which the nitrite-excreting mechanism consumed energy would increase as the nitrite concentration increased in the medium surrounding the cell. Hence, the free-energy efficiency of the cultures would fall with increasing nitrite concentration in the medium, i.e. it would fall as the cultures grew.

We now intend to investigate, by means of the Warburg technique, the respiration of *Nitrosomonas* cultures at various stages in their growth; we also hope to investigate the effect of various concentrations of nitrite on the respiration of the cultures. These experiments should lead to a better understanding of the causes underlying the fall in the free-energy efficiency of *Nitrosomonas* cultures during growth.

SUMMARY

1. The free-energy efficiency of *Nitrosomonas* falls as the amount of carbon assimilated and the amount of ammonia oxidized increase; the free-energy efficiency thus falls during the growth of cultures of the organisms.

2. The low free-energy efficiencies reported by other workers for *Nitrosomonas* are consistent with the high nitrite concentrations at which they were determined.

3. Possible reasons for the observed fall in free-energy efficiency are discussed.

We wish to acknowledge the help, advice, and encouragement of Prof. W. O. Kermack, F.R.S., throughout this work.

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The Ultraviolet Absorption Spectrum of Ribonuclease

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No detailed study of the absorption spectrum of ribonuclease appears to have been made. It is well known that this enzyme contains no tryptophan, while its cystine and phenylalanine contents (Brand, 1948) are such as to make a negligible contribution to its absorption spectrum in the region above 2700 A. (see below). In addition, electrophoretic, ultracentrifugal and solubility studies all indicate homogeneity (Northrop, Kunitz & Herriott, 1948), although recent observations (Martin & Porter, 1951; Hirs, Stein & Moore, 1951) raise anew the question as to what is meant by a homogeneous protein preparation, at least in so far as ribonuclease is concerned.

The quantitative method introduced by Holiday (1936) for the spectrophotometric estimation of the tyrosine and tryptophan contents of proteins has been widely applied and has proved it is useful in a number of instances. There are, however, examples where serious divergences exist between the results obtained by this method, and chemical analyses. If, as appears most likely (Beaven, Holiday & Jope, 1950), absorption due to peptide linkages is negligible in the region of the absorption maxima of the aromatic amino-acids, it would seem reasonable to expect that a study of the differences obtained by the two methods may lead to information regarding the nature of the changes undergone by the aromatic amino-acids upon incorporation into a protein molecule. This assumption is naturally dependent upon the correctness of chemical analyses, regarding which doubts still exist in some instances (Tristram, 1950).

Further interest is lent to a study of ribonuclease by the observation of Kunitz (1940) that it is digested in acid medium by pepsin, with resulting denaturation and loss of enzymic activity. Since pepsin is known to attack linkages between α-carboxylic groups of dicarboxylic amino-acids and the α-amino radical of an aromatic amino-acid (Bergmann & Fruton, 1941), observation of the spectrum both before and after digestion may be

expected to throw some light on the origin of the fine structure spectral shifts observed for aromatic amino-acids in proteins (see Beaven *et al.* 1950, for recent review).

The absorption spectrum of this enzyme was reported by Uber & Ellis (1941), who found the maximum to be in the neighbourhood of 2800 A., with an extinction coefficient of $11{\cdot}4 \times 10^3$ at pH's 2·5 and 4·5, based on a molecular weight of 15000 and a nitrogen content of 16·1%. Their curve shows a minimum at about 2500 A. with a ratio of maximum to minimum extinction coefficient of 1·8.

EXPERIMENTAL

Two Kunitz preparations of ribonuclease (Kunitz, 1940) were kindly made available by Prof. J. Brachet. Cytological tests on one of these (Brachet & Shaver, 1948) indicated freedom from proteolytic activity, a fact confirmed by additional tests conducted during the course of an investigation of the ultraviolet inactivation of this enzyme (Shugar, 1951*a*). A few measurements were also made on a sample kindly supplied by Dr M. Macdonald and prepared according to her method (Macdonald, 1948). Tyrosine was an Eastman Kodak product, recrystallized from water; the nitrogen content agreed with that calculated from the dry weight and it showed only one component chromatographically. The pepsin used was a crystalline preparation from the Worthington laboratories. The lysozyme used in a comparison experiment was prepared according to the method of Alderton & Fevold (1946) and recrystallized four times.

All measurements were made in the 10 mm. cells of the Beckman spectrophotometer (two instruments were used) generally at 10–20 A. intervals except in the neighbourhood of maxima, minima and points of inflexion, where 5 A. intervals were used. The blank cell always contained all the other constituents of the enzyme solution. Special precautions were taken with respect to cell corrections which were measured over the entire spectral range with distilled water in the cells. In order to obtain measurements which could be duplicated to within 1%, it was found necessary to ensure that the same cell face be struck by the incident light beam. Where constituents were added to solutions in the cells following one series of measurements, dilution corrections were made. Enzyme concentrations were so adjusted that most of the readings fell in the range of optical densities from 0·3 to 0·8.

Buffers were made up with double-distilled water and checked with a Beckman pH meter using glass electrodes; 0·1 N-HCl was taken as pH 1, 0·01 N-HCl as pH 2, 0·1 N-NaOH as pH 13. Sørensen phosphate buffers were used in the range 5·4 to 8·7 and 0·1 M-glycocoll buffers above this range.

Results are given as extinction coefficients $\epsilon = d/c$, where d is the optical density for a 10 mm. path length and c the concentration in moles/l.

RESULTS

Curve *A* of Fig. 1 shows the absorption spectrum of ribonuclease at pH 7·2; the principal maximum is at 2775 A. with an extinction coefficient of $11{\cdot}4 \times 10^3$, based on a molecular weight of 15000 (Northrop *et al.* 1948) and a nitrogen content of 16·5% (Brand, 1948). The striking resemblance of the spectrum to that of tyrosine is obvious (Fig. 4, curve for pH 7·2). Curve *B* of Fig. 1 is the spectrum in 0·1 N-NaOH, the maximum being at 2930 A., which is also the position of the maximum of free tyrosine at the same pH (Fig. 4). However, this is not the form of the spectrum obtained immediately upon bringing the enzyme solution to pH 13; the absorption at first increases rapidly, to about 75% of the height of curve *B* during the first 2 min., then

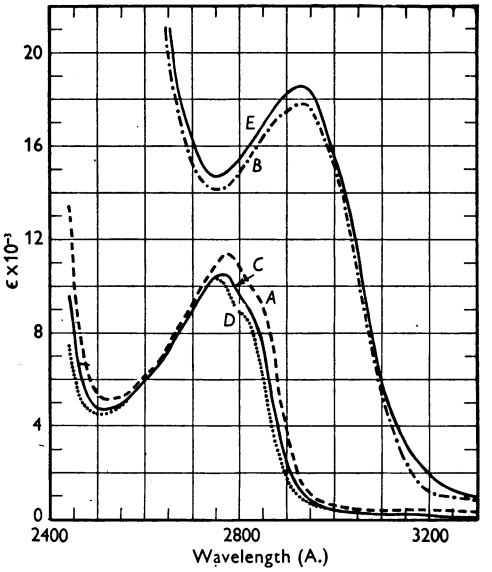

Fig. 1. Absorption spectrum of ribonuclease: *A*, in phosphate buffer, pH 7·2; *B*, in 0·1 N-NaOH; *C*, in 0·1 N-HCl; *D*, following digestion by pepsin in 0·1 N-HCl; *E*, in 0·1 N-NaOH, following digestion by pepsin.

rises slowly to its final value. In one typical experiment where the optical density at 3000 A. attained a final value of 0·439, the optical densities following addition of alkali were, at 2, 3, 4, 5, 6, 7, 9, 13, 19 and 36 min. respectively 0·331, 0·351, 0·375, 0·390, 0·400, 0·409, 0·418, 0·429, 0·439 and 0·439. Warming the solution at this point resulted in no further change. As will be shown below, what is being observed here is the alkaline denaturation of the enzyme. The change in absorption in going from neutral to alkaline solution is due to the dissociation of the phenolic groups of tyrosine (Crammer & Neuberger, 1943) and the partial time-dependence of this change would appear to indicate that at least 25%, and probably more, of these groups in the ribonuclease molecule are not free to ionize at all until denaturation has taken place.

Curve *C* of Fig. 1 shows the spectrum of ribonuclease in 0·1 N-HCl. The absorption maximum has undergone a hypsochromic shift of 10 A. while the extinction coefficient has been reduced 8%. At this point, 20 cu.mm. of a solution of pepsin (2 mg./ml.) were added to the enzyme solution which was then left overnight, since no marked change was immediately obvious. Curve *D* represents the spectrum following the digestion period. It will be seen that the principal maximum is now at 2750 A., which is that for

tyrosine itself; and that the second maximum of tyrosine (Fromageot & Schnek, 1950) noticeable in the original curve *A*, has become more pronounced and is now close to 2800 as in tyrosine, Fig. 4. The ratio of the two maxima in the undigested ribonuclease is 1·13, in the digested enzyme it is 1·16, while for tyrosine itself it is 1·18 (Fig. 4 and Fromageot & Schnek, 1950).

Following digestion, the enzyme solution was brought to pH 13, resulting in curve *E* of Fig. 1. As in the case of the undigested solution it was necessary to wait more than 15 min. for the curve to reach its final form, indicating that, even following digestion, some of the phenolic groups are still not free to ionize.

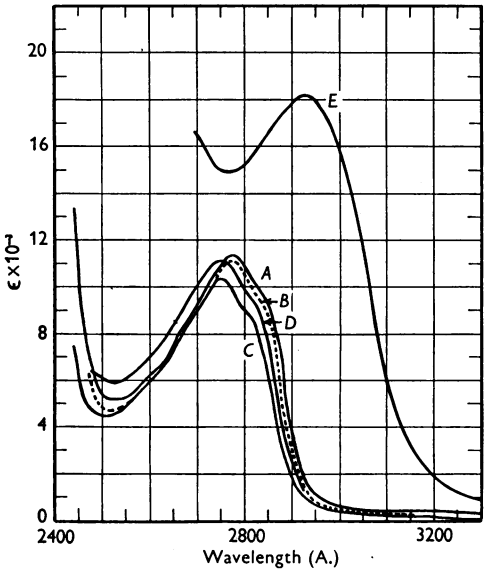

Fig. 2. Absorption spectrum of ribonuclease: *A*, in phosphate buffer, pH 7·2; *B*, in 0·01 N-HCl; *C*, following digestion by pepsin in 0·01 N-HCl; *D*, at pH 7·2, following digestion; *E*, in 0·1 N-NaOH following digestion.

Fig. 2 illustrates another typical experiment in which the digestion was carried out at pH 2. Curve *A* is the original spectrum; curve *B* is that in 0·01 N-HCl, only slightly different from that in neutral solution. Curve *C* is that following digestion and is identical with that obtained at pH 1. However, the rate of digestion was sufficiently rapid at pH 2 to follow the course of the reaction approximately. One hour following addition of pepsin (temperature about 25°) the absorption maximum had shifted to 2765 A. with very little change in extinction coefficient; after another hour the maximum was between 2755 and 2760 A., while the extinction coefficient had dropped 5%. Several hours more were required to reach curve *C*.

Curve *D* resulted when the digested protein was brought to pH 7 with NaOH. While the maximum has remained at 2750 A., the extinction coefficient is now somewhat higher than in the original solution; it did not, however, remain stationary but dropped slowly towards curve *A*, indicating the presence of secondary reactions. Further addition of alkali to bring the pH to 13 resulted in curve *E*, almost identical with that of Fig. 1; in this instance 85% of the height of the curve was attained within 1 min., suggesting that some of the 'bound' phenolic groups had been liberated as a result of digestion. It was still necessary to wait almost 15 min. for curve *E* to be attained.

A plot of the spectrum of the enzyme at various pH values results in the series of curves shown in Fig. 3. It will be seen that up to pH 12·3, all the curves pass through two isosbestic points at 2700 and 2800 A., denoting the presence of a reversible ionic equilibrium due to the dissociation of the phenolic groups of tyrosine. No sooner, however, is pH 12·3 exceeded than the resulting curves break away from the isosbestic points and, in fact, begin to increase in height at a rate which increases with pH. The curve for pH 12·3 itself passes through the isosbestic points only because it was

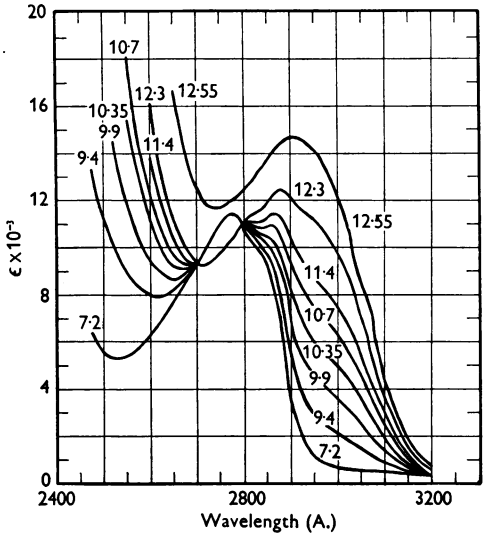

Fig. 3. Absorption spectrum of ribonuclease at pH values indicated. Note isosbestic points at 2700 and 2800 A. Between these two wavelengths, at pH values between 9·4 and 11·4, the curves have not been drawn in, for the sake of clarity.

recorded rapidly; within another half-hour it had already changed slightly. Below pH 12·3, the spectra remained unaltered for periods of some hours at room temperature. It is clear that above pH 12 denaturation takes place; that this latter process is irreversible was demonstrated by the fact that neutralization of the solution resulted in a new spectrum, the maximum of which was quite broad, 2740–2770 A., with an extinction 20% higher than that of the original neutral solution; the minimum was now at 2610 A. while the absorption above 3000 A. was appreciable.

The ionic equilibrium indicated by Fig. 3, if due to the ionization of the phenolic groups of tyrosine, should be shown by tyrosine itself. Fig. 4 shows the absorption spectrum of tyrosine at several pH values. It will be seen that there is one clearly defined isosbestic point at 2670 A., and another at 2775 A., except for the curve at pH 7·2 which does not pass through it. Although the deviation of the pH 7·2 curve from this point is small (about 4%), repeated trials showed that it is real and that, not until pH 9 had been passed, did all the curves intersect at this second

isosbestic point. While the reason for this was at first ascribed to some possible impurity in the tyrosine, it should be recalled that the α-amino group of this amino-acid has a pK of about 9 (Haurowitz, 1950) so that the dissociation of this group may be expected to have a small effect on the spectrum in the neighbourhood of pH 9, even though the phenolic ring is separated from the α-amino radical by two intervening single bonds (Braude, 1949). In the ribonuclease molecule itself, the amino group of tyrosine is bound in a peptide linkage and so this effect would be absent, as it is (Fig. 3).

A comparison of Figs. 3 and 4 shows that the isosbestic points for tyrosine undergo a bathochromic shift of 30 and 25 A. respectively, when this amino-acid is incorporated into the ribonuclease molecule, which corresponds quite well to the 25 A. shift undergone by the maximum of the tyrosine curve.

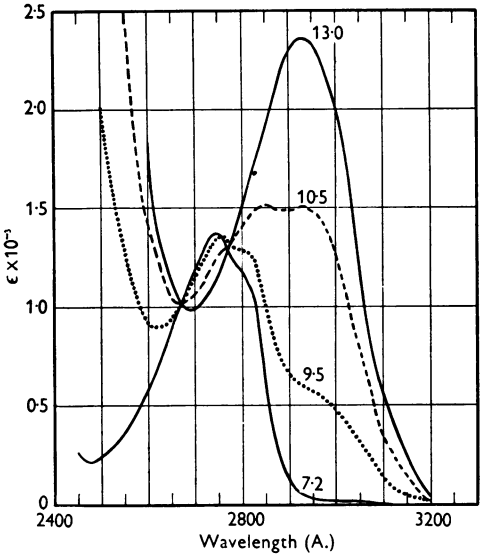

Fig. 4. Absorption spectrum of tyrosine at pH values indicated. Note isosbestic points at 2670 and 2775 A. See text for discussion of isosbestic point at 2775 A.

From the curves in Fig. 4 it is readily shown that the alkaline pK of tyrosine is 10·0 as compared with the values of 10·05 obtained by Crammer & Neuberger (1943) and 10·1 by Fromageot & Schnek (1950).

A plot of the increase in the absorption of ribonuclease at a given wavelength, between 2600 and 3050 A., as a function of pH, yields the solid curve of Fig. 5, from which the apparent pK for dissociation of the phenolic groups is 11·4, if we include also those groups which dissociate only upon denaturation. For purposes of comparison, the analogous results of Crammer & Neuberger (1943) for insulin have been plotted to the same scale and are shown as the dotted line in the same figure. It will be seen that both curves are similar up to pH 12; above pH 12, insulin still bears some resemblance to a normal 'titration' curve (see, however, below), while that for ribonuclease deviates sharply from this behaviour. That the insulin curve represents an equilibrium reversible even at pH 13 is shown by an examination of Fig. 1 of the paper by Crammer & Neuberger (1943); extrapolation of several of their curves shows that, up to and including pH 13, all of them pass through two isosbestic points at about 2700 and 2800 A. Only above pH 13 do the spectra break away from these points.

Applying a correction for the presence of tryptophan, Fromageot & Schnek (1950) showed that the ionization of the tyrosyl phenolic groups of lysozyme follows a completely normal dissociation curve with an apparent pK of 10·8 and that the spectrum was entirely reversible up to pH 13. Elsewhere it has been shown that lysozyme exposed to alkali and brought back to neutral pH suffers no loss in activity (Shugar, 1951*b*). Some of the spectral measurements of the above authors have now been repeated with substantially the same results; in particular, bringing a lysozyme solution directly to pH 13 results in the same curve reported by them, with no time lag perceptible in its attainment, in agreement with their findings, and indicating that the phenolic groups of lysozyme are not partially 'bound' as in ribonuclease.

Some of the principal numerical constants for tyrosine and ribonuclease are listed in Table 1. The values for tyrosine at pH 1 are in good agreement with those of Fromageot &

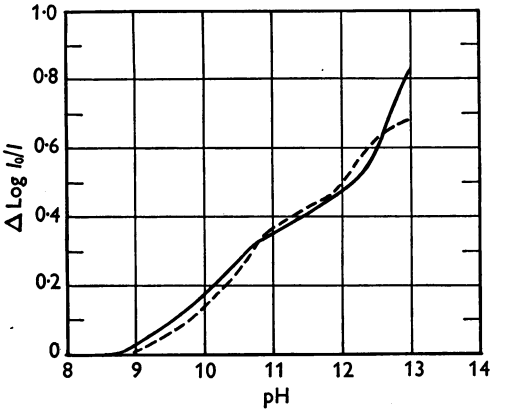

Fig. 5. Relative increase in optical density of ribonuclease at 3000 A. as a function of pH (solid line). The dotted line has been drawn from the observations of Crammer & Neuberger (1943) for insulin, and adjusted to the same scale for purposes of comparison.

Schnek (1950) as are those at pH 13. The latter are also in good agreement with a mean value based on the measurements of a number of observers (Holiday, 1951). However, the extinction coefficient for pH 7·2 is in marked disagreement with that of Fromageot & Schnek (1950), $1{\cdot}23 \times 10^3$ and with that of Holiday (1936), $1{\cdot}24 \times 10^3$. By contrast both Sizer & Peacock (1947) and Kretchmer & Taylor (1950) find no difference in extinction coefficients between pH 1 and 8, as compared to the slight difference found here, and the 11% difference reported by Holiday (1936) and Fromageot & Schnek (1950). The reason for these discrepancies at neutral pH is not clear.

The figures cited for ribonuclease are for the Kunitz preparation proved to be free from proteolytic activity, and based on nitrogen determinations made on solutions measured spectrophotometrically; one determination of

extinction coefficient was also made from the dry weight, the enzyme being dried at 110° for more than 12 hr. as suggested by Brand & Kassell (1942), and indicating a moisture content of 15 %. The value found for the extinction coefficient is quite close to that reported by Uber & Ellis (1941); but the ratio of maximum to minimum extinction coefficients is 2·2 as compared to the value of 1·8 reported by the latter authors. The preparation supplied by Dr Macdonald gave an almost exactly similar spectrum, but with a slightly higher extinction coefficient and a ratio of maximum to minimum absorption of 2·3. The importance of this ratio has already been pointed out (Shugar, 1951*b*) and will be referred to again in the Discussion (see below).

Application of the method of Holiday (1936) to the calculation of the tyrosine and tryptophan contents of ribonuclease from the curve in alkaline solution in Fig. 1 yields (*a*) tryptophan = 0, in agreement with chemical analyses and (*b*) tyrosine = 10 residues/mol. ribonuclease as compared to the 6·6 residues determined chemically by Kunitz (see Northrop *et al.* 1948) and Brand (1948).

If, however, the tyrosine content is calculated more directly from the ratio of the extinction coefficients of ribonuclease in acid solution following digestion and that for tyrosine at pH 1, the result is 7·6 residues/mol. ribonuclease. A similar calculation for the two curves at pH 13 gives 7·5 residues. Even the mean of these two values is 13 % higher than the chemically determined one, suggesting the presence of absorption due to other sources than that of free tyrosine.

Table 1. *Principal absorption bands, extinction coefficients and isosbestic points for tyrosine and ribonuclease*

	Absorption maxima			Isosbestic points		
Substance	pH	λ (A.)	ε (× 10^{-3})	pH range	λ (A.)	ε (× 10^{-3})
Tyrosine	1·0	2750	1·36	9·5–13·0	2670	1·03
	7·2	2750	1·38	9·5–13·0	2775	1·33
	13·0	2930	2·36	—	—	—
Ribonuclease	1·0	2765	10·5	7·0–12·3	2700	9·25
	2·0	2775	11·1	7·0–12·3	2800	11·0
	7·2	2775	11·4	—	—	—
	13·0	2930	17·8	—	—	—
Digested ribonuclease	1·0–2·0	2750	10·4	—	—	—

DISCUSSION

A comparison of the curves for tyrosine and ribonuclease, including the relative heights of their isosbestic points and the minima at about 2500 A., indicates the presence of absorption in the enzyme other than that of free tyrosine, such extraneous absorption increasing with decreasing wavelength. If we accept Brand's (1948) analysis for the cysteine, cystine and phenylalanine contents of ribonuclease, and the extinction coefficients of Fromageot & Schnek (1950) for these amino-acids, their combined absorption accounts for only 15 % of that of the enzyme at 2500 and 2600 A., 2·5 % at 2700 A. and practically zero at 2800 A. and above. It is quite conceivable that the binding of 40 % of the phenolic groups may result in some change in absorption; the fact, however, that calculations in both acid and alkaline media (in the latter of which all the phenolic groups are liberated) leads to the same tyrosine content, argues against this being the cause of the higher absorption.

In view of the low molecular weight of ribonuclease, absorption due to scattered light may be neglected. There remains the possibility of some secondary effects related to the peptide linkages. As already pointed out above, available evidence appears to exclude direct absorption by peptide linkages in this region of the spectrum. This does not, however, exclude the possibility that a non-aromatic amino-acid, bound in a peptide linkage to an aromatic amino-acid, may influence the absorption of the latter, notwithstanding that the aromatic ring is separated from the linkage by two single bonds (Braude, 1949). A secondary effect of this nature has already been postulated to account for the small wavelength shifts observed in peptides containing an aromatic amino-acid (Beaven *et al.* 1950). An examination of the spectra of Fromageot & Schnek (1950) for leucyltyrosine and tyrosylcysteine does indeed show increases in extinction, above that of tyrosine, of the order of 10–20 % at 2750 A. at pH 8 (at pH 13 the difference is smaller); while at 2500 A. that of tyrosylcysteine is about three times as high as for tyrosine, so that the ratio of its maximum to minimum absorption is about 2·2, almost exactly that for ribonuclease in neutral solution (Fig. 1), although too much significance should not be attached to this concordance. It is, however, not without interest to note that this ratio is not markedly altered for ribonuclease following digestion, as would be expected from the above facts if only the linkage at the α-amino group is hydrolysed by pepsin. The decrease in extinction coefficient at 2500 A. accompanying digestion is of the same order as that found for horse serum globulin (Haurowitz & Astrup, 1939) and serum albumin and globulin (Beaven *et al.* 1950).

While the above facts readily account for the 13 % difference between the chemically and spectroscopically determined tyrosine contents, it should not be overlooked that the amino-acid analysis of

ribonuclease leads to a calculated molecular weight somewhat higher than that determined by physical methods (Northrop *et al.* 1948). If the amino-acid analysis of Brand (1948) is really correct for the sample he examined, the question arises as to whether different methods of preparation do indeed result in exactly the same final product. As pointed out in the Results section, a second Kunitz preparation and one prepared by Dr M. Macdonald gave results differing by a few per cent from those listed in Table 1. The increasing precision becoming available for both amino-acid analyses and spectrophotometric measurements make it obviously desirable to remove these doubts by using the same preparation for both series of measurements (see also below).

In a recent communication, Rideal & Roberts (1951) postulate the existence of some absorption by peptide bonds at 2500 A. They find (as is the case for ribonuclease, above) that insulin, bovine serum albumin and gelatin display somewhat higher absorption at this wavelength than that of solutions containing the equivalent concentrations of free amino-acids, and conclude that about 5% of the absorption at this wavelength is due to the peptide bonds. In view of what has been said above, it appears somewhat difficult to accept such a conclusion without further evidence. These authors assume that gelatin contains no tyrosine, whereas in fact gelatin is known to contain small but varying proportions of tyrosine which have been measured both chemically and spectrophotometrically (Beaven *et al.* 1950). As further evidence they cite the fact that the absorption of acetylglycine at 2537 A. is higher than that of glycine, and conclude that, since the absorption of the methyl group is small, the increased absorption must be due to absorption by the peptide linkage; such a conclusion is not in agreement with the widely differing results reported by Fromageot & Schnek (1950) for leucyltyrosyl and tyrosylcysteine, and is at variance with the observation of Edwards (1949) that acetyltryptophan has a lower absorption than that of free tryptophan. Finally, their curve for insulin shows a ratio of maximum to minimum absorption of 2·0, as compared with 2·5 for that of Crammer & Neuberger (1943) and 1·8 for a curve presented by Beaven *et al.* (1950); the differences in absorption between these three curves at 2500 A. are of the same order of magnitude as the difference found by Rideal & Roberts (1951) between their preparation and that of an equivalent solution of free amino-acids.

Very few reliable measurements exist with regard to the changes resulting from peptide binding on the absorption spectra of di- and tri-peptides of aromatic amino-acids, except in so far as the fine structure of these spectra is concerned (Beaven *et al.* 1950). It is, however, clear from the above that further data of this nature are required adequately to explain the differences observed between the absorption spectra of proteins and their constituent amino-acids.

The reversion of the position of the entire absorption band of the enzyme to the position of that for tyrosine, as a result of digestion, is also readily explicable on the basis of the influence of the adjacent bound amino-acids. That it is not the peptide linkage itself which causes the shift is shown by the data of Beaven *et al.* (1950) where the extent of the shift varies with the nature of the amino-acid bound by the aromatic amino-acid. Although they report shifts of the order of only 5–15 A. as a result of peptide combination (compared to the 25 A. shift which occurs in ribonuclease), their measurements were made on peptides, the non-aromatic amino-acids of which were only glycine, cystine or alanine. The mere fact that the shifts caused by these three amino-acids are different, suggests that others may produce more (or less) appreciable shifts. The fact, however, that acidification to pH 1 causes a 10 A. shift in ribonuclease (leaving only 15 A. to be accounted for by digestion, in accord with Beaven *et al.*) does suggest that part of the displacement of the spectrum may be due to reasons other than peptide binding of tyrosine. Tyrosine shows no such shift in acid (Table 1); nor does insulin, although its absorption spectrum is displaced 20–30 A. from that of tyrosine (Crammer & Neuberger, 1943).

The few experiments which have been reported on the effect of digestion upon the displacement of the absorption bands of proteins have generally been conducted on molecules containing major proportions of more than one aromatic amino-acid. Further studies could much more profitably be made upon proteins containing only one main absorbing constituent such as gramicidin (tryptophan only), zein or insulin (tyrosine only). Insulin is of special interest because it is digested by both pepsin and chymotrypsin, each of which attacks different types of bonds; its amino-acid composition is fairly well established (Brand, 1946; Fromageot, 1950); while considerable studies have been made on the digestion products (Sanger & Tuppy, 1951; Stephen, 1951).

If we omit the 40% of the phenolic groups in ribonuclease which dissociate only upon denaturation, the apparent pK of the remaining groups is 10·4. (This value is obtained from Figs. 4 or 5, graphically or by calculation equally well at 2600 as between 2800 and 3100 A.) Crammer & Neuberger (1943) obtained a pK of about 11 for the phenolic groups in insulin (actually 10·8 from the curve in Fig. 5 of this paper, based on their measurements) and ascribed this higher value (as compared to 10·05 for free tyrosine) to the increasing net charge

of the molecule with increasing pH. It should, however, be noted that, from titrimetric measurements the phenolic pK of leucyltyrosine is 10·2; that of tyrosylcysteine 10·5; glycyltyrosine 10·4 and tyrosylarginine 9·6 (Cohn & Edsall, 1943). The values for leucyltyrosine and tyrosylcysteine have been confirmed spectrophotometrically by Fromageot & Schnek (1950). It is not at all unlikely that, in tripeptides containing the tyrosine in the middle, the variation of the phenolic pK from that of free tyrosine may be even more accentuated, so that the phenolic pK values observed in proteins may be related principally to the nature of the amino-acids to which the tyrosine residues are directly bound. In so far as ribonuclease is concerned, the pK value of 10·4 for the 'free' phenolic groups can hardly be considered anomalous. An examination of the data of Table 5 of the paper by Crammer & Neuberger (1943) for insulin suggests, indeed, the presence of at least two sets of phenol groups, one of which dissociates much more readily than the other, as would be expected from the above considerations if the tyrosine residues were linked to more than one other type of amino-acid. The same result may be deduced from the form of the dotted curve of Fig. 5 of this paper.

With regard to those phenolic groups not free to dissociate prior to denaturation, the situation is somewhat analogous to that for egg albumin, where very few of these groups are able to ionize below pH 13 (Crammer & Neuberger, 1943), a result confirmed titrimetrically by Cannan, Kibrick & Palmer (1941). It is difficult to visualize an explanation for this phenomenon other than that advanced by Crammer & Neuberger (1943), namely, some type of hydrogen bonding of the phenolic groups. However, their suggestion that it is these bonds which tend to endow the native protein with a certain degree of stability is in contradiction with the observed facts for ribonuclease which has fewer 'bound' phenolic groups, but which is so much more resistant to heat denaturation than is egg albumin.

Crammer & Neuberger also report (1943) that denaturation of egg albumin, as measured spectrophotometrically, is instantaneous at pH 13. While this may very well be true, they did not employ a spectrophotometer and were obliged to use a spectrographic technique which is not as readily amenable to the rapid study of spectral changes. In so far as ribonuclease is concerned, it appears that the alkaline denaturation (or inactivation) may be followed quantitatively by the above procedure.

The finding that digestion at pH 2 does liberate some of the 'bound' phenolic groups is similar to the situation prevailing for bovine serum albumin (Sizer & Peacock, 1947), a protein which also contains an appreciable percentage of 'bound' phenolic groups. Further confirmation for the existence of these bound groups is forthcoming from the work of Sizer (1946, 1947), who showed that the tyrosyl groups of insulin are more readily attacked by tyrosinase than those of serum albumin; more recent studies (Haas, Sizer & Loofbourow, 1951) suggest some modification of this interpretation.

The results of Tanford (1950) on human serum albumin are rather curious. Spectrophotometric measurements show an apparent pK for the phenolic OH groups of 11·7, with no signs of dissociation below pH 10. From titrimetric data, the phenolic pK is calculated to be 9·6. Tanford (1950) discounts the validity of his spectrophotometric results because of the presence of tryptophan. However, the tryptophan content of serum albumin is very small compared to its tyrosine content (Haurowitz, 1950), and its change with pH is much smaller than that of tyrosine at wavelengths in the neighbourhood of 3000 A., so that the spectrophotometric results look much more convincing than is suggested by the author, and suggest the need for a re-examination of the titrimetric results.

SUMMARY

1. The ultraviolet absorption spectrum of ribonuclease has been studied over the pH range 1–13.

2. The tyrosine content calculated from the spectral data corresponds to 7·5 residues per molecule ribonuclease.

3. The dissociation of the phenolic groups of tyrosine in ribonuclease is shown to correspond to the dissociation of the same groups in tyrosine; 40 % of these groups in the ribonuclease molecule are bound and can ionize only after denaturation of the molecule; the apparent pK of the remaining 60 % is 10·4.

4. Digestion of the enzyme with pepsin accentuates the resemblance of the ribonuclease spectrum to that of tyrosine.

5. The differences between the ribonuclease and tyrosine spectra are accounted for qualitatively on the basis of secondary effects resulting from peptide linkages. The nature of these effects is discussed.

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Further Observations on the Preparation and Composition of the Hyaluronic Acid Complex of Ox Synovial Fluid

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Ogston & Stanier (1950) concluded that hyaluronic acid occurs in synovial fluid in combination with protein. The undegraded state of this complex in solution, prepared by ultrafiltration, was judged by viscosity measured in an Ostwald viscometer, and by sedimentation in the ultracentrifuge. It was not possible to account satisfactorily for the composition of the complex in terms of the analytical values for its components.

The main object of the present work was to make better and more extensive analyses. As a preliminary, methods of preparation were examined in the hope of finding one which would give undegraded material more conveniently than by ultrafiltration. In this search, a more sensitive test of degradation, based on the variation of viscosity with velocity gradient, was used. It is concluded that ultrafiltration remains the method of choice and improved analyses of material thus prepared are reported.

The results of this work generally confirm the previous conclusions, though altering some of them in detail. Nearly the whole weight of complex is now accounted for in terms of protein, acetylglucosamine, glucuronic acid and mineral content.

In the course of the work, the zero-concentration value of the sedimentation constant of the complex has been re-determined and the action of hyaluronidase has been briefly investigated. The values given by Ogston & Stanier (1951*a*) for the physical dimensions of the particle of complex have been adjusted in the light of the new results.

METHODS

The methods for collection and storage of synovial fluid, preparation of the mucin clot from 1 ml. of fluid and determination of dry weight described by Ogston & Stanier (1950) were used.

The ultrafilter residue. This was prepared by the method described by Ogston & Stanier (1950), using a sintered-glas