(c) Glucose also led to breakdown of glutamine, and the accumulation in streptococcal cells was found to be transitory only. This applied to glutamine added as such, as well as to that produced from ammonium glutamate and glucose. The maximum observed rate of accumulation of synthesized glutamine was about $0.01 \mu\text{mol./mg./hr.}$ in cells capable of decomposing added glutamine at the rate of $0.6 \mu\text{mol./mg./hr.}$ The rate was much lower, and probably zero, in organisms requiring added glutamine in growth.

(d) α -Amino- γ -methylsulphonylbutyric acid inhibited the accumulation of synthesized glutamine in non-exacting streptococci. In one strain, $2.8 \times 10^{-2} \text{ M}$ -

solutions of the sulphoxide lowered the initial rate of accumulation from $2.8 \times 10^{-3} \text{ M}$ -ammonium glutamate by 50%. These concentrations of reagents led to partial inhibition of growth of the organism, and the action of other concentrations on growth and metabolism were similarly correlated. The compound was without action on growth in the presence of glutamine and its action on growth in the presence of glutamate was presumably due to inhibition of glutamine synthesis. Both the synthesis and breakdown of glutamine are thus of functional value to the organisms.

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REFERENCES

- Archibald, R. M. (1945). *Chem. Rev.* **37**, 162.
 Baddiley, J. & Gale, E. F. (1945). *Nature, Lond.*, **155**, 727.
 Borek, E., Miller, H. K., Scheiness, P. & Waelsch, H. (1946). *J. biol. Chem.* **163**, 347.
 Buchanan, R. E. & Fulmer, E. I. (1930). *Physiology and Biochemistry of Bacteria*, **3**, 156. London: Baillière, Tindall and Cox.
 Fildes, P. & Gladstone, G. P. (1939). *Brit. J. exp. Path.* **20**, 334.
 Gale, E. F. (1945). *Biochem. J.* **39**, 46.
 Gale, E. F. (1947). *J. gen. Microbiol.* **1**, 53.
 Gale, E. F. & Epps, H. M. R. (1944). *Biochem. J.* **38**, 232.
 Gunsalus, I. C., Bellamy, W. D. & Umbreit, W. W. (1944). *J. biol. Chem.* **155**, 685.
 Hale, J. H. & McIlwain, H. (1947). *Brit. J. exp. Path.* **28**, 346.
 Hills, G. M. (1940). *Biochem. J.* **34**, 1057.
 Lichstein, H. C., Gunsalus, I. C. & Umbreit, W. W. (1945). *J. biol. Chem.* **161**, 311.
 McIlwain, H. (1939). *Biochem. J.* **33**, 1942.
 McIlwain, H. (1946a). *Biochem. J.* **40**, 67.
 McIlwain, H. (1946b). *Biochem. J.* **40**, 460.
 McIlwain, H. (1946c). *J. Path. Bact.* **58**, 322.
 McIlwain, H., Fildes, P., Gladstone, G. P. & Knight, B. C. J. G. (1939). *Biochem. J.* **33**, 223.
 McIlwain, H. & Hughes, D. E. (1944). *Biochem. J.* **38**, 187.
 Melville, J. (1935). *Biochem. J.* **29**, 179.
 Pollack, M. A. & Lindner, M. (1942). *J. biol. Chem.* **143**, 655.
 Roper, J. A. & McIlwain, H. (1948). *Biochem. J.* **42**, 485.
 Utter, M. F., Reiner, J. M. & Wood, H. G. (1945). *J. exp. Med.* **82**, 218.
 Waelsch, H., Owades, P., Miller, H. K. & Borek, E. (1946). *J. biol. Chem.* **166**, 273.
 Wiggert, W., Silverman, M., Utter, M. F. & Werkman, C. H. (1940). *Iowa St. Coll. J. Sci.* **14**, 179.

The Estimation of the Leucine Isomers in Protein Hydrolysates by Infrared Analysis

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Certain of the monoamino-acid constituents of protein hydrolysates may be estimated by partition chromatography (Martin & Synge, 1941; Gordon, Martin & Synge, 1943; Tristram, 1946). This method, however, does not effect any separation of leucine $(\text{CH}_3)_2\text{CH}.\text{CH}_2.\text{CH}(\text{NH}_2)\text{COOH}$ and isoleucine $\text{CH}_3.\text{CH}_2.\text{CH}(\text{CH}_3).\text{CH}(\text{NH}_2)\text{COOH}$, the acetyl derivatives of which have identical properties under the conditions of the Martin & Synge technique. The present investigations were directed towards obtaining a rapid and accurate spectroscopic method for analyzing the isoleucine/leucine

ratio in the leucine fractions separated from protein hydrolysates by partition chromatography.

The possibility of analyzing a mixture of leucine and isoleucine by means of differences in their vibration spectra was first suggested by Edsall (1938) in work on the Raman spectra of amino-acids, but, as Edsall noted, the Raman technique presents many difficulties in such a problem. In the course of spectroscopic work on amino-acids, peptides and proteins, we recorded the infrared spectra of leucine and isoleucine and their acetyl derivatives, the latter for the first time. As might be anticipated from the

differences in the hydrocarbon skeletons of such isomers, marked differences were found between the spectra of leucine and isoleucine, and between those of their acetyl derivatives. The spectra of the latter are illustrated in Fig. 1, from which it is clear that the 714 cm^{-1} band of acetyl-L-leucine and the 736 cm^{-1} band of acetyl-L-isoleucine should provide a ready means of distinguishing these two isomers and estimating their relative abundance in a mixture. Using partition chromatography (Gordon *et al.* 1943; Tristram, 1946) it is possible to isolate a representative mixture of the leucine isomers as their acetyl derivatives. For this reason, the infrared analyses have been carried out on the acetamido acids rather than on the free amino-acids.

The applicability of an infrared analysis was first tested on a number of synthetic mixtures of acetyl-L-leucine and acetyl-L-isoleucine. The method was then extended to the examination of the leucine isomers obtained from various control mixtures, and finally leucine fractions from the hydrolysates of various proteins were studied.

ANALYTICAL PROCEDURE

Chemical

The leucine isomers were isolated by the method of Martin & Synge (1941). In the early stages of this work the procedure was to isolate the isomers on the quantitative column, using c. 25 mg. of protein. For a total leucine content of 10% this entailed the working up of some eight fractions. Now the R_F values of acetyl leucine and acetyl isoleucine are identical so that as long as the isolated samples are not submitted to recrystallization there is no need to attempt their quantitative isolation. Accordingly, the later samples were prepared qualitatively using 100–200 mg. of protein, the aim being to obtain about 20 mg. of mixed acetamido acids.

Procedure. The hydrolysate, equivalent to 100–200 mg. of protein, was acetylated in the usual manner (Gordon *et al.* 1943) using c. 20 ml. 6N-NaOH and 5.0 ml. acetic anhydride. After acidification to pH 1 with 10N- H_2SO_4 , the mixture was adsorbed on silica gel, transferred to a large column (4.0 cm. diameter) and extracted with 1 l. chloroform-17% butanol (Tristram, 1946). The acetamido acids were then prepared for analysis and the leucine isomers isolated by the procedure outlined by Tristram (1946). In order to eliminate any acetylphenylalanine and acetylvaline, only the centre portion of the leucine band was collected. It was freed from colouring matter and indicator by passing it through water-saturated silica gel using cyclohexane-5% propanol. The solvent was then removed and the acetamido acids allowed to crystallize in a vacuum desiccator.

Physical

Preparation of specimens for analysis. For accurate analytical work using infrared spectra, the sample should be either in the gaseous or liquid state, although solids which can be obtained as thin plates or films of which the thicknesses can be accurately determined are acceptable. Solvents having no intense absorption over the spectral

regions investigated are very scarce in infrared work, and none of these was suitable for this problem. Estimations were accordingly made on the solid material suspended as a paste in liquid paraffin (Nujol). The acetamido acids were finely ground in a mortar and well mixed with an approximately equal quantity of Nujol until a smooth homogeneous paste was obtained. This paste was then pressed into a thin layer (approx. 30μ) between two rocksalt plates and placed in the spectrometer beam. With this technique, the total amount of acetamido acids suspended in the radiation beam is not accurately known. The method accordingly leads to estimates of the relative rather than the absolute concentrations of the two isomers.

Quantities of materials used. The amount of material used in the analyses was limited by the yield of leucines obtained from the chromatographic separation; experiments were originally made on about 13–15 mg. of material, but later, smaller quantities, c. 7–10 mg., were found to be satisfactory for an assay if the sample was well dried and crystalline. The economy was principally achieved by having a suspension layer of very small area, placed close to the entrance slit of the spectrometer; a further reduction in quantity could probably be obtained by bringing the incident beam of the spectrometer to a subsidiary focus of small area. The concentration of the acetamido acid in the suspensions was such that the relevant absorptions were near the optimal percentage for accurate estimation.

Spectroscopic details. The earlier experimental work was done on a Hilger D. 88 spectrometer equipped with a rock-salt prism. The later work, on which all the data in this paper are based, was performed on a double-beam spectrometer (Hilger D. 209) with automatic pen recording (Sutherland & Thompson, 1945). At 700 cm^{-1} , the rock-salt prism begins to absorb radiation, and the small amount of energy available makes accurate quantitative work difficult; accordingly we used a potassium bromide prism, which transmits to much lower frequencies in the infrared.

The resolving power of the D. 88 spectrometer is comparable with that of the D. 209 spectrometer at 700 cm^{-1} when the latter is equipped with a KBr prism. Recording of both spectrometers was automatic, and the spectral range $760\text{--}700\text{ cm}^{-1}$ was scanned several times for each specimen, to check reproducibility. In this way errors in the shape of the bands and the exact height and location of the maxima could be minimized.

Choice of bands for analysis. The infrared absorption spectra of acetyl-L-leucine, acetyl-L-isoleucine, acetyl-DL-leucine and acetyl-DL-isoleucine are shown in Fig. 1, in the region $1800\text{--}650\text{ cm}^{-1}$. The absorption bands are listed in Table 1. Although the spectra of the laevo compounds differ in many respects, it is advisable to choose two bands for analytical work which lie fairly close together. This is because these bands can then be examined under identical spectroscopic conditions (e.g. slit width).

It is clear from the spectra (Fig. 1) that the band of acetyl-L-leucine at 714 cm^{-1} and that of acetyl-L-isoleucine at 736 cm^{-1} satisfy this condition, and are yet sufficiently separated to be well resolved by the spectrometer. These bands do in fact overlap to a slight extent so that the intensities of absorption at 736 and 714 cm^{-1} are not entirely due to

acetyl-L-isoleucine and acetyl-L-leucine respectively. However, this can be taken into account in the calculations.

that no appreciable amount of racemization had occurred. The absorption spectra of the DL-acetamido acids are seen (Fig. 1 and Table 1) to be some-

Fig. 1. Infrared absorption spectra of acetylleucines, 1800–650 cm^{-1} . (Broken lines indicate Nujol absorption.)

The effects of racemization. The estimations were made on the assumption that the acetamido acids were present entirely in the laevo configuration and

what different from those of the corresponding laevo compounds. This effect had been shown earlier by Wright (1937, 1939) for many amino-acids. The

presence of an appreciable proportion of racemized material in the chromatographic fraction may therefore render the estimation inaccurate. Acetyl-DL-leucine has an absorption peak at 714 cm^{-1} with approximately the same extinction coefficient as that of acetyl-L-leucine, but acetyl-DL-isoleucine gives rise to a broad absorption band centered at 726 cm^{-1} which would contribute to the absorptions measured at 736 and 714 cm^{-1} . The spectra of corresponding L- and DL-leucines are not sufficiently different in the region $3600\text{--}750\text{ cm}^{-1}$ to enable a convenient check to be made on the presence of low proportions of DL-isomers in the fractions, but it is believed that the proportion of racemates in the specimens assayed is quite low.

Table 1. *Absorption maxima of the acetylleucines*
1800–650 cm^{-1}

Acetyl-L-leucine	Acetyl-DL-leucine	Acetyl-L-isoleucine	Acetyl-DL-isoleucine
1695	1703	1703	1720
1624	1625	1622	1608
1554	1567	1547	1566
(1460) Nujol	(1460) Nujol	(1460) Nujol	(1460) Nujol
(1380) Nujol	(1380) Nujol	(1380) Nujol	(1380) Nujol
1347	1316	1364	1329
1298	1271	1339	1298
1276	1247	1270	1270
1244	1166	1238	1232
1164	1131	1170	1157
1127	1099	1150	1108
1103	1035	1105	1072
1034	976	1043	1042
974	920	993	996
917	864	965	835
863	815	876	820
822	714	823	793
796	681	803	726
714	—	736	701
678	—	665	—

Corrections for spurious and scattered radiation. It is important in quantitative analytical work in the infrared region to make allowance for spurious radiation. This is usually of higher frequency than the spectral range under examination, and may therefore have an appreciable magnitude in comparison with the energy being estimated. This is particularly important in the present case where the spectral region $750\text{--}700\text{ cm}^{-1}$ is so far removed from the region of maximum energy of the Nernst source. Spurious radiation of frequency greater than about 900 cm^{-1} is allowed for by placing a fluorite plate in the spectrometer beam to measure the effective zero energy at $750\text{--}700\text{ cm}^{-1}$ (fluorite is opaque for frequencies less than 900 cm^{-1}). This zero setting was found to be appreciably different from that obtained when the beam was cut off with a metal shutter, showing that a small proportion of high-frequency radiation greater than 900 cm^{-1} was being received by the detector when the $750\text{--}700\text{ cm}^{-1}$

region was being scanned. That the amount of radiation scattered by the solid particles in suspension is probably very small in this region of the spectrum has been shown by comparing the degree of transmission of the sample, at a frequency well removed from an absorption band, with the degree of transmission of a layer of Nujol between rocksalt plates at that same frequency. Scattering effects have therefore been neglected.

Methods of calculating concentrations

We assume the validity of Beer's law, viz.

$$I_\nu = I_{0\nu} e^{-kc}, \quad (1a)$$

relating the intensity of the transmitted radiation I_ν , the incident intensity $I_{0\nu}$ of the same frequency, the number of gram molecules c placed in the spectrometer beam and the molecular extinction coefficient k . We further assume that Beer's law may be extended additively for mixtures of two components,

$$I_\nu = I_{0\nu} e^{-(kc + k'c')}. \quad (1b)$$

The incident intensity $I_{0\nu}$ is measured experimentally as the intensity of radiation of frequency ν , transmitted through the absorption cell containing Nujol only. This procedure allows for radiation reflected from the cell faces, and radiation absorbed and scattered by the cell and the Nujol.

Let c = number of gram molecules of acetyl-L-leucine, c' = number of gram molecules of acetyl-L-isoleucine, I_{01} = the incident intensity at 714 cm^{-1} , I_1 = transmitted intensity at 714 cm^{-1} , I_{02} = incident intensity at 736 cm^{-1} , I_2 = transmitted intensity at 736 cm^{-1} , k_1 = molecular extinction coefficient of acetyl-L-leucine at 714 cm^{-1} , k_2 = molecular extinction coefficient of acetyl-L-leucine at 736 cm^{-1} , k_1' = molecular extinction coefficient of acetyl-L-isoleucine at 714 cm^{-1} , k_2' = molecular extinction coefficient of acetyl-L-isoleucine at 736 cm^{-1} . Then, from Beer's law,

$$\log_e \frac{I_{01}}{I_1} = k_1 c + k_1' c', \quad (2a)$$

$$\log_e \frac{I_{02}}{I_2} = k_2 c + k_2' c'. \quad (2b)$$

$$\text{Let } d_1 = \text{optical density at } 714\text{ cm}^{-1} = \log_e \frac{I_{01}}{I_1}, \quad (3a)$$

$$\text{and } d_2 = \text{optical density at } 736\text{ cm}^{-1} = \log_e \frac{I_{02}}{I_2}. \quad (3b)$$

Then, solving (2a) and (2b) for c and c' , we have

$$c = \frac{d_1 k_2' - d_2 k_1'}{k_1 k_2' - k_2 k_1'}, \quad (4a)$$

$$c' = \frac{d_1 k_2 - d_2 k_1}{k_2 k_1' - k_1 k_2'}, \quad (4b)$$

or

$$\frac{c'}{c} = \frac{d_1 k_2 - d_2 k_1}{d_2 k_1' - d_1 k_2'}. \quad (5)$$

The terms of the right-hand side of equation (5) are homogeneous in d , so that common logarithms may be used throughout. We may rewrite this

$$\frac{c'}{c} = K \frac{d_2 - d_1 k_a}{d_1 - d_2 k_b}, \quad (6)$$

where K , k_a , k_b , are constants related to the molecular extinction coefficients, and which are easily determined from the experimental data, i.e.

$$K = \frac{k_1}{k_2}, \quad k_a = \frac{k_2}{k_1}, \quad k_b = \frac{k_1'}{k_2'}.$$

This equation enables the ratio of the quantities of the two isomers to be calculated in a manner independent of the total quantity of material present.

maxima), and the conditions studied under which the assumptions of Beer's law stated in the previous section, are justified. The relevant absorption bands of some of these mixtures are shown in Fig. 2.

It was found that, for a given mixture, the value of $(d_2 - d_1 k_a)/(d_1 - d_2 k_a)$ is constant, within the limits of experimental error, for a moderate range of d_1 and d_2 . For path lengths giving greater than about

Fig. 2. Absorption spectra of some mixtures of known composition of acetyl-L-leucine and acetyl-L-isoleucine, 745–710 cm^{-1} .

Evaluation of K , k_a , k_b : k_a , k_b are evaluated from the absorption curves for pure acetyl-L-leucine and acetyl-L-isoleucine. For the pure leucine isomer, $c' = 0$, and from equation (6), $k_a = \left[\frac{d_2}{d_1} \right]_0$, the subscript 0 indicating that d_1 and d_2 are the optical densities for the pure leucine isomer.

Similarly, for the pure isoleucine isomer, $k_b = \left[\frac{d_1}{d_2} \right]_0$.

If a series of isomeric mixtures of known proportions is examined and the function $\frac{d_2 - d_1 k_a}{d_1 - d_2 k_b}$ plotted graphically against c'/c , a straight line should be obtained, the value of the slope being the constant K . Having determined the line and relation between c'/c and the function of the optical densities d_1 and d_2 , the value of c'/c may be obtained directly from the graph for the value of

$$(d_2 - d_1 k_a)/(d_1 - d_2 k_b)$$

given by any mixture of unknown composition.

RESULTS

The calibration data

Synthetic mixtures of the pure crystalline acetyl-L-leucines were examined over a range varying from 0 to 50 % isoleucine; mixtures containing more than 50 % isoleucine were not examined, analytical data for the proteins indicating that isoleucine is invariably found in smaller amounts than leucine. Each synthetic mixture was examined at different path lengths (giving different absorptions at the

85 % absorption, the intensity of absorption of the weaker band is apparently enhanced, giving high values of c'/c . When the bands are weak (the stronger band being less than about 40 % absorption), the intensities cannot be measured with sufficient precision to enable c'/c to be determined accurately. We have therefore examined samples such that the absorption intensities of the stronger band lie between about 40 and 80 %, both in the calibration and in the estimation of mixtures of unknown composition.

The evaluation of the calibration data is set out in Table 2 (a). Substituting the values $k_a = 0.36$ and $k_b = 0.40$, from this table, into equation (6),

$$c'/c = K(d_2 - 0.36d_1)/(d_1 - 0.40d_2). \quad (7)$$

But $d_1 = \log_{10} 100/I_1$ and $d_2 = \log_{10} 100/I_2$, so that $c'/c = K(1.28 - \log_{10} I_2 + 0.36 \log_{10} I_1)/(1.20 - \log_{10} I_1 + 0.40 \log_{10} I_2)$. (7a)

Equation (7a) is the form of equation (7) which is used in the numerical calculations.

The absorption data for mixtures of known composition are given in Table 2 (b); the values of $(d_2 - 0.36d_1)/(d_1 - 0.40d_2)$ calculated from these give a straight line when plotted against the corresponding values of c'/c (Fig. 3); this is taken as the working curve in the analysis of unknown mixtures. When $c' = 0$ (pure leucine), $(d_2 - 0.36d_1)/(d_1 - 0.40d_2)$ is equal to zero, so that the curve passes through the origin.

Table 2. Calibration data

(a) Evaluation of k_a and k_b				
	Percentage transmission at 714 cm. ⁻¹ (I_1)	Percentage transmission at 736 cm. ⁻¹ (I_2)	k_a	k_b
Acetyl-L-leucine	38.5	71.0	0.358	—
	36.0	69.5	0.357	—
Acetyl-L-isoleucine	71.0	43.0	—	0.406
	75.0	48.5	—	0.398
Mean value of k_b = 0.36.		Mean value of k_b = 0.40.		

(b) Transmission of synthetic mixtures

Percentage ratios leucine : isoleucine	c'/c	Transmissions		$\frac{d_2 - 0.36 d_1}{d_1 - 0.40 d_2}$	Mean value
		I_1	I_2		
51 : 49	0.96	31.5	30.5	1.14	1.15
		56.5	55.0	1.18	
		60.5	59.5	1.15	
66 : 34	0.515	19.0	28.0	0.59	0.59
		20.5	29.5	0.59	
		21.5	31.0	0.58	
		42.0	52.5	0.56	
75 : 25	0.33	24.0	39.0	0.41	0.40
		61.5	73.0	0.39	
		66.0	78.0	(0.31)	
		40.0	55.0	0.39	
78 : 22	0.28	61.0	73.0	0.36	0.38
		60.0	71.5	0.40

Fig. 3. Calibration curve for leucine-isoleucine analysis.

The calibration data in Table 2 are given in detail to enable an estimate to be made of the 'spread' of the results obtained by this method of analysis.

Control mixtures

Two control experiments were performed to test the validity of the chemical procedure.

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(i) Known proportions of the acetyl-L-leucines were evaporated together from solution in the same way as the leucine fractions from the chromatograph column. The mixture had not been treated chromatographically. The results of the spectroscopic analysis are shown in Table 3. The agreement between the analytical values and the original composition indicates that the spectra of the isomers after these have been crystallized together from solution are still additive.

Table 3. Estimation of isoleucine in a control mixture of leucines

Isoleucine as % total leucine isomers	
Present in original mixture	Estimated spectroscopically
32.2	30.3

(ii) A mixture of acetamido acids, in the proportions in which they occur in an insulin analysis (Tristram, 1946), and having an isoleucine/leucine ratio of 1 : 4, was passed through the chromatograph column. The leucine fraction was isolated and the isomer ratio estimated spectroscopically (Table 4).

Table 4. Control experiment on 'synthetic insulin hydrolysate'

Isoleucine as % total leucine isomers	
Present in original 'hydrolysate'	Estimated spectroscopically
19.8	18.7, 18.7, 17.5

The estimated ratios agree within 2% with the ratio originally present, the proportion of isoleucine estimated being low. This control experiment indicates that the leucine isomers are not differentiated in the chromatograph column.

Proteins

The isoleucine/leucine ratio has been estimated in the hydrolysates of three proteins, casein, edestin and insulin (two samples from independent hydrolysates). The ratios are given in Table 5. Several

Table 5. *The isoleucine/leucine ratio as determined spectroscopically in some proteins*

Protein	c/c	Isoleucine as % of total leucines
Casein	0.46	32
Edestin	0.24	19
	0.20	17
		} Two assays
Insulin (first sample)	0.205	17
	0.19	16
		} Two assays
Insulin (second sample)	0.23	18.5
	0.17	14.5
	0.18	15.3
		} Three assays (mean value 15-18%)

assays were made in each sample at varying optical densities; consistent values of the isoleucine/leucine ratio were obtained from assays having absorptions near the optimal value for accurate measurements. The spectra of these samples were examined carefully in the range 1800-700 cm^{-1} and compared with those of similar synthetic mixtures of acetyl leucine and isoleucine. Agreement throughout the spectral range was good and no evidence of extraneous bands, arising from impurities, was found. The spectra of acetyl-L-valine, and the acetyl-DL-leucines were particularly examined in this connexion.

DISCUSSION

The reliability and accuracy of the method

The assumption made in these calculations is that the relative absorption intensities of the isomers at two fixed frequencies can be expressed by Beer's law, in a form which is independent of the total amount of absorbing material. The two isomers are examined together as a physical mixture of the solids. The possibility that intermolecular interaction (such as might arise by crystallizing the isomers together) could invalidate the use of Beer's law has been shown to be absent. The validity of these assumptions is confirmed by the experimental data from the synthetic mixtures (Fig. 3), which show a constant value for K , the slope of the curve.

Reproducibility of the percentage absorptions which are measured is checked by scanning each mixture twice or more; the percentage absorptions

are then considered accurate to $\pm 0.5\%$ of absorption. The corresponding error in the percentage of isoleucine in the mixture is plotted in Fig. 4 against the percentage of isoleucine. It is thus seen, for example, that when the percentage of isoleucine is near 19%, this figure is expressed as $(19 \pm 1.5)\%$. In order not to overstate our accuracy, the errors in measurement of the two bands have been assumed to be cumulative. The independence of total concentration is ascertained by examining each sample at two or more optical densities. Reasonably good agreement is obtained when the absorption of the stronger component is within the range 40-80%, and when the weaker band is not too feeble to be measured accurately.

Fig. 4. Error curve for isoleucine estimation, assuming errors of $\pm 0.5\%$ in measuring absorption bands.

The method is capable of refinements and by adopting a standard procedure of working, with adequate controls, it can probably be made a very reliable method of amino-acid analysis. Results are only reproducible if identical spectroscopic working conditions both for the calibration and assays are maintained.

As discussed earlier, racemization would cause errors in the present spectroscopic estimations of the isoleucine/leucine ratio.

Whether or not the leucine fraction is fully recovered from the chromatograph column, the spectroscopic method of analysis should yield a reliable value of the isoleucine/leucine ratio.

Comparison of the method with microbiological assay

The greatest difference between the spectroscopic method and that of microbiological assay is the relatively large amount of material needed for the former. For a spectroscopic analysis, we require at present 5-10 mg., compared with the few $\mu\text{g.}$ required for a microbiological assay. Moreover, the

spectroscopic method, as used at present, gives the relative concentrations of the isomers, whereas absolute concentrations of the amino-acids are given by microbiological assay.

The advantage of the spectroscopic method over microbiological assay is that a highly characteristic physical property of the molecule (its vibration spectrum) is being used. The microbiological method is subject to doubts, particularly regarding lack of specificity of the organism, and interference of other constituents in a mixture. The microbiological method is rendered inaccurate by racemization, the D-form in general not being estimated (see Dunn, Camien, Shankman & Block, 1947), and the assay therefore being low in such a case. The effect of racemization in spectroscopic estimation has already been discussed, but there is little doubt that a more refined technique could make due allowance for this. We would stress that the present investigations have been of an exploratory nature, and that the method is capable of greater accuracy with a carefully standardized routine.

Discussion of analysis

The mean values of percentages of isoleucine in total leucine fractions shown in Table 5 are considered reliable to $\pm 1.5\%$ (see Fig. 4), although the control experiments suggest that there may be a tendency for the present results to be systematically low. The comparison with microbiological values is given in Table 6. It will be observed that the spectroscopic values for casein and insulin are close to those previously obtained by microbiological assay, but the isoleucine proportion is slightly low in both cases.

Table 6. Comparison of spectroscopic values of the isoleucine/leucine distribution with microbiological values

	Percentage of isoleucine in total leucine fraction	
	Spectroscopic	Microbiological assay
Casein	32	38.5*, 39.5†, 39.2‡, 36.2§
Edestin	18	45.5
Insulin	15-18	17.8¶

* Hier, Graham, Friedes & Klein (1945).

† Kuiken, Norman, Lyman, Hale & Blotter (1943).

‡ Dunn, Camien, Rockland, Shankman & Goldberg (1944).

§ Stokes, Gunness, Dwyer & Caswell (1945).

|| Smith, Greene & Bartner (1946); Smith & Greene (1947).

¶ Brand (1946).

An earlier spectroscopic value for the ratio of the leucine isomers, which was given in a publication by Tristram (1946), was in error, due to imperfect calibration data obtained in the preliminary stages of the investigations on insulin.

The value of comparing one method with another based upon different principles is indicated very strikingly in the case of the ratio of the leucine isomers of edestin. Smith *et al.* (1946) and Smith & Greene (1947) reported that the leucine (*Lactobacillus arabinosus*) and isoleucine (*Streptococcus faecalis*) contents of edestin were 4.25 and 3.55 % respectively (i.e. 54.5 % of leucine). Infrared analysis suggests that the distribution is nearer 80 % leucine (Table 5).

Fig. 5. Comparison of spectra of acetyl leucines from edestin hydrolysate with mixtures of known composition.

It has been shown that the chromatographic separation of the acetyl leucines fraction does not effect any concentration of isoleucine, and whatever the recovery of the total leucine isomers, the ratio is unaltered. Two of the calibration mixtures (Table 2b), containing respectively 51 and 78 % leucine, are near to the distributions suggested respectively by microbiological and spectroscopic methods, and the absorption curves of these mixtures are compared in Fig. 5 with that obtained from the leucines fraction of edestin.

SUMMARY

1. The infrared absorption spectra of leucine and isoleucine and of their acetyl derivatives have been examined in the range 1800-650 cm^{-1} . The spectra of each pair of isomers show marked differences which make it easy to distinguish the isomers.

2. A spectroscopic method of analyzing isoleucine/leucine mixtures has been investigated (using as indicators characteristic bands near 700 cm^{-1} of the acetyl derivatives) and tested on synthetic mixtures of the L-isomers.

3. The applicability of the spectroscopic method of analysis for estimating the isoleucine/leucine ratio in the leucine fraction of protein hydrolysates has been tested by control experiments on mixtures of leucine and isoleucine subjected to the treatment employed in protein analyses.

4. The study of the method in the determination of the isoleucine/leucine ratio in the leucine fraction from the hydrolysates of casein, insulin and edestin gave results which agree with those obtained by microbiological methods except in the case of edestin where there is a very large discrepancy:

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REFERENCES

- Brand, E. (1946). *Ann. N.Y. Acad. Sci.* **47**, 187.
 Dunn, M. S., Camien, M. N., Shankman, S. & Block, H. (1947). *J. biol. Chem.* **168**, 43.
 Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S. & Goldberg, S. C. (1944). *J. biol. Chem.* **155**, 591.
 Edsall, J. T. (1938). *Cold Spr. Harb. Symp. quant. Biol.* **6**, 40.
 Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
 Hier, S. W., Graham, C. E., Friedes, R. & Klein, D. (1945). *J. biol. Chem.* **161**, 705.
 Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F. & Blotter, L. (1943). *J. biol. Chem.* **151**, 615.
 Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.
 Smith, E. L. & Greene, R. D. (1947). *J. biol. Chem.* **167**, 833.
 Smith, E. L., Greene, R. D. & Bartner, E. (1946). *J. biol. Chem.* **164**, 159.
 Stokes, J. L., Gunness, M., Dwyer, L. M. & Caswell, M. C. (1945). *J. biol. Chem.* **160**, 35.
 Sutherland, G. B. B. M. & Thompson, H. W. (1945). *Trans. Faraday Soc.* **41**, 174.
 Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
 Wright, N. (1937). *J. biol. Chem.* **120**, 641.
 Wright, N. (1939). *J. biol. Chem.* **127**, 137.

Studies on Vitamin A

5. THE PREPARATION OF RETINENE₁—VITAMIN A ALDEHYDE

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The state of knowledge concerning photoreception and the role of rhodopsin (visual purple) has been surveyed recently (Granit, 1947) so that only a brief introduction is necessary here.

Wald (1935-6) found that dark-adapted retinas, which are pinkish purple in colour owing to the presence of relatively large amounts of rhodopsin, turn yellow on illumination. Light petroleum will extract from retinas exposed to light a material which Wald called *retinene*. The petroleum solution, freed from solid matter by centrifuging, shows an ultraviolet-absorption maximum near 365 m μ , displaced to 385 m μ when chloroform is the solvent. When the antimony trichloride reagent, used in vitamin A studies, is added to a chloroform solution of retinene, a blue colour is produced. The blue solution exhibits a well-defined absorption band with a maximum at 664 m μ . Wald postulated for scotopic vision a cyclic process into which entered vitamin A, retinene and visual purple.

In later studies, Wald (1937, 1938-9 *a, b*) found that a similar extraction procedure applied to retinas of fresh-water fish, in which rhodopsin is largely replaced by porphyropsin (visual violet), yielded retinene₂. This substance is similar to retinene₁ but its absorption spectrum is slightly displaced in the direction of longer wave-lengths, as also is the characteristic absorption maximum of the colour test. The discovery that vitamin A₂ (Edisbury, Morton & Simpkins, 1937; Lederer & Rosanova, 1937) tends

to take the place of vitamin A in fresh-water fish as compared with sea fish and mammals indicated that retinene₂ bears the same relationship to vitamin A₂ as retinene₁ to vitamin A. This is not surprising, as there was already ample evidence of a connexion between vitamin A deficiency and night blindness associated with retarded formation of rhodopsin (visual purple) in the dark-adaptation process.

The discovery of the retinenes was a notable advance, but paucity of material has delayed its exploitation. The amounts obtainable from eyes are too minute to permit orthodox constitutional investigations and for nearly a decade the substances have remained known only by qualitative spectroscopic characteristics quoted in Table 1.

Table 1. *Absorption maxima of substances concerned in visual processes*

Substance	λ_{max} (m μ)	Solvent	SbCl ₃ colour test (in chloroform) λ_{max} (m μ)
Vitamin A	326	cycloHexane	617 inflexion 582
Vitamin A ₂	345-350	cycloHexane	693
Rhodopsin	502	1% aqueous digitonin	
Porphyropsin (visual purple)	520-530		
Retinene ₁	369-5	Light	664
	389	petroleum	
Retinene ₂	390	Chloroform	
	405	Petrol	>705
		Chloroform	