which possesses the retention volume of the original corticotrophin.

2. The final product has been characterized and it appears to differ from the original corticotrophin only in possessing a *N*-terminal glycine residue in place of serine.

3. Preliminary observations that the product has the same corticotrophic activity as the native hormone are quoted.

We are grateful to Miss M. B. Thomas for her skilled assistance throughout the work, and to Dr R. G. Sheperd, Dr G. W. Crosbie, Dr J. I. Harris and Dr R. I. T. Cromartie for most helpful advice and discussion. We thank Mrs B. Brown for the amino acid analyses and Dr F. L. Engel for the gift of corticotrophin concentrates and him and Dr H. E. Lebovitz for permission to quote their results. We also thank the Medical Research Council for a grant for expenses.

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The Determination of the Tyrosine Content of Gelatins

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(*Received* 26 *March* 1962)

The low and variable tyrosine contents of gelatins and collagens suggest that tyrosine may be present, at least in part, as an impurity. Orekhovitch, Tustanovskit, Orekhovitch & Plotnikova (1948) and Russell (1958) have claimed that samples of procollagen and gelatin respectively can be obtained substantially free from tyrosine. However, Harkness, Marko, Muir & Neuberger (1954) have found tyrosine in purified rabbit-skin collagen, and Schmitt, Gross & Highberger (1955) were unable, even with highly purified collagen, to decrease the tyrosine contents much below 0·41 %. Eastoe (1955) reported that fractionation of gelatin to remove impurities decreased the tyrosine content to about 0·2 %. Leach (1960*a*) could not confirm Russell's results, but was able to isolate 0·36 % of a minor component from gelatin, which, although considerably richer in tyrosine than the original gelatin, contributed only about 0·01 g. of tyrosine/100 g. of the parent material.

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The small amounts of tyrosine in whole and fractionated gelatins have now been determined by two independent methods. The methods, which are discussed separately, have been critically examined and compared with recalculated chromatographic data of Eastoe (1955, 1960).

METHODS

Colorimetric assay

Tyrosine reacts with α-nitroso-β-naphthol in the presence of nitric acid to give a coloured derivative which is unstable to light. Many workers have modified the reaction conditions to improve the stability, and these methods were examined. Disadvantages were found with these procedures, the most serious being the unreliability of the colour-forming reaction with gelatins. The occasional absence of colour development in conjunction with the heterogeneous mode of reaction in the method of Ottaway (1958) suggested that an induction period followed by an autocatalytic reaction was involved. Accordingly, in the present method the reaction in fresh solutions was promoted by 'seeding' with a solution that had previously been caused to react (and thus was coloured). Ceriotti & Spandrio (1957) had found that, in addition to nitric acid, hydrochloric acid was necessary to obtain the colour reaction. With the present method it was advantageous to combine these acids in the promoter solution and thus avoid a number of pipetting operations.

Reagents. α-Nitroso-β-naphthol (A.R.) was used as 0·03% solution in 0·03N-NaOH. Solution was effected by shaking, any residue being filtered off. Fresh solution, prepared daily, was used.

Standard L-tyrosine (British Drug Houses Ltd.) solution, containing 100 μg./ml., was prepared by dissolving the amino acid in a little 5N-HCl and diluting. This solution was stable for some weeks at 4°. Subsidiary standards containing 20, 40, 60 and 80 μg./ml. were obtained by dilution as required.

Approx. 1·5% (w/v) gelatin solutions were accurately prepared in water.

Promoter solution was prepared by mixing, in order, 2 ml. of nitrosonaphthol solution, 1 ml. of standard tyrosine solution, 6 ml. of water, 1 ml. of 2·5N-HNO_3 and 3 ml. of conc. HCl. The solution was heated at 70° for 3 min. to give a bright-red liquid. (Alternatively this solution could be prepared by heating the nitrosonaphthol and tyrosine solutions with 10 ml. of the promoter solution used previously.) The promoter solution was completed by mixing the coloured solution with 50 ml. of 2·5N-HNO_3, 200 ml. of water and 150 ml. of conc. HCl, and making up to 500 ml. with water. The promoter solution was kept in the dark and could be used as long as it was detectably pink (about 7 days).

Procedure. Each reaction mixture was prepared in duplicate in a dry, rimless, 6 in. × 1 in. glass test tube. Nitrosonaphthol solution (1 ml.), standard tyrosine solution or a gelatin solution (2 ml.) and the promoter solution (5 ml.) were mixed, heated in a 40° thermostat bath for 15 min. and cooled to room temperature in cold water. It was advisable to add the first reagent to all the tubes, then the tyrosine-containing solution and finally the promoter solution.

Immediately on cooling, the extinctions of the solutions were measured against water with a Hilger Uvispek spectrophotometer at 510 mμ in 2 cm. cells. Since tyrosine standards were always included (see below) the need for blank determinations was eliminated and these were usually omitted. From the tyrosine standards a linear plot was obtained of extinction against tyrosine content, for concentrations of the standards up to 100 μg./ml. (25 μg./ml. in the reaction mixture). The tyrosine content of the gelatin solution was then interpolated. Variations in the absorption occurred between successive experiments, and thus measurements of standards were always carried out concurrently with gelatin or unknown solutions.

Discussion of the colorimetric method. The use of the promoter technique has ensured that the colour reaction always occurs and has eliminated the necessity for high temperatures and correspondingly short reaction times. At 40° differences in the colour development between tyrosine and gelatin, which occur in the early part of the reaction, largely disappear in 15 min., and after this interval the rate of change of the pink colour is small (Table 1).

The extinction of the cooled solutions changes by about 6%/hr. For this reason, the number of test tubes used at any one time must be limited to allow all the extinction readings to be completed in 15 min. As a precaution, exposure to light was minimized since the coloured solutions were sensitive, particularly to direct sunlight.

The internal reproducibility of any experiment was good with both the standards and the gelatin solutions. The recovery of tyrosine (Table 2) was about 95%, though this decreased when the original solution contained above 100 μg./ml. Provided that gelatin solutions contained tyrosine below this concentration, results were reproducible to within ±6%. The good recovery indicates that the presence of gelatin has little effect on the colour yield from free tyrosine. Similar recoveries were obtained with hydrolysed tyrosine and gelatins.

The colorimetric determination was also carried out on a number of representative amino acids: histidine, lysine, hydroxyproline, aspartic acid, threonine and phenylalanine. The absence of significant colour yields indicated that interference with the tyrosine estimation from these sources was negligible.

Some gelatins contain preservatives, and interference due to these was considered. The colour yield from sodium pentachlorophenate and sodium hydroxyphenylphenate

Table 1. *Development and decline of colour in tyrosine and gelatin solutions with increase in reaction time*

Reaction time (min.)	Proportion of colour yield to maximum value (%)	
	Tyrosine	Gelatin
7	64·1	79·2
10	82·4	96·0
13	92·2	100
14	94·3	99·0
15	96·1	97·6
16	97·5	96·4
17	98·0	95·6
21	100	92·0
26	96·1	88·8

was not appreciable, whereas that from *p*-chloro-*m*-cresol amounted to 16% of that for an equivalent amount of tyrosine.

Spectrophotometric assay

The tyrosine content of proteins, including gelatin, may be determined from their ultraviolet-absorption spectra, provided that interfering substances are absent, or, if present, that their effect can be eliminated. This method of estimation has been discussed by Goodwin & Morton (1946), Fromageot & Schnek (1950/1951), Beaven & Holiday (1952) and Bencze & Schmid (1957).

The relatively high proportion of phenylalanine in gelatin ensures that in the gelatin absorption spectrum measured in neutral or acid solution the contribution due to the phenylalanine spectrum will obscure the tyrosine absorption maximum. This interference can be avoided by carrying out the spectrophotometric measurements on solutions in N-NaOH where the ionization of the tyrosyl hydroxyl group causes a displacement of the absorption maximum of the tyrosine sufficient to free it from the phenylalanine spectrum. Interference by tryptophan was regarded as negligible for the reasons detailed below, and the effect of cystine, present only as a trace in gelatin (Eastoe, 1955), could be ignored.

The spectrum of pure tyrosine could be closely superimposed on spectra of acid-processed gelatins over the wavelength range 290–350 mμ (Fig. 1, curve *A*). With alkali-processed gelatins, however, the spectra could be brought into coincidence for wavelengths above 300 mμ but not at the maximum. Compared with the superimposed tyrosine spectrum, the gelatin maximum occurred at a shorter wavelength and showed higher absorption (Fig. 1, curve *B*). Both these results would be expected if a further absorbing substance is present with the tail of its absorption overlapping the tyrosine maximum. The inability to secure coincidence of the two curves at the maximum indicated that tyrosine contents calculated from the readings at this point would be in error. The normally less-accurate procedure of using a non-maximum value for the determination has been adopted to avoid this difficulty. The wavelength chosen was 304 mμ as this was near the lower limit of superimposition.

Procedure. Extinctions were measured in 2 cm. cells against the solvent with a Hilger Uvispek spectrophotometer, readings being taken between wavelengths of 270 and 350 mμ. The method advocated by Goodwin & Morton (1946) and by Beaven & Holiday (1952) for correcting for extraneous absorption resulting from haze and traces of pigment in the solution was used. The absorption in the wavelength range 330–350 mμ was linearly related to wavelength and this line was extrapolated to below the tyrosine absorption peak. Extinctions were then corrected by measuring from this base line. Beaven & Holiday (1952) state that if the true base line has been drawn the absorption spectra derived from the tyrosine-containing material dissolved in acid and in alkali respectively will intersect at 277 mμ. This intersection has been obtained with gelatins.

Gelatin (about 4 g./l.) and tyrosine (about 20 μg./ml.) solutions were prepared in N-NaOH, though some earlier experiments were performed on 0·1 N-NaOH solutions. With the 0·1 N-NaOH the ionization of the tyrosyl hydroxyl groups in gelatin was apparently not always complete, so that subsequently N-NaOH was used. Similar observations have been made on ribonuclease by Shugar (1952). Spectra determined for tyrosine in 0·1, 0·3 and 1·0 N-NaOH agreed to better than 1%. Tyrosine contents in gelatin were determined from readings at 292 mμ (gelatins gave a maximum at about this wavelength) and 304 mμ, the specific extinction coefficients $E^{1\%}_{1\,cm.}$ for tyrosine at these wavelengths being 13·0 and 8·05 respectively. The ratio of these values was 1·62. Variations in this ratio were ob-

Table 2. *Recovery of tyrosine in the colorimetric method*

To 1 ml. of nitrosonaphthol solution was added 1 ml. of gelatin solution together with 1 ml. of a solution containing 0–80 μg. of tyrosine. The normal procedure was then carried out.

Tyrosine added to 1 ml. of gelatin soln. (μg.)	Total tyrosine content found (μg.)	Tyrosine recovered (μg.)	Recovery (%)
0	46	—	—
20	65	19	95
40	84	38	95
60	102	56	93·3
80	119	73	91·3

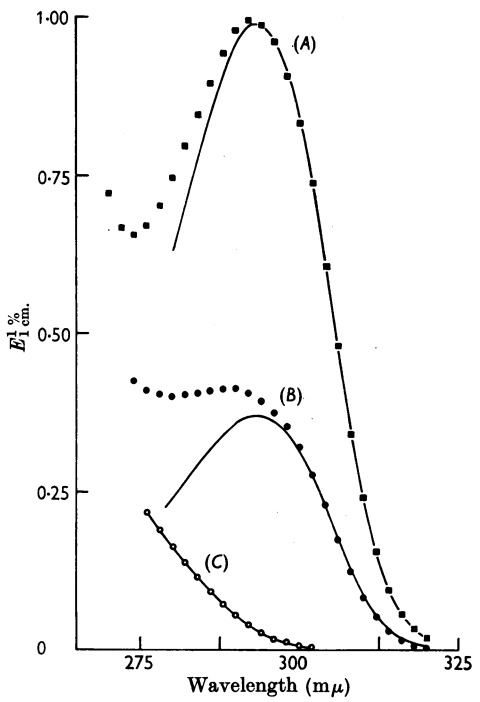

Fig. 1. (*A*) and (*B*): absorption spectra of gelatin, with tyrosine spectra (continuous lines) superimposed. ■, Pig-skin gelatin, acid-processed; ●, ox-hide gelatin II, alkali-processed. (*C*): ○, Difference spectrum (the spectrum of ox-hide gelatin II less the superimposed tyrosine spectrum).

tained with gelatins; acid-processed materials gave 1·61–1·65 and alkali-processed gelatins 1·65–1·80.

Hydrolysates of tyrosine and gelatin gave spectra in which the maxima were displaced about +1 mμ. As a result the specific extinction coefficients $E^{1\%}_{1\,cm.}$ became 12·7 (292 mμ) and 8·83 (304 mμ) and their ratio 1·44. The tyrosine in hydrolysates was estimated from these latter data.

Tyrosine determinations were reproducible to within ±5% and a recovery of 101% of added tyrosine was obtained in the presence of gelatin. Similar reproducibility was found with gelatin hydrolysates. Recoveries of hydrolysed tyrosine are shown in Table 3.

Interference by tryptophan. Tryptophan has not frequently been identified in gelatins, and estimates of its content have varied (Bowes & Kenten, 1947). Spectrophotometric data of Holiday (1936) and Goodwin & Morton (1946) suggest that gelatin contains about 0·05% of tryptophan. If tryptophan at this concentration was present in acid-processed gelatin, the tyrosine spectrum would not superimpose on that for the gelatin. Also, since tryptophan is destroyed in the hydrolysis, the spectrum for hydrolysed gelatin would differ largely from that for the intact material. In view of the different absorption curve obtained for alkali-processed gelatins, the evidence for the absence of tryptophan in these materials is less conclusive. If allowance were made for a content of 0·05% of tryptophan, the spectrophotometric values for tyrosine would differ substantially from those obtained by the colorimetric method. Further, the spectrum obtained as the difference between that for gelatin and for tyrosine is a smooth curve (Fig. 1, curve *C*), with no shoulder to indicate the presence of tryptophan. For these reasons, it was considered that insufficient tryptophan was present in the gelatins studied to affect significantly the spectrophotometric results.

Interference by phenolic preservatives. Some technical gelatins contain phenolic preservatives, the spectra of which will interfere with tyrosine estimations when the spectrophotometric method is used. Five preservatives of this type were examined and their influence on gelatin spectra was determined. The ratio of extinction coefficients, measured at 292 and 304 mμ, was altered as shown in Table 4.

Where the presence of interfering preservatives was suspected from the abnormal extinction ratio and apparently high tyrosine values, continuous ether extraction of the gelatin was used to remove the contaminant before repeating the extinction measurements. It should be added that edible gelatins never and photographic gelatins rarely contain phenolic preservatives.

Interference in alkali-processed gelatins. An alkali-processed ox-hide gelatin was fractionated by alcohol coacervation (see Results section), and the absorption spectra of the fractions were determined. The tyrosine spectrum could be closely superimposed on the spectra for the later fractions, but those for the first two fractions showed little resemblance to the tyrosine spectrum (Fig. 2). The interference obscuring these two spectra is attributed to traces of impurities that are collected in these fractions (Stainsby, 1954; Courts, 1959*a*, *b*). This interference accounts for the inability to superimpose the tyrosine spectrum on that of the whole gelatin except above 300 mμ.

Chromatographic assay

The majority of tyrosine estimations previously reported from these Laboratories (Eastoe, 1955, 1957, 1960; Leach, 1957) have been obtained as a part of a complete amino acid determination by the method of Moore & Stein (1951). Owing to the overlap in the elution of phenylalanine and tyrosine there was considerable uncertainty in estimating these amino acids separately. Subsequent chromatographic techniques will resolve these amino acids (e.g.

Table 3. *Recovery of tyrosine in hydrolysates*

Tyrosine (100 μg.) was added to a gelatin solution, and the whole was hydrolysed by the procedure of Eastoe (1955) for 24 hr. Tyrosine was determined spectrophotometrically at two wavelengths, 292 and 304 mμ.

Tyrosine added (μg.)	Calc. total tyrosine content (μg.)		Tyrosine recovery (%)	
	At 292 mμ	At 304 mμ	At 292 mμ	At 304 mμ
—	126	118	—	—
100	217	214	91	96
100	228	215	102	97

Table 4. *Influence of phenolic preservatives on the extinction ratio of gelatins*

Values are stated for both acid- and alkali-processed gelatins, for 0·1% (w/w) of preservative based on the gelatin.

Preservative	$E_{292}/E_{304\,m\mu}$ Acid-processed gelatin	$E_{292}/E_{304\,m\mu}$ Alkali-processed gelatin
—	1·64	1·73
Phenol	2·0	2·3
β-Naphthol	2·0	2·3
p-Chloro-*m*-cresol	1·5	1·5
Sodium *o*-phenylphenate	1·4	1·3
Sodium pentachlorophenate	1·5	1·4

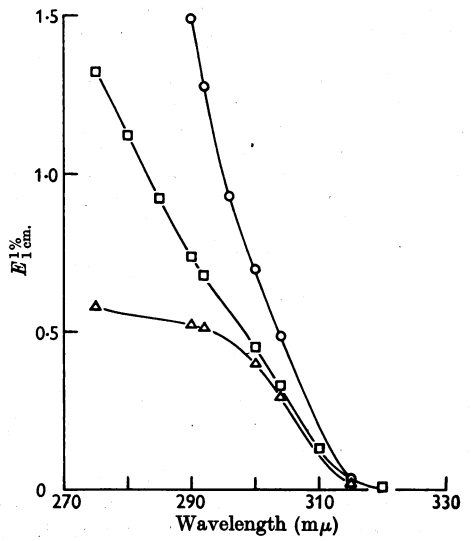

Fig. 2. Absorption spectra of fractions of ox-hide gelatin II: ○, fraction 1; □, fraction 2; △, fraction 3.

Table 5. *Comparison of tyrosine and phenylalanine data calculated by the empirical and mathematical methods*

Empirical (1) and mathematical (2) methods were used with Moore & Stein analytical figures of Eastoe (1955, 1960) for calculation of data. Values are expressed as g. of amino acid/100 g. of protein.

Material	Duration of hydrolysis (hr.)	Tyrosine		Phenylalanine	
		1	2	1	2
Ox-hide gelatin I					
(*a*) First extraction	24	0·27	0·38	2·29	2·26
(*b*) Third extraction	24	0·35	0·41	2·13	2·28
	24	0·29	0·38	2·21	2·18
	48	0·37	0·44	2·26	2·30
Ox-hide gelatin II					
Untreated	48	0·29	0·26	2·24	2·32
Purified	48	0·18	0·23	2·23	2·33
Commercial bone gelatin	24	0·23	0·36	2·49	2·47
Pig-skin gelatin	24	0·60	0·62	2·56	2·58
Atypical calf-skin gelatin	48	0·14*	0·15	2·20*	2·24

* Eastoe (1960). All other empirical data are given by Eastoe (1955).

Stein & Moore, 1954; Moore, Spackman & Stein, 1958). Eastoe (1955) adopted an empirical method, constructing the two elution peaks by inspection. This was justified by its agreement with the more accurate mathematical method of separating the peaks in the two instances where the full calculation was carried out. In view of the importance attached by Russell (1958) to tyrosine data, this latter method has been applied to all gelatins on which the Moore & Stein technique had been performed and reported by Eastoe (1955, 1960).

The method involves fitting the equation

$$y = A(1 \pm Bx^2)\, e^{-x^2/C^2}$$

to the phenylalanine curve, regions being used where the tyrosine contribution is negligible. The fraction number (x) is zero when the extinction (y) is a maximum; A, B and C are constants, A being equal to the maximum value of y. $+B$ is used for the trailing side of the peak and $-B$ for the leading side to give the required asymmetry. The equation is then used to predict the phenylalanine values in the region of the tyrosine peak, and subtraction from the experimental figures gives the whole tyrosine peak. The recalculated values, together with those for phenylalanine, are compared with the previous data in Table 5.

Results for the ox-hide gelatin I*b* (Table 5) indicate the reproducibility ($\pm 8\%$) of the chromatographic method is better than the original data of Eastoe showed. Recovery of tyrosine and phenylalanine from a synthetic mixture with the same column and technique was 100% (Bender, Palgrave & Doell, 1959).

RESULTS

The tyrosine contents of a number of gelatins, determined by the three methods described, are recorded in Table 6.

Ox-hide gelatin II has been fractionated by the alcohol coacervation method of Stainsby (1954), modified for use with propan-2-ol by A. M. Kragh and P. R. Saunders (personal communication). Eleven fractions were obtained, the tyrosine in each fraction being determined by both the colorimetric and spectrophotometric methods. The values are compared in Table 7.

DISCUSSION

Tyrosine determinations by the three methods are in good agreement and little difference is shown between values derived from intact and hydrolysed samples of the same gelatin. Intact gelatins will incur some degradation under the conditions of the colorimetric and spectrophotometric methods, though this is negligible compared with the hydrolysis used before the chromatographic analysis. The spectrophotometric and colorimetric methods offer accurate and convenient means of determining tyrosine in gelatins. The chromatographic separation of amino acids is a more elaborate method, but it is equally accurate. The colorimetric method is reliable and less subject to interference than the spectrophotometric estimation, and the latter is simple and to be preferred where large numbers of samples have to be analysed. These latter two methods should be applicable to hydrolysed collagens, to soluble collagens provided that they remain in solution under the conditions obtaining, and possibly to other proteins.

The balance sheet for the fractionated gelatin (Table 7) shows little loss of tyrosine during the fractionation. Fractions representing about 80% of the original gelatin were obtained with tyrosine contents equal to or below that of the parent sample. Significant concentration of tyrosine-containing material occurs in the first fraction, and a very much larger amount, almost one-fifth of the total tyrosine present, collects in the last (residual) fraction. It has been reported that the impurities in gelatins are concentrated by fractionation into

Table 6. *Comparison of results for tyrosine contents of gelatins*

Values are expressed as g. of tyrosine/100 g. of protein.

Material	Method ...	Tyrosine content: Colorimetric	Spectrophotometric	Chromatographic
Ox-hide gelatin I				
(*a*) First extraction	Intact	0·38	0·69–0·87*	—
	Hydrolysed	—	—	0·38
	Preservative removed	0·37	0·37	—
(*b*) Third extraction	Intact	0·39	0·75–0·95*	—
	Hydrolysed	—	—	0·42
	Preservative removed	0·42	0·39	—
Ox-hide gelatin II (untreated)	Intact	0·31	0·29	—
	Hydrolysed	0·24	—	0·26
Commercial bone gelatin	Intact	0·36	0·37	—
	Hydrolysed	—	—	0·36
Pig-skin gelatin	Intact	0·74	0·78	—
	Hydrolysed	—	—	0·62
Atypical calf-skin gelatin	Intact	—	0·19	—
	Hydrolysed	—	—	0·15
Limed ossein gelatin	Intact	0·33	0·33	—
Acid-treated pig-skin gelatin	Intact	0·76	0·76	—
	Hydrolysed	0·68	0·74	0·56

* From measurements at 292 and 304 mμ. Data are high and not in agreement as these gelatins contained *p*-chloro-*m*-cresol.

Table 7. *Tyrosine contents of ox-hide gelatin II fractions*

Fraction	Wt. of gelatin (g. dry, ash-free)	Tyrosine (g./100 g. of protein): Spectrophotometric method	Colorimetric method	Total tyrosine by colorimetric method (mg.)
1	1·5	0·61*	0·47	7
1a	6·4	0·36*	0·30	19
2	11·0	0·42	0·38	42
3	19·8	0·36	0·31	62
4	29·2	0·32	0·30	88
5	18·2	0·33	0·25	46
6	27·4	0·24	0·21	58
7	21·8	0·26	0·23	50
8	21·5	0·22	0·21	45
9	20·2	0·25	0·25	51
10	16·4	0·66	0·67	110
Totals	193	—	0·30 (mean)	580
Original gelatin II	204	0·29	0·31	630

* In error, owing to interference from impurities collected in this fraction.

the first and last fractions (Stainsby, 1954; Courts, 1959*a*, *b*; Leach, 1960*b*; Williams, 1961), though the figures are difficult to reconcile with the tyrosine contents and amounts of the known impurities in gelatins.

Eastoe (1960) has analysed an alkali-processed calf-skin gelatin of high purity to give a tyrosine content of 0·15–0·19 % (Table 6). The later fractions (excluding the last) of the ox-hide gelatin II, reported here, contained 0·21–0·25 % of tyrosine, and refractionation by the same coacervation technique did not decrease this concentration. These values support the view that about 0·2 g. of tyrosine/100 g. of gelatin is attached firmly to the gelatin molecule, and that, when higher contents are observed, the excess is derived from impurities. A figure of 0·2 % of tyrosine corresponds approximately to one tyrosine residue in a molecular weight of 90 000, a feasible value for a minor constituent.

SUMMARY

1. A colorimetric method for determining tyrsine has been developed for use with gelatins. The reliability of the α-nitroso-β-naphthol reaction has been improved by using a 'promoter' solution, which made possible the use of more reproducible reaction conditions.

2. The determination of tyrosine in gelatins by spectrophotometric and chromatographic methods has been discussed. The former has been considered with special reference to the elimination of interference by certain constituents of gelatins.

3. The tyrosine contents of a number of gelatins have been determined by the three methods. Results are comparable; in addition similar values are obtained from intact and hydrolysed samples of the same gelatins. The gelatins studied contained 0·2–0·8 % of tyrosine.

4. Fractionation of gelatin by alcohol coacervation concentrates impurities into the first and last fractions, which have a considerably enhanced tyrosine content. The intermediate fractions have tyrosine contents that do not exceed that of the original gelatin, no fraction containing less than 0·2 % of tyrosine.

5. The results support the view that a proportion of the tyrosine in gelatins is combined in the protein structure, together with a tyrosine-containing impurity.

The authors wish to thank Dr J. E. Eastoe of the Royal College of Surgeons for making available the original chromatograms of his Moore & Stein analysis, and Dr A. A. Leach of The British Gelatine and Glue Research Association for helpful discussions. This paper is published by permission of the Director and Council of The British Gelatine and Glue Research Association.

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The Form of Vitamin C Released by the Rat Adrenal

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(*Received* 12 *April* 1962)

The adrenal gland of the rat loses part of its very high concentration of vitamin C (ascorbate and dehydroascorbate) under a variety of conditions. ['Dehydroascorbate' is used in this and the next paper as a conventional term for the reversibly oxidized form of vitamin C: it is not meant to imply that 'dehydroascorbic acid' ionizes in water as do ascorbic acid and dioxogulonic acid (cf. Lloyd & Sinclair, 1953).] Slusher & Roberts (1957) reported that the loss of vitamin C by the adrenal gland equals the gain by the adrenal-vein blood, and Salomon (1957, 1958) concluded that ascorbate is not the principal component of this loss and gain. Slusher & Roberts (1957) used the method of Roe & Kuether (1943), which estimates a total of ascorbate, dehydroascorbate and dioxogulonate, and their work therefore did not specify the nature of the material leaving the gland. In the present work parallel estimations have been made of ascorbate [by the method of Mindlin & Butler

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