange at the β position was about the same as that at the α position strongly suggests that the dissociation of a proton from the β-carbon atom is an essential step in the mechanism of transaminase action.

Herbst & Rittenberg (1943) observed an incorporation of deuterium from the heavy water of the medium into the α and β positions of alanine formed in an organic reaction between pyruvic acid and α-aminophenylacetic acid. Taking into consideration the widely accepted idea that the primary step in the transaminase reaction is the formation of a Schiff base from the amino acid and pyridoxal phosphate, the most probable course of events may be pictured as shown in Scheme 1.

According to this Scheme, the Schiff base dissociates a proton from its β-carbon atom, contrary to the prevailing idea that the base dissociates a proton from its α-carbon atom (Schlenk & Fisher, 1947; Braunstein & Shemiakin, 1953; Metzler, Ikawa & Snell, 1954).

The dissociation at the β position is more conceivable than that at the α position also in view of the present knowledge of electronic theory. The dissociation of a proton from the α-carbon atom would be difficult because of the effect of lone-pair electrons on the nitrogen atom, whereas the dissociation from the β-carbon atom may be facilitated by the effect of formation of a hyperconjugated double-bond system in the proposed intermediate.

Almost all the transaminases known to react on amino acids require the presence of at least one hydrogen atom at the β position of their substrate molecules (Meister, 1955). The only exceptions are



Scheme 1

transaminases of glycine and of *tert.*-leucine (Heyns & Koch, 1951). It is also known that the amino acids having electron-withdrawing groups such as carboxyl or phenyl at their β positions are good substrates of transaminases, whereas the amino acids having long side chains at the same position react more slowly than alanine.

The formation of a Schiff base from pyridoxal phosphate and an amino acid is considered to be a common step in various pyridoxal phosphate-dependent enzymic reactions. The β-dissociation concept proposed here may be applicable to most of these reactions, and the compound [designated as (I) in the scheme] may be the key intermediate which undergoes various kinds of reactions.

SUMMARY

1. The four hydrogen atoms attached to the α- and β-carbon atoms of L-alanine exchanged with hydrogen atoms of medium water during the course of transaminase action. The exchange was ascertained by mass spectrometry and infrared spectrometry by following the incorporation and release of deuterium atoms into and from these positions.

2. The rate of exchange at the β position was about the same as that at the α position.

3. Based on the above findings, a new mechanism of transaminase reaction is proposed in which the Schiff base formed from the amino acid and pyridoxal phosphate dissociates a proton primarily from the β-carbon atom rather than from the α-carbon atom. This concept is discussed.

The authors are grateful to Dr D. Rittenberg of Columbia University for helpful suggestions and to Dr G. Chihara and Dr T. Shimanouchi for their co-operation in the infrared studies. Thanks are also extended to the Rockefeller Foundation and to Dr A. E. Mirsky for providing a mass spectrometer. The authors are indebted also to Mr I. Chiba for his technical assistance.

REFERENCES

Braunstein, A. E. & Shemiakin, M. M. (1953). *Biokhimiya*, **18**, 393.
Fukushima, K., Onishi, T., Shimanouchi, T. & Mizushima, S. (1959). *Spectrochim. acta.* **14**, 236.
Graff, J. & Rittenberg, D. (1952). *Analyt. Chem.* **24**, 878.
Green, D. E., Leloir, L. F. & Nocito, V. (1945). *J. biol. Chem.* **161**, 559.
Herbst, R. M. & Rittenberg, D. (1943). *J. org. Chem.* **8**, 380.
Heyns, K. & Koch, W. (1951). *Hoppe-Seyl. Z.* **288**, 272.
Konikova, A. S., Dobbert, N. N. & Braunstein, A. E. (1947). *Nature, Lond.*, **159**, 67.
Konikova, A. S., Kritzmann, M. G. & Teiss, R. V. (1942). *Biokhimiya*, **7**, 86.
Meister, A. (1955). *Advanc. Enzymol.* **16**, 185.
Metzler, D. E., Ikawa, M. & Snell, E. E. (1954). *J. Amer. chem. Soc.* **76**, 648.
Oshima, T. & Tamiya, N. (1959). *J. Biochem., Tokyo*, **46**, 1675.
Schlenk, F. & Fisher, A. (1947). *Arch. Biochem.* **12**, 60.
Suzuki, S., Oshima, T., Tamiya, N., Fukushima, K., Shimanouchi, T. & Mizushima, S. (1959). *Spectrochim. acta*, **14**, 969.
Tamiya, N. (1960). *Analyt. Chem.* **32**, 724.
Wilson, A. N. & Harris, S. A. (1951). *J. Amer. chem. Soc.* **73**, 4693.