THE STRUCTURE OF THE ULTRAVIOLET ABSORPTION SPECTRA OF CERTAIN PROTEINS AND AMINO ACIDS*

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The ultraviolet absorption of the proteins in the region above 2500 A. comprises a broad band with its maximum about 2800 A. The spectrophotometric studies of Judd Lewis (1), Smith (2), and Smith and Marrack (3) have indicated the main quantitative features of this band without, however, disclosing evidence of finer structure. Indeed, with the exception of Spiegel-Adolf and Krumpel (4), who were able to distinguish several maxima in the absorption band of serum albumin, those investigators who have used the arc between metallic electrodes, in air, as the source of radiation have not discovered any details of structure. Vlès and Prager (5) on the other hand found a band system consisting of nine narrow bands in serum albumin, when using the hydrogen discharge tube, which emits a continuous spectrum in the ultraviolet. With such a tube as source Ross (6) has observed a similar resolution in the absorption bands of gelatin and several other proteins, and Lavin and Northrop (7) have found narrow bands in the absorption of crystalline pepsin.

The ultraviolet absorption of the amino acids has likewise been extensively investigated. Although the aliphatic amino acids do not show appreciable absorption above 2500 A., the aromatic amino acids tyrosin, tryptophan, and phenylalanine give absorption in the same spectral region as the proteins, as shown by Dhéré (8), Ward (9), Smith (2), Gróh and Hanák (10) and others. The absorption band of the proteins has in consequence been generally attributed to the content of these amino acids.

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The existence of narrow component bands in the absorption of the aromatic amino acids has been demonstrated as with the proteins only with the use of a source which gives a continuous spectrum. Stenström and Reinhard (11), with the under-water spark, and Ross, and Lavin and Northrop, with the hydrogen discharge tube have found two bands in both tyrosin and tryptophan in the region 2700–2900 A. Ward, who used nickel electrodes in air, reported a group of bands in phenylalanine. Although Smith could not confirm this, Ross found five, and Lavin and Northrop found six narrow bands lying between 2400 and 2700 A. in this amino acid. Ross noted the correspondence in position of these bands with the band elements of several proteins.

EXPERIMENTAL

The primary object of the present work was the discovery of the spectral characteristics which may be associated, in serum protein, with immunologic activity. It was necessary first to provide a background against which might be discerned any peculiarities associated with biological activity. This we have attempted to do by investigating a number of familiar and important proteins. Independent observations were also made on the aromatic amino acids and a number of other amino acids.

The proteins[1] studied and the methods of their preparation were as follows:

1. Serum albumin, from horse serum. Crystallized three times after removal of the globulin, as described by Adair and Robinson (12), and dialyzed free from sulfate.

2. Crystalline egg albumin. Prepared according to Heidelberger (13) and crystallized three times.

3. Serum pseudoglobulin, and

4. Euglobulin. The precipitate obtained from normal horse serum on half saturation with ammonium sulfate was separated and reprecipitated twice by the same procedure. The solution of protein was then dialyzed free from salt. The resulting precipitate represented the fraction which we have considered as euglobulin, while the protein

[1] The authors wish to thank Dr. Michael Heidelberger of the Department of Medicine for several of the proteins used in this work.

which remained in solution after dialysis yielded the pseudoglobulin fraction.

5. Thyroglobulin, from the hog. Prepared according to Heidelberger and Palmer (14).

6. Crystalline insulin. Supplied to us by Dr. Oskar Wintersteiner of the Department of Biochemistry.

7. Pneumococcus Type I antibody. Prepared by the method of Felton (15) from immune horse serum. Two preparations (B76 and B77) were used. These contained about 65 per cent specifically precipitable nitrogen as determined by the quantitative precipitin reaction with Type I acetyl polysaccharide.

The amino acids studied were the following: tyrosin, cystine, tryptophan, phenylalanine, and histidine, the two latter supplied to us by the Department of Biochemistry; and proline and oxyproline kindly supplied by Dr. Randolph West of the Department of Medicine. All had been purified by recrystallization.

Solutions of the different proteins and amino acids were made up to contain 5 mg. of the test substance per cubic centimeter. Progressive dilutions were then made, the concentration in each successive step being one-half that in the preceding. Spectrographic exposures were made on a single photographic plate of each series of dilutions, the time of exposure and the width of the slit, for each series, being kept constant. The slit openings used were between 0.050 and 0.100 mm. In these series it was possible in many instances to distinguish narrow absorption bands that could not be made out in any single exposure. The spectrograms of the series of graded dilutions also disclosed differences, in each substance, in the relative intensities of the narrow bands.

The solutions were maintained at pH 7.0 throughout the work by the use of M/10 phosphate buffer for the primary solutions and for all of the subsequent dilutions. In the cases of tyrosin and insulin, however, the substances were dissolved in 0.01 M HCl, on account of their low solubility at pH 7.

The temperature of the solutions at the moment of exposure was that of the room (21–26°C.). In a few experiments glycerol was used as the solvent and the exposure made when the solution had been brought to a low temperature by immersing the fused quartz cell, in which it was contained, in liquid air. The technical difficulties of such low temperature work are very great, however, as Lavin and Northrop have pointed out in a paper which appeared subsequent to the completion of our experiments. Almost immediately after solidification the glycerin begins to become opaque in consequence of "cracking" and it becomes impossible to admit sufficient light to affect the photographic plate. The information obtained from the low temperature experiments was meager and is not included in the data presented in Table I and Fig 2.

The spectrograph which was used was one of the Littrow type, and was constructed by one of us (C.B.C.). The single quartz lens is 56 mm. in diameter with a focal length for λ2536 of 450 mm. The spectrum given by this lens is of sufficient length to give a distinct separation of the fine absorption bands without the loss of contrast which occurs when a lens of greater focal length is used. This lens proved somewhat more satisfactory than one of shorter focus, however, so that the selection of the focal length stated cannot be regarded as fortuitous. The lens was "figured" by zonal polishing for the correction of longitudinal spherical aberration, and during this process was tested by monochromatic light (Hg 5461) provided by light from the mercury arc passing through the optical system of the spectrograph itself. Exposures were made on 3¼ × 4¼ Wratten M plates. That portion of the spectrum which is in sharp focus at a given setting of prism and lens, is short, with a single lens of large relative aperture and of high degree of aspheric correction, and with our lens did not exceed 80 mm., so that plates of larger size would not have been justified. The Hg arc spectrum was photographed on each plate immediately adjacent to the first and last exposures of each absorption series.

The source of radiation was an hydrogen discharge tube operated on 4400 volts. The capillary portion of the tube was water-cooled; its dimensions were 6 mm. inside diameter by 250 mm. length. The crystal quartz end-window was sealed on with hard deKhotinsky cement. The tube was alternately exhausted, with a Hyvac pump through a sealed-on P_2O_5 trap and filled with hydrogen through a palladium thimble until the lines of atomic hydrogen disappeared in the region 2000–3000 A. We are greatly indebted to Prof. Harold Urey of the Department of Chemistry of this University for invaluable assistance in the design and construction of the hydrogen tube.

A quartz condenser was used to focus the radiation from the hydrogen tube upon the slit of the spectrograph. During this adjustment a fluorescent screen was used to indicate the convergence of the ultraviolet rays upon the slit; if visible light is brought to a focus at the slit, the illumination of the latter by ultraviolet radiation is very likely to be found far from uniform along its length.

The narrow bands which can be distinguished in the ultraviolet absorption band of the proteins and amino acids which we have investigated vary considerably in distinctness. While some are prominent, others are exceedingly faint and can be made out only under certain conditions of illumination and visual acuity. The measurement of the position of many of the narrow bands is quite impossible with the usual type of micrometer microscope, since the magnification reduces the visual contrast too greatly, and it becomes impossible to locate the center of a band. We have been able, however, to surmount to a considerable extent the difficulties of measurement by equipping the stage of the micrometer microscope with a long cross-hair, which extends across the full width of the plate, and is carried on a slide so that it may be moved over the plate to a position directly above the center of an absorption band. This maneuver is made with the free hand while the plate is under observation by the unaided eye. The plate is first

moved out of the optic axis of the microscope to a position where it can be readily observed, and the substage mirror adjusted to give the optimum intensity and direction of illumination for the detection of the narrow bands. The desired portion of the plate is now brought into the field of the microscope and the stage moved longitudinally by means of the micrometer screw until the long cross-hair, which has not been disturbed after being placed over an absorption band, is brought into coincidence with the cross-hair in the ocular. The Hg lines on the

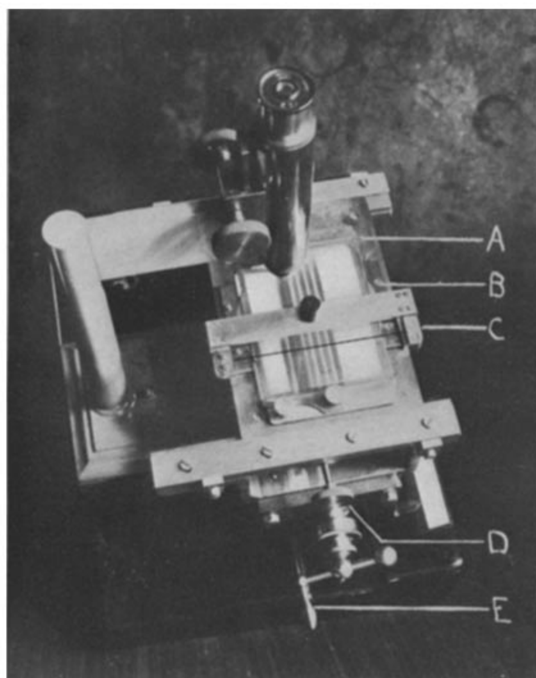

FIG. 1. Micrometer microscope with long cross-hair. *A*, stage; *B*, guide for rotation of plate-carrier; *C*, movable slide with cross-hair; *D*, milled head for rack and pinion motion; *E*, crank for micrometer screw.

plate serve as reference lines from which the wavelengths of the absorption bands can be calculated by means of the Hartmann formula.

The complete micrometer microscope, which was constructed by one of us, (C.B.C.) is shown in the photograph, Fig. 1. The plate-carrier which rests upon the stage has a mechanism by which it can be rotated about its center for orientation of the plate. The stage is movable transversely by rack and pinion at right angles to the direction of longitudinal motion which is controlled by the

micrometer screw. A human hair was used as the cross-hair; this was adjusted to lie parallel with the direction of transverse motion of the stage.

Ten or more settings of the long cross-hair were made in the measurement of each of the narrow bands. The differences between individual readings were naturally greater with faint than with strong bands. With the latter duplicate readings were frequently made to within 1 A. Even with the faintest bands the error of measurement probably does not exceed 5 A. Several plates of each of the test substances were examined. The average values of wavelength for the bands which could be distinguished in the various substances are given in Table I.

TABLE I

Table of Wavelengths of Narrow Bands in Proteins and Aromatic Amino Acids

Serum albumin.......				2533	2583	2613	2645		2688	2733	2788	2847	2900
Egg albumin.........				2532	2587		2650		2680	2742	2799	2855	2923
Thyroglobulin.......				2534	2581	2614	2645		2682	2743	2796	2841	2909
Euglobulin..........			2487	2529		2616	2640		2680	2749	2795	2849	2915
Pseudoglobulin.......					2587		2649		2691	2747	2794	2849	2916
Pneumococcus antibody.............					2591		2649		2685	2768		2850	2911
Gelatin.............				2529	2584		2644		2679	2745		2839	
Insulin..............				2530	2586		2645		2683	2766		2839	2898
Tyrosin.............									2672	2747		2816	
Tryptophan.........									2694		2794		2888
Phenylalanine........	2366	2418	2466	2517	2574	2606	2635	2671	2714				

It is evident in this that among the proteins the positions of the narrow bands correspond with one another very closely. Vlès and Prager noted this relation with serum albumin, egg albumin, and casein. Bands at certain positions are present in some of the proteins while apparently wanting in others. Even if the error in measurement is as great as 5 A., it seems clear that certain bands depart considerably from the "group" position, as for example the band at 2766 A. of insulin, and that at 2855 of egg albumin.

The relative intensities of the narrow bands are indicated in the curves, Fig. 2, which were traced by a recording microphotometer. We are indebted to Dr. Garman of Washington Square College for his kindness in making these records. The curves do not reveal the presence of numerous bands which can be distinguished on direct examination, but show those that appear most distinct visually. Since the various solutions to which the microphotometer records correspond

were of different molalities, the heights of the curves may not be used to compare the amounts of absorption at a particular wavelength given by the different substances. The curves do give, however, a measure of the relative intensities of the more distinct bands within the spectrum of a given substance.

It is evident on examination of these curves, as well as on direct examination of the absorption series spectrograms, that the proteins differ among themselves in the relative intensities of the narrow bands. The differences are sufficient to give each absorption curve a characteristic configuration.

The narrow bands show with great distinctness in serum albumin (Curve 1, Fig. 2). They are somewhat more sharply defined in our curve than in that of Vlès and Prager, who made their spectrograms at 0°C., while ours were made at 21–26°C. No tracing was made for egg albumin; its absorption, however, in its fine structure resembles that of serum albumin. The narrow bands appear almost uniformly distinct in thyroglobulin also, but the absorption curve as a whole shows a close resemblance only to that of euglobulin, from which it can hardly be distinguished except for the prominence in the latter of the band at 2680 A. The intensity of this band varies, however, in different spectrograms of euglobulin. In several it has appeared more intense than the broad band which has its peak at 2795 A.

In the curve of pseudoglobulin the broad band about 2794 A. is relatively more intense than in the preceding proteins. The narrow bands, however, are less distinct than in these; this fact is less evident in the photometric curve (Curve 4, Fig. 2) than in the spectrograms themselves.

Except in the region of the strongest band (2700–2850 A.) the curve for the Pneumococcus Type I antibody runs almost exactly parallel with that of pseudoglobulin. The suggestion given by this resemblance that this antibody is a modified pseudoglobulin is supported by the recent observations of Chow and Goebel (16), which leave little room for doubt that the pneumococcus antibody is carried by the pseudoglobulin fraction and not as was earlier supposed, by the euglobulin of serum. These authors have found that antibody shows chemical differences from normal pseudoglobulin. We have found that there are likewise spectrographic differences: the total absorption

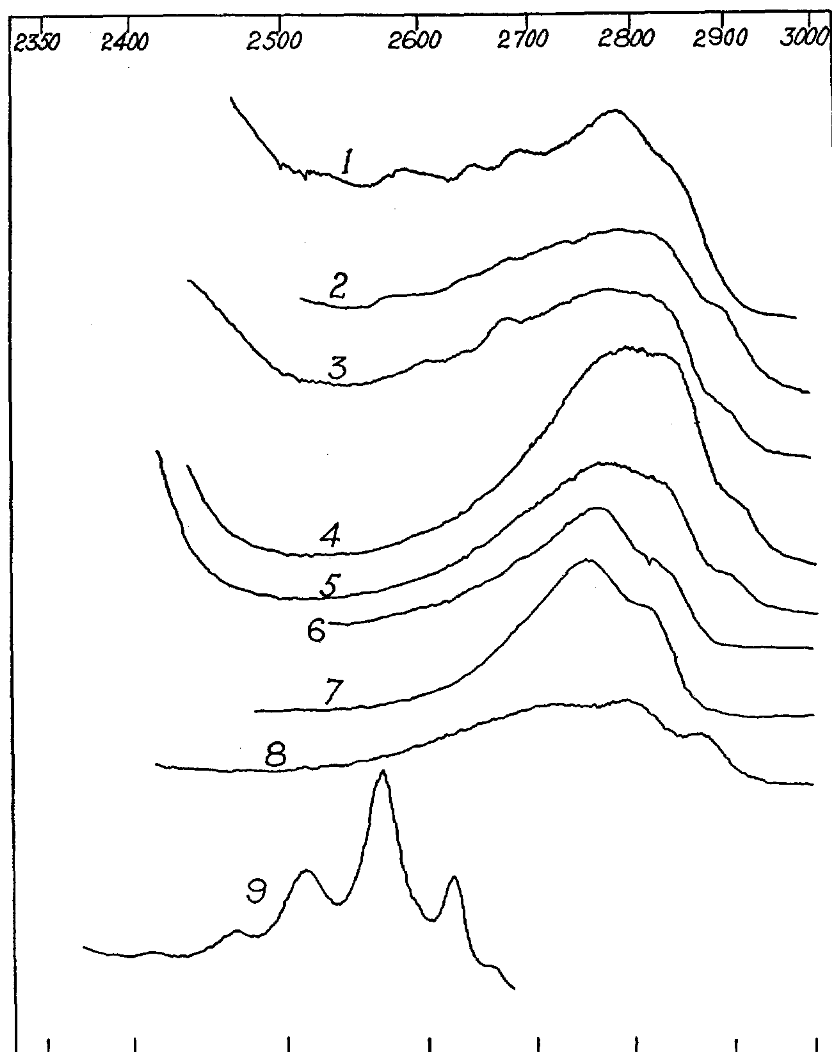

FIG. 2. Microphotometric curves of ultraviolet absorption of proteins and aromatic amino acids.

between 2700 and 2850 A. is relatively smaller in antibody than in the "normal" protein, and the point of maximum absorption is at 2768 A. rather than 2795 A.

No microphotometer tracing was made of gelatin. It is evident

in the absorption series that the maximum of absorption is at 2584 A. The broad band about 2795, and the band about 2900, which are found in most of the proteins, are wanting. We were able to distinguish clearly in gelatin the four narrow bands found by Ross and attributed by him to phenylalanine, as well as two other bands at longer wavelengths.

The curve for insulin (at pH 4, Curve 6, Fig. 2) shows the resemblance to that of tyrosin which Kuhn, Eyer, and Freudenberg (17) have noted for this protein. Neither their absorption curves, however, nor those of Graubner (18) show the narrow band structure which is evident in our spectrograms as four narrow bands on the short wave side of the peak at 2766 A. Some of these are faintly indicated by points of inflection in the curves given by the latter author.

Among the amino acids, we have found no evidence of absorption of wavelengths longer than 2200 A. by proline or oxyproline. The sharply delimited end-absorption of histidine comprises a broad band which has its maximum about 1950 A. It gives no indication of resolution into components. Cystine shows in addition to end-absorption, which extends with concentrated solutions to 2450 A., a band with its maximum about 2490 A. This appears on our plates much less distinct than one would be led to expect from the descriptions of Foster, Anslow, and Barnês (19). It could not be resolved.

The relatively sharp and narrow band of tryptophan (Curve 8, Fig. 2) which lies on the long wavelength side of the broad central region of absorption has its center according to our measurements at 2888 A. The values of 2875 and 2900 A. have been given by Stenström and Reinhard, and by Ross, respectively. We find the center of the absorption maximum of this amino acid at 2795 A. This band can be distinguished clearly from one almost equally strong with maximum at 2694. When these two bands are not distinguished as separate, the absorption maximum appears about 2750, as found by Stenström and Reinhard, Ross, and Lavin and Northrop. The maximum of absorption by tryptophan has been given moreover by Gróh and Hanák as 2775, by Ward as 2800, and by Smith as 2790. The values of the two latter observers agree closely with ours; their method appears to have given the position of the absorption maximum accurately, although it did not reveal the lesser bands. The band of tryptophan which Smith

has described at 2180 A. and which appears on Ward's curve is evident on our plates, but is not resolved.

The absorption maximum of tyrosin (Curve 7, Fig. 2) in solution at pH 2.2, is found on our plates at 2747 A. All observers have agreed on the position of this band. The relatively narrow band which we find at 2816 has been observed only by those who have used a source giving a continuous spectrum. Several observers (Dhéré, Stenström and Reinhard, and Gróh and Hanák) have noted that the spectral absorption of tyrosin is affected by the pH, the maximum of absorption lying farther toward the red in alkaline than in acid solutions. It seems probable that this shift is due to change in position and intensity of the narrow band just described. In our curve at pH 2.2 and in that of Stenström and Reinhard at pH 4 this band is small and lies near 2800. In alkaline solution (pH not stated) Ross found it at 2840, together with the band at 2750. Gróh and Hanák at pH 11.0 and Stenström and Reinhard at pH 12.7 (incorrectly quoted by Ross) found only one large band near 2900. There is no evidence in these cases that the band at 2750 had disappeared and it seems probable from study of the curve of Stenström and Reinhard that it was still present but obscured by the more intense band at 2900. A third band of low intensity is evident on our plates at 2672. It can barely be made out on the photometric curve. The large band found by Smith at 2240 affords on our plates no indication of resolution.

In the spectrum of phenylalanine (Curve 9, Fig. 2) we have found nine bands between 2366 A. and 2714 A. Five of these correspond to the bands observed by Ross, but lie from 5 to 14 A. deeper in the ultraviolet than the values given by this observer. There appears to be somewhat closer agreement between our results and those of Lavin and Northrop as far as can be made out from their figure. It is evident from the photometric curve that the band at 2574 is the dominant one. The bands at 2366, 2606, and 2714 are feeble and do not appear in the reproduction although the two latter are evident in the original tracing.

DISCUSSION

The theory that the ultraviolet absorption of the proteins, in the region above 2500 A., is due to the aromatic amino acids is supported by the observations reported here.

The narrow bands which appear between 2530 and 2690 in all of the proteins may be assigned with some confidence to phenylalanine, as Ross and Lavin and Northrop have previously suggested. These bands occupy slightly longer wavelengths in the proteins than in the amino acids (Table I). The average amount of this shift is 12 A.; within the limit of accuracy of measurement it appears to be the same for each of the bands and to be uniform throughout the proteins. The relative intensities of the bands are not the same, however, in the proteins as in the amino acids. The band at 2574 for example which is by far the strongest in phenylalanine is not more marked in the proteins than the bands corresponding to those at 2517 and 2635 of the amino acid. On the other hand the band about 2680 which is one of the strongest in serum albumin and euglobulin is represented in phenylalanine by a relatively feeble band.

Only a small part of the ultraviolet absorption of the proteins, in terms of amount rather than spectral extent, may be referred to phenylalanine. Much the larger part, which occurs between 2700 and 2850 to 2900, may be attributed to tryptophan and tyrosin.

The band of tryptophan at 2794 may be held responsible for the occurrence of the maximum of absorption at this wavelength in several of the proteins. The band of this amino acid at 2694 cannot be identified in the proteins and indeed is so broad that it cannot be distinguished readily in the amino acid itself. The sharp band at 2888 A., however, corresponds to a band which appears between 2898 and 2923 in all of the proteins which have been examined except gelatin. This band was found by Lavin and Northrop in crystalline pepsin and attributed by them to tryptophan. In the proteins this band appears shifted by 10 to 35 A. toward the red from its position in the amino acid.

The absence from the gelatin spectrum of this band and the one at 2794 is clearly correlated with the lack of tryptophan in this protein (20).

The large band of tyrosin at 2747 gives origin apparently to the absorption maxima of insulin and pneumococcus antibody near 2766 A. Tryptophan appears for this reason to have a smaller share in the absorption of these two proteins than it has in other proteins which contain this amino acid. This suggests that these two proteins have a

relatively small content of tryptophan, although it is not at all certain that the amount of spectral absorption is in general proportional to the content of amino acid.

The band present in all of the proteins between 2839 and 2855 A. corresponds to the band which is found in tyrosin in acid solution at 2816, and in alkaline solution, at 2840 A. or longer wavelengths, depending on the pH. The band about 2850 is very prominent in all of the proteins. Its exact position is probably influenced, as in the case of the 2747 A. band of tyrosin, by its proximity to a band of the more strongly absorbing tryptophan, the adjacent band in this case being that at 2888, which would tend to shift the apparent maximum of the tyrosin band to a longer wavelength. In gelatin and insulin in which the tryptophan influence is small, this tyrosin band lies at 2839 A., having thus a definitely shorter wavelength than in most of the other proteins.

The position of this band is of interest in connection with the theory of Stenström and Reinhard, who have sought to relate the shift in this band, in tyrosin itself, with change in pH, to the ionization at the —OH group which is attached directly to the benzene ring. An effect upon the ionization at this point results, they believe, in the protein molecule, from the proximity of this —OH group to the $-NH_2$ group of an adjacent amino acid. The pneumococcus antibody is known from the work of Felton (21) and Chow and Goebel to possess an increased basicity as compared with the normal pseudoglobulin. On the theory of Stenström and Reinhard this greater basicity should occasion an even wider displacement of this tyrosin band than in the normal protein. However, the tyrosin band has the same position in the pneumococcus antibody as in the normal pseudoglobulin. The increased basicity of the antibody is thus left unexplained, in view of the fact that Chow and Goebel have found no essential difference, beyond a slightly higher lysine content, in the distribution of the basic amino acids in antibody and normal protein.

The experiments at low temperatures were inconclusive. We have been led to expect, from our observations (22) on the metal-porphyrin compounds at the temperature of liquid air, marked changes in the relative intensities of the bands. We did not, however, observe such changes. Vlès and Prager on the other hand noted a change in the

relative intensity of one of the bands of serum albumin between 0° and 37°C.

The magnitude of the displacements to longer wavelengths, which are observed in the narrow bands of the proteins, from their position in the amino acids,—from 10 to 35 A.—suggests that the effect of polypeptid linkage is not upon the electronic energy of the amino acid, but rather upon its vibrational energy.

It might at first sight appear surprising that the aromatic amino acids should retain their spectral character to so great an extent when combined in the protein molecule. It may be remarked, however, that the parts of the amino acid molecule which are involved in polypeptid linkage are separated by two or more carbon atoms from the benzene ring. Arnold and Kistiakowsky (23) have found that the mutual influence upon their absorption spectra of two chromophore groups within a molecule extends through a carbon chain only when this is two atoms or less in length, the spectra being unaffected when the separation is greater than this.

CONCLUSIONS

1. The absorption spectra of a number of proteins in the region 2500 to 3000 A. have been found to comprise from six to nine narrow bands. In consequence of variation in the relative intensity of these bands from protein to protein, the absorption curve has a characteristic configuration for each protein.

2. These bands correspond closely in position with the narrow bands which appear in the absorption spectra of tryptophan, tyrosin, and phenylalanine. Tryptophan and tyrosin each present three bands, phenylalanine shows nine.

3. The bands in the proteins are accordingly attributed to these amino acids. In the proteins the bands are displaced from the positions which they occupy in the uncombined amino acids, in most instances, by 10 to 35 A. toward longer wavelengths.

4. The absorption spectrum of Pneumococcus Type I antibody resembles that of normal pseudoglobulin but shows characteristic differences.

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