The riboflavin content of the cabbage is probably 41.5–41.7 $\mu\text{g.}/100\text{ g.}$ raw cabbage, the potato is probably nearer the higher value of the two presented.

DISCUSSION

It is of interest that Gillespie & Rubbo [1940] reported that biotin was not essential for *Cl. acetobutylicum* whereas the reverse was claimed by Park & Wood [1942]. There are marked differences in the mineral constituents in the media used by these two sets of investigators; in general the mineral content of the latter workers was 1/10 that of Gillespie & Rubbo. Park & Wood's concentrations would certainly be suboptimal for the *Lactobacillus* and it is again interesting to note that they report an enhancement of growth with ammonium phosphate instead of with asparagine. Assuming that both sets of results are correct it would appear that a change in the mineral balance of a medium has enabled the organism to dispense with biotin. Though this may seem unlikely, it does suggest that the amount of a growth factor necessary for optimum action is profoundly affected by the mineral balance. This lends additional emphasis to the main theme of this paper.

It should be added that a series of additions have been made to the basal medium by a variety of workers. We have tested many of these compounds and found them inactive. Thus, adenine, guanine,

uracil, thymine and adenylic acid were all tried separately and together, and found to be inactive. Since these components may be of natural origin it seems likely to us that their reported activity has been due to the adsorption on to the compound tested of small but adequate amounts of a growth factor or growth factors.

SUMMARY

Earlier work had revealed a number of irregularities in the application of the microbiological method of assay for riboflavin, using *Lactobacillus casei* ϵ . Evidence is presented to show that acid production can be altered by a variation in the concentrations of Fe^{++} , Ca^{++} , K^+ , PO_4^- and NaCl in the basal medium. Optimum concentrations for these constituents have been demonstrated and in addition it was shown that further increases in Fe^{++} , K^+ , PO_4^- and NaCl concentrations do not result in a great decrease in acid production. Further addition of Ca^{++} , however, results in a serious decrease. The necessity for, and the effect of, the addition of a liver eluate to the basal medium was shown. The bearing of these results on the assay of foodstuffs for riboflavin has been discussed.

We wish to acknowledge the generous gift of liver extract from Messrs Boots, Ltd., a grant from the Medical Research Council and a Research Scholarship in Biochemistry, held by one of us (M. S.), kindly provided by Messrs Rowntree, York.

REFERENCES

- Chattaway, F. W., Happold, F. C., Lythgoe, B., Sandford, M. & Todd, A. R. [1942]. *Biochem. J.* **36**, v.
 Evans, W. C., Handley, W. C. R. & Happold, F. C. [1942]. *Biochem. J.* **36**, 311.
 Gillespie, J. M. & Rubbo, B. D. [1940]. *Nature, Lond.*, **146**, 838.
 Hutchings, B. L., Peterson, W. H. & Shull, G. N. [1942]. *J. biol. Chem.* **142**, 913.
 Johnson, E. R. & Mueller, J. H. [1941]. *J. Immunol.* **40**, 33.
 Lindner, M. & Pollack, M. A. [1942]. *J. biol. Chem.* **143**, 655.
 Park, R. C. & Wood, W. B. [1942]. *Johns Hopkins Hosp. Bull.* **70**, 19.
 Snell, E. E. & Strong, F. M. [1939]. *Industr. Engng Chem. (Anal. ed.)*, **11**, 346.
 — & Wright, L. D. [1941]. *J. biol. Chem.* **139**, 675.

The State of Tyrosine in Egg Albumin and in Insulin as Determined by Spectrophotometric Titration

By J. L. CRAMMER AND A. NEUBERGER, *From the Biochemical Laboratory, Cambridge*

(Received 13 January 1943)

Numerous studies on the reactions between protein and hydrogen and hydroxyl ions have yielded important information as to the number and character of ionizable groups in the protein molecule. The detailed interpretation of the titration curves is, however, in many cases ambiguous. The assumption is often implicitly made that the dissociation constants of the various polar groups in proteins are not very different from those found in amino-acids

or peptides. In this general form, such an assumption cannot be correct. Above and below the isoelectric point (I.E.P.) the protein carries a net positive or negative charge and the number and distribution of these charges will greatly influence the ionization of different groups. A considerable displacement of dissociation constants can therefore be expected as the net valency of the protein increases. Near the I.E.P. the electrostatic effect

will be small, if the charge distribution is symmetrical. Investigations of the effect of changes of temperature [Wyman, 1939], of addition of formaldehyde [Kekwick & Cannan, 1936] or of iodination [Neuberger, 1934] have in some cases been of assistance in apportioning parts of titration curves to certain groups. But the overlapping of the titration ranges of different ionizable groups renders a quantitative evaluation of dissociation constants difficult, and sometimes impossible. It occurred to us that the ionization of the phenolic group of tyrosine in the protein might be investigated by a spectroscopic method, without interference from other groups ionizing in the same region. This would indicate how far charge effects in general can influence the intrinsic dissociation constants of the relevant amino-acids.

Most organic acids and bases which absorb light in the visible or ultra-violet region have absorption spectra which differ considerably from those shown by their respective anions or cations. *pH* measurements by indicators are based on such shifts in absorption in the visible spectrum. Similar changes in absorption spectra in the ultra-violet have been noted with phenol, and with phenol derivatives such as tyrosine [Stenström & Goldsmith, 1926], and the dissociation constants thus obtained agree well with the values established by other means [Flexsner, Hammett & Dingwall, 1935]. Proteins absorb ultra-violet light, the maximum absorption occurring with light of wave-length between 2750 and 2850 Å., and it has been assumed that the absorption above 2750 Å. is due to tyrosine and tryptophan [Holiday, 1936]. The spectrum of the latter amino-acid changes very little with *pH*, while the ionization of the phenolic group of tyrosine is accompanied by an increase of the molecular extinction coefficient, and a shift in the long wave maximum of about 200 Å. It was thought therefore that the change of the absorption spectrum of

a protein with change of *pH* could be used to determine the *pK* of the phenolic group in the native protein molecule.

EXPERIMENTAL

The tyrosine and tryptophan samples used had been recrystallized several times; 3:5-diiodotyrosine was kindly supplied by Dr C. R. Harington. Insulin was a recrystallized sample obtained from Messrs Boots; we used two preparations of thrice recrystallized egg albumin which were kindly put at our disposal by Prof. A. C. Chibnall, and by Dr K. Bailey, respectively.

The water content of insulin was assumed to be 10% (personal communication from Prof. Chibnall); the concentrations of the egg albumin solutions were estimated by the Kjeldahl method, assuming a N content of 15.7%. All measurements, including the *pH* estimations (hydrogen electrode), were made at $20 \pm 2^\circ$.

The optical measurements were made with a Hilger medium spectrograph and Spekker photometer, using a tungsten-steel spark as light source. The wave-length scale was checked against the iron spectrum, and the maximum error in the relevant region was found to be 5 Å.

RESULTS

The dissociation constant of the phenolic groups of tyrosine and 3:5-diiodotyrosine

The absorption curves for tyrosine and tryptophan obtained by us (Table 1) differ slightly from those reported by Holiday [1936] and Holiday & Ogston [1938]. The maximum absorption of 3:5-diiodotyrosine was (Table 1) at 2910 Å. in acid solution, with a molecular extinction coefficient of 2.31×10^3 ; thus substitution of the two ortho positions by iodine produces an increase in the extinction of the phenolic group, and also a shift in the maximum of about 160 Å. In alkaline solution the increase in absorption, as compared with that of phenol, is even more marked; two maxima were observed, one at 3160 Å. ($\epsilon = 5.26 \times 10^3$) and the other at 3060 ($\epsilon = 5.17 \times 10^3$). The values reported here for diiodo-

Table 1. *Molecular extinction coefficients of tyrosine, tryptophan and 3:5-diiodotyrosine ($\epsilon \times 10^{-3}$)*

Substance	λ (Å.)								
	2600	2650	2700	2750	2800	2850	2900	2950	3000
Tyrosine at <i>pH</i> 13	1.66	1.14	0.96	1.14	1.50	1.80	2.15	2.30	1.97
Tyrosine at <i>pH</i> 1	0.53	0.76	1.09	1.29	1.21	0.75	0.15	0.02	—
3:5-Diiodotyrosine at <i>pH</i> 13	—	—	—	—	—	1.65	2.21	2.91	3.88
3:5-Diiodotyrosine at <i>pH</i> 1	1.14	1.32	1.46	1.63	2.01	2.21	2.30	2.09	1.52
Tryptophan at <i>pH</i> 13	2.95	3.82	4.32	4.88	5.43	4.87	4.10	2.29	1.25
Tryptophan at <i>pH</i> 1	3.25	4.10	4.72	5.16	5.49	4.52	3.41	1.40	0.58

Substance	λ (Å.)							
	3050	3100	3150	3200	3250	3300	3350	3400
Tyrosine at <i>pH</i> 13	1.25	0.70	0.35	0.09	—	—	—	—
Tyrosine at <i>pH</i> 1	—	—	—	—	—	—	—	—
3:5-Diiodotyrosine at <i>pH</i> 13	5.12	4.69	5.18	4.37	3.41	2.37	1.30	0.34
3:5-Diiodotyrosine at <i>pH</i> 1	0.62	0.23	—	—	—	—	—	—
Tryptophan at <i>pH</i> 13	0.52	0.14	0.05	—	—	—	—	—
Tryptophan at <i>pH</i> 1	0.09	—	—	—	—	—	—	—

tyrosine differ slightly from those obtained by Ginsel [1939].

The phenoxide ion of tyrosine absorbs very strongly between 2900 and 3100 Å., while the phenolic form absorbs little or no light in this region (Table 1). We have therefore used the extinction at the three wave-lengths of 2950, 3000 and 3050 Å. as a measure of the concentration of the phenoxide ion. Table 2 shows that mixtures of the two forms behave in accordance with the expectation that the absorption spectra of the two molecular species are additive and independent of each other.

Table 2. *Spectroscopic estimation of the phenolic dissociation constant of tyrosine*

Partly neutralized solutions of tyrosine were made up by mixing solutions of tyrosine in 0.01 N HCl with appropriate glycine buffer mixtures, to a final ionic strength of 0.04, care being taken to avoid contamination with CO₂. The concentrations of the phenoxide ion at different pH values were calculated from the extinctions at three different wave-lengths, with the aid of the values given in Table 1.

	pH ...	...	9.50	9.72	9.98	10.15	10.33	10.50
$\epsilon \times 10^{-1}$	(a) at 2950 Å.		50	69	106	127	150	170
	<i>pK</i> calc.		10.05	10.03	10.05	10.06	10.06	10.05
	(b) at 3000 Å.		41	59	90	107	123	141
	<i>pK</i> calc.		10.07	10.07	10.02	10.05	10.08	10.06
	(c) at 3050 Å.		28	38	58	72	81	93
	<i>pK</i> calc.		10.04	10.08	10.04	10.02	10.07	10.04

The values obtained for the dissociation constant of the phenolic group of tyrosine vary little for different wave-lengths and at different pH values. The mean value for the *pK* is 10.05; the dissociation exponent for zero ionic strength—*pK*₀—can be extrapolated with the aid of the simplified Debye equation: $pK_0 = pK + 0.5 \sqrt{\mu}$. A value of 10.15 is thus obtained. Hitchcock [1924], by a combination of solubility and hydrogen electrode measurements, obtained two *pK* values for the alkaline dissociation of tyrosine. A *pK* of 9.2 was assigned to the amino-group, whilst the higher *pK* of 10.1 was assumed to correspond with the dissociation of the phenolic group. This interpretation is supported by our spectroscopic measurements.

The dissociation constant of the phenolic group in 3:5-diiodotyrosine was measured in a similar manner. The extinctions of partly neutralized diiodotyrosine solutions at the wave-lengths 3300, 3250, 3200 and 3150 Å. were determined and the *pK* calculated. A mean value of 6.42 was obtained. This is in good agreement with the value of 6.48 obtained by Dalton, Kirk & Schmidt [1930].

The state of tyrosine in insulin

The applicability to proteins of the spectrophotometric method, thus established for tyrosine and diiodotyrosine, depends mainly on the validity of the assumptions indicated above. Can the absorption spectra of proteins quantitatively be interpreted in terms of those of their constituent amino-acids? It has been shown that the introduction of

peptide linkages changes the absorption spectrum of tyrosine very little [Fruton & Lavin, 1939], and Holiday [1936] has been able to estimate spectroscopically the tyrosine and tryptophan contents of several proteins, with results which are in fair agreement with those by other methods. For other proteins it has been necessary to introduce a rather unsatisfactory correction for 'pigment', which has also been discussed by Morton [1942]. Moreover gelatin, which contains only traces of tyrosine and no tryptophan, as shown by chemical methods, exhibits a small but definite band at 2800 Å., which

is shifted towards the red in alkaline solution [Anslow & Assar, 1940]; these authors ascribe this band, which they also find in egg albumin, to peptide bonds. This interpretation, however, appears to be based on insufficient evidence.

Insulin has an amino-acid composition which simplifies the interpretation of spectroscopic data. It contains 12.5 % of tyrosine [du Vigneaud, 1938] and no tryptophan; 12.5 % of cystine, which is all present in the disulphide form; and some phenylalanine. The ultra-violet absorption spectrum of insulin has been examined by several authors, and was found to resemble that of tyrosine [Kuhn, Eyer & Freudenberg, 1931; Coulter, Stone & Kabat, 1936; Graubner, 1938].

Tables 3–5 and Fig. 1 give the experimentally determined molecular extinction coefficients of insulin at different pH values. With a molecular weight of 46,000 [Miller & Anderson, 1942], insulin contains 32 tyrosine and 24 cystine groups. The absorption of cystine in the relevant part of the spectrum is quite small; in order to calculate the tyrosine groups we have corrected, however, for cystine, using the values given by Ley & Arends [1932]. In acid solution (Table 3 and Fig. 2) the absorption of insulin at the maximum, i.e. between 2700 and 2800 Å., agrees quite well with the calculated value. At shorter wave-lengths the absorption can obviously not be explained by tyrosine alone; phenylalanine may be largely responsible for this excessive absorption. At the longer wave-lengths, i.e. at 2850 Å. and above, however, the absorption

of insulin is considerably greater than the calculated value, which is based on the assumption that tyrosine in insulin has the same absorption as the free amino-acid. The curve is very steep in this region and a slight shift of the absorption band of tyrosine incorporated in protein would greatly change the extinction coefficients. A shift of the whole band

Absorption of insulin in alkaline solution

In alkaline solution there is an increase of absorption below 2650 Å., which is probably not connected with the ionization of the phenolic group, since it occurs at pH 9. The absorption in the region from

Fig. 1.

Fig. 1. Absorption spectrum of insulin. Curve no. 1 was obtained at pH 1, the figures 2-9 correspond to the pH values in Table 5, whilst curve 10 was obtained at pH 13.4.

Fig. 2.

Fig. 2. Absorption spectrum of egg albumin.

by about 20 Å. appears quite possible, and would account very satisfactorily for the whole absorption curve of insulin. It can be concluded that in acid solution the absorption of insulin above 2700 Å. is quantitatively explained in terms of that of tyrosine and that of cystine bound in disulphide linkage, and it appears unnecessary to postulate the presence of other absorbing groups, or of any linkage of the phenolic group of tyrosine.

2650 to 2800 Å. does not change appreciably if the pH is increased to 13. A further increase of alkalinity, however, is associated with a greatly increased absorption between 2650 and 2850 Å., and the maximum at 2950 Å., which is mainly due to the phenoxide ion, becomes less distinct. This 'unspecific' absorption which is connected with an unknown change in the molecule appears to spread to longer wave-lengths and at pH 13.8 the extinc-

Table 3. *Molecular extinction coefficients of insulin at pH 1, with the number of tyrosine groups thus calculated*

λ (Å.)	...	2450	2500	2550	2600	2650	2700	2750	2800	2850	2900
$\epsilon \times 10^{-4}$		2.36	1.95	2.08	2.46	3.07	3.89	4.50	4.22	3.02	1.05
No. of tyrosine groups calculated		—	—	—	35.6	34.1	32.0	32.2	32.4	37.6	60.6

Table 4. *Molecular extinction coefficients of insulin at alkaline pH, with the number of tyrosine groups thus calculated*

	λ (Å.)	...	2750	2800	2850	2900	2950	3000	3050	3100	3150
$\epsilon \times 10^{-4}$ (a)	at pH 13.0		3.72	5.05	5.90	6.90	7.30	6.70	4.30	2.69	1.81
	No. of tyrosine groups calculated		29.1	31.5	31.2	31.2	30.4	33.5	34.0	36.4	50.4
" (b)	at pH 13.4		—	—	6.85	7.40	7.60	6.98	4.78	—	—
	No. of tyrosine groups calculated		—	—	36.1	33.1	32.0	35.0	37.5	—	—
" (c)	at pH 13.8		—	—	7.75	7.89	7.76	7.41	5.21	—	—
	No. of tyrosine groups calculated		—	—	41.1	34.9	33.6	37.0	41.0	—	—

tion coefficients at 3000 and 3050 Å. substantially exceed the calculated value.

It will be shown later that the phenolic groups in insulin dissociate at a higher pH than do those in tyrosine, and the ionization will therefore not be quite complete at pH 13.0. It can be calculated that approximately 30 groups out of 32 are dissociated at that pH . The absorption at pH 13.0 is fully explained if we assume again the slight shift postulated above. At higher alkalinity the values found are much too high, which must be due to 'unspecific' absorption. Generally speaking, however, it can be concluded that the absorption curve of insulin between the limits of pH 1 and 13 can be completely accounted for by its amino-acid composition, if we assume a slight shift of the curve for tyrosine incorporated in protein, in comparison with that for the free amino-acid.

*Change of absorption of insulin
with change in pH*

Having thus established a quantitative correlation between absorption spectrum and stoichiometric composition, we studied the change of the spectrum as a function of the pH in greater detail (Table 5 and Fig. 1). The desired pH was obtained

of Table 1 are not very different, but the agreement at the same pH and different wave-lengths is less satisfactory. A slight error in the first assumption will not significantly affect the results.

It can be seen from Table 5 that agreement at any one pH for different wave-lengths is satisfactory, but that the apparent pK increases considerably as the pH rises. The mean pK found for the phenolic group in insulin is about 11, as compared with 10.1 for that of tyrosine. The pK is calculated as if insulin were a monobasic acid so far as tyrosine is concerned; such a method of calculation would be correct if the tyrosine groups were placed so far from each other, and from any other group which could influence the intrinsic pK of the phenolic group, as to render such an influence negligible. This is obviously not the case. We may safely assume that the effect of charged groups will be the most important factor; substitution by polar but uncharged groups will not affect the pK greatly, unless such substitution were to take place in the phenolic ring itself. Calculated for a molecular weight of 46,000, insulin has 8 arginine, 32 histidine, 4 lysine and 24 free α -amino-groups [Chibnall, 1942]. The number of free carboxyl groups is not known with any accuracy, but we deduce from the titration data of Harington & Neuberger [1936]

Table 5. *Molecular extinction coefficients of insulin at different pH values, with the pK values for the phenolic groups thus calculated*

pH values at which determinations were made ...	9.9	10.2	10.45	10.8	11.35	11.8	12.3	13.0
(a) $\epsilon \times 10^{-4}$ at 2950 Å.	1.46	2.27	2.76	3.88	4.56	5.04	6.44	7.30
No. of phenolic groups ionized	5.4	8.6	10.6	15.5	18.4	20.4	26.3	30.0
pK calc.	10.63	10.63	10.75	10.83	11.25	11.55	11.64	11.82
(b) $\epsilon \times 10^{-4}$ at 3000 Å.	1.12	1.87	2.34	3.39	4.11	4.53	5.87	6.7
No. of phenolic groups ionized	4.6	8.0	10.2	15.4	18.2	20.1	26.3	30.0
pK calc.	10.67	10.68	10.78	10.83	11.23	11.57	11.65	11.82
(c) $\epsilon \times 10^{-4}$ at 3050 Å.	6.7	1.21	1.76	2.47	2.94	3.25	3.96	4.30
No. of phenolic groups ionized	4.4	8.1	12.4	17.0	20.3	22.5	27.5	30.0
pK calc.	10.69	10.67	10.65	10.75	11.12	11.43	11.52	11.82
$pH_0 - pH_s$	0.55	0.55	0.65	0.7	1.1	1.4	1.5	1.7

by mixing CO_2 -free $NaOH$ with a portion of a concentrated solution of insulin made up in 0.01 N HCl , and diluting to the required volume; the ionic strength in most instances was less than 0.01. The time interval between the addition of alkali, the completion of the spectroscopic measurements, and the estimation of pH , was less than 10 min. The calculation of the pK was based on the assumption that 94% of the tyrosine groups in insulin are ionized at pH 13, which is equivalent to an effective pK of 11.8 over the very alkaline range. It is further assumed that the differences between the absorption spectra at pH 1 and 13, within the limits of 2950 and 3050 Å., are entirely due to tyrosine; the shift in the tyrosine spectrum is thus taken into account. The pK values calculated from the data

that there are approximately 50 such groups. At pH 9.9 all the iminazole and most of the α -amino-groups will be ionized, while all the guanidino and most of ϵ -amino-groups will still retain their positive charges. The total net charge of the insulin molecule at that pH will be approximately -40. At pH 12.3 most of the tyrosine groups will have dissociated, most of the lysine groups become unionized, and the net charge increased to about -70. The increase in apparent pK of the phenolic group runs parallel with this increase of net charge of the molecule, and it is concluded that the shift of the apparent pK of tyrosine in the protein, as compared with that of free tyrosine, is largely accounted for by the increase in the negative charge of the protein as the pH is raised. On this 'net

electrostatic effect' there may be superimposed a specific local effect of some substituents, say of neighbouring guanidino groups, on particular tyrosine groups, but such specific local effects are probably of minor importance compared with that of the total net charge. It has so far been assumed that the charge distribution on the surface of the protein molecule is symmetrical; there is no definite evidence that this is so in the case of insulin. The low dipole moments found for many proteins [Oncley, 1942] seem to indicate, however, that proteins in general do not show any marked asymmetry of charge distribution and, in the absence of any evidence to the contrary, this generalization is considered to apply to insulin too.

Instead of considering the pH as constant throughout the solution, and treating the pK of the phenolic group as a variable dependent upon the net charge, we might equally well, and perhaps more usefully, consider the intrinsic ionization tendency of the ionizing group as constant, and the pH on the surface of the protein— pH_s —as variable and depending on the bulk pH — pH_b —and on the net charge [Danielli, 1937; 1941; Hartley & Roe, 1940]. Because of its negative charge, the surface of the protein at an alkaline pH will contain more protons than the bulk of the solution, and the ultra-violet 'colour' of the phenolic groups will serve 'as an indicator of pH_s '. It must be remembered, however, that the pH of the surface as defined by our measurements is not quite the same as the pH_s as used by Danielli [1941] in his study on the 'protein error' of indicators. In the latter case the pH of a volume element of the solvent adjoining the surface is considered, while we estimate the pH at a point on the actual surface of the molecule itself. The differences between bulk pH and surface pH , which are numerically identical with the differences between the pK of the phenolic group in the protein, and that of free tyrosine, are recorded in Table 5; the values are not strictly accurate, since no correction for the variation of ionic strength was made, but they give an indication of the magnitude of the shift of pK with increasing net charge, according to the first view, or of the difference between pH_s and pH_b according to the Hartley-Danielli definition.

If the generalization be accepted that the surface of protein molecules is electrically uniform, then

the change of the dissociation constants of ionizable groups in the protein is entirely dependent on the size, shape and net charge of the molecule. Spherical molecules of the same molecular-weight class, like egg albumin and insulin, should therefore show an identical behaviour if the net charge is the same. It can also be predicted that in the neighbourhood of the I.E.P. the dissociation constants will be approximately the same as those found in the simple amino-acids or peptides, whilst in very acid media, or in most cases even more in alkaline solution, a considerable shift is to be expected.

Most of the spectroscopic data refer to a molecule outside the stability range of insulin, as defined by the sedimentation method [Miller & Anderson, 1942]. The changes occurring at moderate alkalinity are, however, reversible [Sjögren & Svedberg, 1931] and we believe that our data, which were obtained at pH values of 12 or less, represent characteristics of the unchanged, or reversibly-changed, insulin molecule.

Absorption spectrum and composition of egg albumin

Egg albumin presents a much more complicated picture. In acid solution there is a point of maximum absorption at about 2800 Å.; at pH 13 there is very marked increase of absorption between 2950 and 3150 Å. and the maximum is shifted to about 2900 Å. (Fig. 2). Qualitatively this behaviour is to be expected for a molecule containing both tyrosine and tryptophan. The quantitative analysis of the spectrum in terms of these two amino-acids is, however, not quite satisfactory.

There is a certain amount of disagreement about the molecular weight of egg albumin; we have accepted Chibnall's [1942] figure of 43,000 which is in fair agreement with most recent data. We have also used his data for tyrosine and tryptophan, i.e. we assume that egg albumin contains 10 tyrosine and 3 tryptophan groups per molecule. Table 6 gives the molecular extinction coefficients found at different pH values, and also the calculated extinction coefficients. The latter data were obtained by use of the figures for extinction coefficients given in Table 1, with the assumption that no phenolic groups are dissociated at pH 9.5 and that all 10 groups are ionized at pH 13.

Table 6. *Molecular extinction coefficients of egg albumin*

$\epsilon \times 10^{-4}$		λ (Å.) ...	2500	2550	2600	2650	2700	2750	2800	2850	2900	2950	3000	3050	3100
(a) at pH 4.9	Found		1.12	1.13	1.45	1.81	2.26	2.51	2.85	2.55	1.90	0.86	0.48	—	—
	Calc.		—	—	1.50	1.99	2.50	2.84	2.86	2.11	1.17	0.44	0.18	—	—
(b) at pH 9.5	Found		1.48	1.27	1.70	1.90	2.54	2.83	2.83	2.66	2.00	1.16	0.68	0.35	0.19
	Calc.		—	—	1.42	1.91	2.39	2.84	2.84	2.21	1.38	0.71	0.38	0.16	0.04
(c) at pH 13.0	Found		—	—	—	—	2.65	3.28	3.28	3.42	3.50	3.28	2.73	1.51	0.95
	Calc.		—	—	—	—	2.25	3.13	3.13	3.26	3.38	3.04	2.34	1.41	0.74

At pH 4.9 the agreement between calculated and found values is quite satisfactory at the maximum, i.e. at 2800 Å.; at the shorter wave-lengths the experimental values are too low by about 10%, while at 2850 Å. and higher the extinction coefficients found are much larger than those calculated. These very high values at longer wave-lengths are also observed at pH 9.5, and to a lesser extent at pH 13. This discrepancy, which is here more marked than is the case with insulin, may be due to a number of causes. The absorption bands of tyrosine and tryptophan present in the protein may differ from those of the free amino-acids. Such a shift would be expected if these groups were linked to other groups by hydrogen bonds [Morton & Stubbs, 1940]. An examination of the fine structure of the tryptophan band in the egg albumin spectrum has revealed such a shift towards longer wave-lengths [Holiday, 1942]. It is also possible that another group is responsible for the increased absorption, as has been suggested by Anslow & Assar [1940]. A third possibility is that some of the increased 'absorption', particularly that above 3200 Å., is actually due to scattering of light, but an extrapolation to shorter wave-lengths with Rayleigh's formula, which was attempted by Custers, de Boer & Dippel [1933] for gelatin, appears unsatisfactory, since the refractive index of the protein for the light which is absorbed by the molecule, will probably be different from that found for light to which the protein is transparent. It seems unlikely that the discrepancy found can be explained by scattering. If allowance is made for the shift of absorption bands in the protein, the absorption spectrum of egg albumin between pH 1 and 13 can be almost quantitatively explained in terms of tyrosine and tryptophan, so that the change observed between pH 9.5 and 13.0 must be mainly due to the ionization of the phenolic group. An exact quantitative estimation on the basis of the present data is, however, somewhat uncertain.

Change of absorption spectrum of egg albumin on denaturation

If the pH of an egg albumin solution is increased from 9.5 to 12 little change in the absorption spectrum occurs, but if the pH is raised to 13 the spectrum changes very markedly (Table 6 and Fig. 2). If a solution exposed to pH 13 be brought back to pH 12 the absorption between 2900 and 3150 Å. is very much larger than that of a solution of pH 12 and the same ionic strength not previously exposed to pH 13 (Fig. 3). Exposure to pH 13 also denatured the protein, and it appeared likely that the irreversible change of the absorption spectrum, and the denaturation, were interrelated phenomena. This was shown to be the case. Denaturation by acid, urea, alkali and heat all pro-

duced the marked increase of absorption at pH 12, for wave-lengths above 2850 Å. (Table 7). Denaturation was demonstrated by the appearance of a precipitate on adjusting the pH to about 5, and also by a positive nitroprusside reaction. Exposure to pH 12 for 72 hr. at room temperature produced no denaturation and no change of absorption

Fig. 3. Absorption spectrum of native and denatured egg albumin at pH 12.

spectrum. Denaturation at pH 13, however, appeared to be instantaneous. This is in agreement with the findings of Cannan [1942], who noted that the speed of denaturation of egg albumin by alkali is proportional to the fourth power of the hydroxyl ion concentration.

The extinction coefficients of preparations of the same protein which have been denatured by different means are approximately the same, and the increase in absorption must be connected with the mechanism of denaturation. The lack of ionization of the phenolic groups in the native protein, demonstrated here spectroscopically, is also confirmed by the titration data of Cannan, Kibrick & Palmer [1941]. The absorption spectrum indicates that on denaturation the phenolic groups are able to ionize at a pH lower than 12. This interpretation is supported by the change of absorption of the denatured protein with pH. Our data do not permit of an accurate estimation of the dissociation constant of the phenolic group in the denatured protein; the pK appears, however, to lie between 11.4

Table 7. *Absorption spectra of native and of denatured egg albumin*

The stock egg albumin solution contained 7.59 g. of protein/100 ml. of solution. For urea denaturation 1.89 g. of urea was added to 2 ml. stock solution, and the mixture was allowed to stand for 45 min. Acid denaturation was produced by adding 3 ml. 0.1 *N* HCl to 1 ml. of stock solution and keeping the mixture at 35° for 6 hr. Heat-denatured protein was obtained by adjusting the protein solution first to *pH* 7.5, and then heating it to 85° for 8 min. At that *pH* the solution remained clear. After dilution of these solutions, their *pH* was suitably adjusted by the addition of acid or alkali.

Egg-albumin solution	λ (Å.)	$\epsilon \times 10^{-4}$
(a) Urea-denatured at <i>pH</i> 10.75	2950	1.51
	3000	0.96
	3050	0.55
(b) Urea-denatured at <i>pH</i> 11.55	2950	2.01
	3000	1.53
	3050	0.96
(c) Urea-denatured at <i>pH</i> 12.0	2950	2.78
	3000	1.89
	3050	1.25
(d) Alkali-denatured at <i>pH</i> 12.0	2950	2.75
	3000	1.84
	3050	1.21
(e) Acid-denatured at <i>pH</i> 12.0	2950	2.70
	3000	1.78
	3050	1.18
(f) Heat-denatured at <i>pH</i> 11.3	2950	1.60
	3000	0.97
	3050	0.60
(g) Heat-denatured at <i>pH</i> 12.0	2950	2.60
	3000	1.71
	3050	1.18
(h) Native at <i>pH</i> 12.0	2950	1.49
	3000	0.95
	3050	0.50

and 11.8, a value not very different from that found for insulin. The difference in extinction coefficients between native and denatured protein at *pH* 12 corresponds to the ionization of 5–6 tyrosine groups, and from the probable value of the *pK* it must be concluded that at least 7–8 phenolic groups are concerned. It is possible that about 2 phenolic groups can ionize in the native protein, and this may account for the slight increase of absorption observed between *pH* 9 and 12; but this is uncertain.

The interpretation of the spectroscopic changes in terms of the acquired ionization of the phenolic groups on denaturation appears well founded, but the cause of this is, at present, a matter for speculation. It may be that phenolic groups in the native protein have *pK* values larger than 12.5, due to the specific configuration of the molecule, and that on denaturation the unfolding of the protein causes a change in *pK*. Such an assumption implies a highly asymmetrical charge distribution in the native protein which is very unlikely. It is also possible that

the tyrosine groups are not on the surface of the molecule and are thus not able to react with hydroxyl ions. This interpretation also seems improbable; it is unlikely that the interior of the molecule is inaccessible to small ions like OH⁻. Furthermore this theory would claim a position for tyrosine groups different from that of all other ionizable groups, which can react normally with H⁺ and OH⁻. It seems most probable that the phenolic OH groups are bound, in the native protein, in a linkage which is broken by denaturation. This interpretation is supported by the behaviour of native and denatured egg albumin to reagents such as ferricyanide [Mirsky & Anson, 1936], Folin's phenol reagent [Herriott, 1938] and porphyrindine. [Brand & Kassell, 1940].

Mirsky & Pauling [1936] have postulated hydrogen bonds in proteins and have suggested that the breaking of such bonds may be largely responsible for denaturation. Phenols are known to form comparatively strong H bonds, but most relevant observations have been made either in non-polar solvents or on crystals. One of the few examples of H bond formation in water of phenols with groups other than those of the solvent molecules is that with salicylic acid or the salicylate ion, and a similar type of bond between the phenolic group of tyrosine and a carboxyl group of another amino-acid residue may exist in native proteins. The negative charge of the COO⁻ ion would stabilize the bond appreciably, and the bond would definitely increase the acid strength of this COOH, as in the case of salicylic acid [Branch & Yabroff, 1934]. SH groups may also be masked by the formation of weaker hydrogen bonds [Hopkins & Hunter, 1942]. Moreover, unknown groups may be concerned in a similar bond formation. The large number of such bonds, of which those formed by tyrosine will probably be the strongest and therefore the most important, would endow the native configuration of the protein with a certain degree of stability.* Strongly alkaline solution will cause ionization of the phenolic groups and thus break the hydrogen bonds; in order to effect denaturation it may be necessary to break several of these bonds simultaneously and thus the speed of denaturation will increase with a high power of the OH⁻ concentration [Cannan, 1942]. The mechanism of denaturation by heat at neutral *pH* may be very similar, and may also involve ionization of the phenolic hydroxyl group. Denaturation by acid may be due to a different mechanism. Addition of H⁺ to COO⁻ will transform a strong H bond, stabilized by the negative charge, into a weaker bond, although no definite figures can be given for the relative bond energies in the acid and in the ion. Such a picture

* Brand & Kassell [1942] have very recently suggested a similar theory for the denaturation of lactoglobulin.

of denaturation, in which the H bonds formed by the phenolic group are largely, but not exclusively, responsible for the configuration of the native protein, is in accordance with other known facts. If a protein is irradiated with ultra-violet light it becomes either denatured, or changed in such a way that it becomes insoluble on a very slight increase of temperature [Clark, 1935]. It has also been found by Svedberg & Brohult [1938] that haemocyanin is split by irradiation with light of $\lambda = 2800 \text{ \AA}$. If hydrogen bonds formed by phenolic groups are present in these native proteins, then these effects of irradiation with light which is absorbed by the tyrosine grouping, are not surprising. Insulin cannot be denatured according to the usual meaning of the word; its tyrosine groups also appear to be free and able to ionize. We may assume that the large number of —S—S— bonds present in the molecule are largely responsible for the stability of this protein.

SUMMARY

1. The change of the absorption spectrum with pH has been used to estimate the dissociation constants of the phenolic groups of tyrosine

and of 3:5-diiodotyrosine. pK values of 10.15 and 6.4 respectively were obtained, which are in good agreement with those obtained by other methods.

2. The absorption spectrum of insulin can be accounted for by the tyrosine and cystine present in the molecule if a slight shift of the tyrosine band in the protein be assumed. The change of the insulin spectrum with pH indicates that the pK of the phenolic group increases as the pH is raised. This change in the dissociation constant is assumed to be due mainly to the increasing net charge of the protein.

3. The absorption spectrum of egg albumin cannot be quantitatively interpreted in terms of its content of tyrosine and of tryptophan. The changes of absorption with pH, however, clearly indicate that in the native protein few or none of the phenolic groups are able to ionize, whilst after denaturation the occurrence of ionization can be demonstrated spectroscopically. The bearing of these results on the mechanism of denaturation is discussed.

The authors wish to express their thanks to the Medical Research Council for personal grants to both of them.

REFERENCES

- Anslow, G. A. & Assar, S. C. [1940]. *J. opt. Soc. Amer.* **31**, 118.
 Branch, G. E. K. & Yabroff, D. L. [1934]. *J. Amer. chem. Soc.* **56**, 2568.
 Brand, E. & Kassell, B. [1940]. *J. biol. Chem.* **133**, 437.
 ——— [1942]. *J. biol. Chem.* **145**, 365.
 Cannan, R. K. [1942]. *Chem. Rev.* **30**, 395.
 ——— Kibrick, A. C. & Palmer, A. H. [1941]. *Ann. N.Y. Acad. Sci.* **41**, 243.
 Chibnall, A. C. [1942]. *Proc. roy. Soc. B*, **131**, 136.
 Clark, J. H. [1935]. *J. gen. Physiol.* **19**, 199.
 Coulter, C. B., Stone, F. M. & Kabat, E. A. [1936]. *J. gen. Physiol.* **19**, 739.
 Custers, J. F. H., de Boer, J. H. & Dippel, C. J. [1933]. *Rec. Trav. chim. Pays-bas*, **52**, 193.
 Dalton, J. B., Kirk, D. L. & Schmidt, C. L. A. [1930]. *J. biol. Chem.* **88**, 589.
 Danielli, J. F. [1937]. *Proc. roy. Soc. B*, **122**, 155.
 ——— [1941]. *Biochem. J.* **35**, 470.
 Flexsner, L. P., Hammett, L. P. & Dingwall, A. [1935]. *J. Amer. chem. Soc.* **51**, 2103.
 Fruton, J. S. & Lavin, G. I. [1939]. *J. biol. Chem.* **130**, 375.
 Ginsel, L. A. [1939]. *Biochem. J.* **33**, 428.
 Graubner, W. [1938]. *Z. ges. exp. Med.* **63**, 527.
 Harington, C. R. & Neuberger, A. [1936]. *Biochem. J.* **30**, 809.
 Hartley, G. S. & Roe, J. W. [1940]. *Trans. Faraday Soc.* **36**, 105.
 Herriott, R. M. [1938]. *J. gen. Physiol.* **21**, 501.
 Hitchcock, D. I. [1924]. *J. gen. Physiol.* **6**, 742.
 Holiday, E. R. [1936]. *Biochem. J.* **30**, 1795.
 ——— [1942]. Personal communication.
 ——— & Ogston, A. G. [1938]. *Biochem. J.* **32**, 1166.
 Hopkins, G. & Hunter, L. [1942]. *J. chem. Soc.* p. 638.
 Kekwick, R. A. & Cannan, R. K. [1936]. *Biochem. J.* **30**, 235.
 Kuhn, W., Eyer, H. & Freudenberg, K. [1931]. *Hoppe-Seyl. Z.* **202**, 97.
 Ley, H. & Arends, B. [1932]. *Z. phys. Chem. B*, **17**, 177.
 Miller, G. L. & Anderson, K. J. L. [1942]. *J. biol. Chem.* **144**, 459.
 Mirsky, A. E. & Anson, M. L. [1936]. *J. gen. Physiol.* **19**, 451.
 ——— & Pauling, L. [1936]. *Proc. nat. Acad. Sci., Wash.*, **22**, 439.
 Morton, R. A. [1942]. The Application of Absorption Spectra to the Study of Vitamins and Hormones, 2nd ed. London: Adam Hilger.
 ——— & Stubbs, C. A. [1940]. *J. chem. Soc.* p. 1347.
 Neuberger, A. [1934]. *Biochem. J.* **28**, 1982.
 Oncley, J. L. [1942]. *Chem. Rev.* **30**, 433.
 Sjögren, B. & Svedberg, T. [1931]. *J. Amer. chem. Soc.* **53**, 2657.
 Stenström, W. & Goldsmith, N. [1926]. *J. phys. Chem.* **30**, 1683.
 Svedberg, T. & Brohult, S. [1938]. *Nature, Lond.*, **142**, 830.
 du Vigneaud, V. [1938]. *Cold Spr. Harb. Symp. Quant. Biol.* **6**, 275.
 Wyman, J. Jr. [1939]. *J. biol. Chem.* **127**, 1.