THE DEPENDENCE OF DIFFERENCE SPECTRA OF DPN AND OF RELATED SUBSTANCES UPON HYDROGEN ION CONCENTRATION*,**

by

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The effect of pH on the spectra of purines and their derivatives has long been recognized[1,2,3] and has been widely used as a means of characterizing compounds of this type[4]. However, these pH-dependent spectral changes are difficult to study since they occur in a spectral region which is characterized by a large pH-independent absorption and since, by the usual methods of measurement, no discrete spectral peaks can be detected. A chance observation of the difference spectrum of diphosphopyridine nucleotide (DPN) at different pH values suggested the possibility that differential spectroscopy might be a useful method for isolating the spectral effects which do vary with the pH of the medium. The results indicate that this technique may be generally useful in the characterization of resonating structures containing dissociable groups. An accompanying paper describes the estimation of trypsin and chymotrypsin by this method.

EXPERIMENTAL

Solutions for spectral measurements were prepared by titrating aliquots of a standard solution of the compound being investigated to the desired pH range with dilute hydrochloric acid or sodium hydroxide. The aliquots were then transferred to volumetric flasks and diluted to volume. The pH of each solution was redetermined after dilution. Measurements of pH were made with a Beckman model G pH meter.

The difference spectra were measured at 25° in 1 cm quartz cells in a Beckman model DU spectrophotometer equipped with a photomultiplier attachment and with dual thermospacers and a water-jacketed cell compartment. One solution was arbitrarily selected to serve as a reference and the difference spectra of the solutions at other pH values were determined with respect to the reference solution as a blank. By this technique the absolute spectrum of the reference solution is reduced to zero and pH dependent effects appear as difference spectra. Optical density differences were measured at 2.5 mμ intervals except in the vicinity of the maxima where the interval was decreased to 1 or 0.5 mμ. For many of the measurements in solutions of high absolute optical density it was necessary to "zero" the instrument and to make the readings with the selector switch set in the 0.1 position.

Materials. DPN of a purity estimated by the supplier to be greater than 95% was obtained from the Pabst Laboratories. Adenine, adenylic acid (adenosine-3-phosphate), 2,6-diaminopurine and ribonucleic acid were supplied by Nutritional Biochemicals Corporation. Nicotinic acid was an Eastman product and nicotinamide, prepared by recrystallizing a commercial product from benzene, was a gift from Mr. IRWIN FRIDOVICH. Trigonelline sulfate was a gift from Dr. HENRY KAMIN.

* This investigation was supported by research grant RG-2941 (C2) from the National Institutes of Health, U.S. Public Health Service.

** Part of a thesis submitted by Miss MAIRE T. HAKALA to the Graduate School of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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RESULTS

The difference spectra of 0.001 M solutions of DPN, adenylic acid, and adenine are shown in Fig. 1. The measurements were made on solutions having concentrations adequate to give readily measurable differences and the results were reduced to a common concentration to illustrate the relative magnitudes of the observed effects. In the case of DPN the difference spectra were measured with $2.58 \cdot 10^{-3}$ M solutions. Since the absolute optical density of these solutions is about nine at 280 mμ, the usefulness of the photomultiplier detector is apparent.

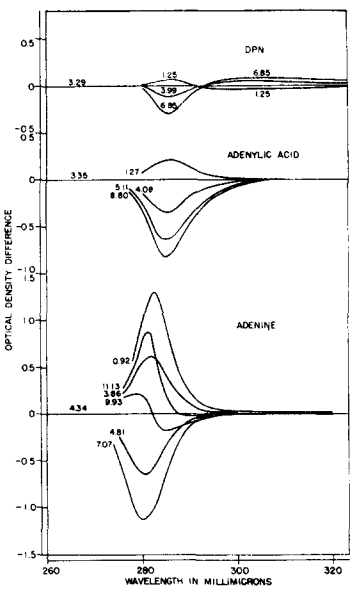

Fig. 1. Representative difference spectra ofo.001 M solutions of DPN, adenosine-3-phos-phoric acid and adenine. The pH of the solution giving rise to the spectral curve is indicated by the number adjacent to the curve.

As is shown in Fig. 1, there is a shift in the wavelength of the maximal optical density difference with pH. In the case of adenine the maxima and minima between pH 0.92 and pH 6.26 lie on a straight line between 282.5 and 280 mμ. Between pH 6.26 and pH 9.93 the wavelengths at which the minima occur shift, again in a linear manner, from 280 to 285 mμ. The relative maximum at pH 9.93 occurs at 279 mμ and shifts to 281 at pH 11.13.

As an aid in visualizing the nature of difference spectra, one simple hypothetical case is shown in Fig. 2. In the lower part of this figure the spectrum of the acid form of a dissociable resonant compound is represented by the higher of the two peaks outlined by solid lines. The basic form of the compound is the higher of the two peaks outlined by dashed lines. If the acidic form is half-neutralized and if Beer's law is obeyed by each form, it is clear that the height of each peak should be reduced to half that shown by the pure acidic or basic form. The half-neutralized forms are represented by the lower curves in Fig. 2 and their geometric sum, representing the spectrum of the solution when pH is equal to the pK of the dissociating group, is outlined by the longer dashes. The difference spectrum of the acidic form relative to the half-neutralized form is represented by the area shaded with lines of negative slope; the difference spectrum of the basic form relative to the half-neutralized form is the area shaded with lines of positive slope. In the upper part of the figure the same conventions of shading are used to indicate the hypothetical observed spectra which would result if the optical density of the half-neutralized solution were arbitrarily set at zero at every wave length. It is clear that this model approximates the difference spectra observed with DPN. The shift in the

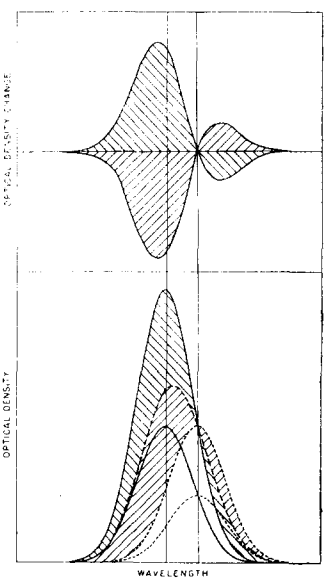

Fig. 2. Illustration of the hypothetical origin of difference spectra. See text for discussion.

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wavelength at which the maxima and minima occur can be formally accounted for by a similar but more complex model.

Although the nature of difference spectra, as illustrated in Fig. 2, makes it appear improbable that any rigorous relation could be established between the spectra and pH, there seems to be a consistent relation between the areas under the spectral curves and pH. This hypothesis was tested by measuring the areas under the spectral curves, drawn to a suitable scale, with a planimeter. In order to minimize the error of measurement, the trailing portions of the curves were neglected and areas were measured over a span of wavelengths sufficient to include the maxima and minima but not the edges of the curves. In the case of adenine the limiting wavelengths selected were 276 and 286 mμ. The obvious algebraic signs were assigned to these areas and the value of the largest negative area was added to each of the other values to obtain a consistent series of positive numbers. The resultant values were fitted to the Henderson-Hasselbalch equation in the form:

$$\mathrm{pH} = \mathrm{p}K' + \log \frac{\text{Maximum area} - \text{Measured area}}{\text{Measured area}}$$

or where appropriate, to the form in which the logarithmic term is the reciprocal of that shown. The fit of the results to theoretical dissociation curves is shown in Fig. 3.

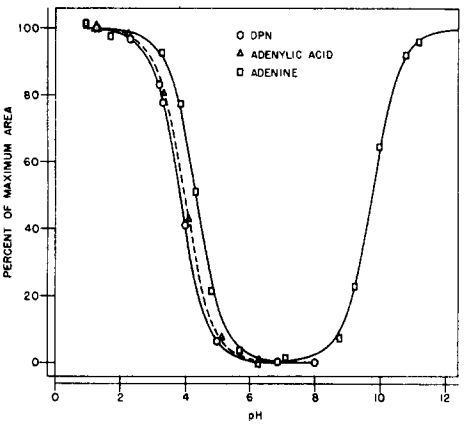

Fig. 3. Relation between the areas under difference spectral curves, expressed as percent of maximum area, and pH. The curves are calculated from the mass law for pK values of 3.85 for DPN, 3.95 for adenylic acid, and 4.35 and 9.72 for adenine.

In the case of DPN the areas between 280 mμ and the isobestic point at 293 mμ and between the isobestic point and 320 mμ were measured independently to test the hypothesis, apparent from Fig. 2, that these areas vary with pH in the same manner. The hypothesis was confirmed. The results shown in Fig. 3 were derived by summing corresponding areas lying above and below the baseline, no regard being taken of the geometric signs of the separate areas. Consistent algebraic signs were then assigned to the sums of areas.

Similar measurements were made on 0.001 M solutions of 2,6-diaminopurine using a solution at pH 4.10 as the reference solution. Qualitatively the spectra resemble those shown in Fig. 1 for adenine. The wavelength at which the maxima and minima occur shifts from 305 mμ at pH 0.93 to 300 mμ at pH 6.72. Identical curves are obtained between pH 6.72 and pH 9.32. Spectral curves at pH 11.07 and 12.01 resemble those shown for adenine at pH 9.93 and 11.13 with the exception that the maximum at pH 12.0 occurs at 300 mμ in the case of 2,6-diaminopurine. Although a detailed analysis of the data was not made, a plot of the areas under the spectral curves against pH indicated apparent pK' values of the order of one, five and eleven.

The difference spectra of solutions of ribonucleic acid having an optical density of 13 at 260 mμ in neutral solution were determined with respect to a solution at pH 3.95. The insolubility of this material in solutions acid to pH 2.40 prevented measurements in this range. The spectral curves found are similar to those shown by adenine with the

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exception that, since there are no unsubstituted imino groups in the 9 position of the purine rings, there is no reversal of the trend of the curves in the alkaline pH range. As would be expected from the variety of dissociable groups present in nucleic acid, a plot of the areas under the curves against pH gives a continuous sigmoid curve from pH 2.40 to pH 11.58.

Although the change in the extinction coefficient of adenine nucleotides at 280 mμ with pH has been associated with the ionization of the 6-amino group of adenine, it was of interest to determine whether any contribution to the difference spectra of DPN might arise from the nicotinamide moiety. Difference spectra of nicotinic acid, nicotinamide, and trigonelline are shown in Fig. 4. It should be noted that the dimensions of the axis of ordinates are not the same for the three sets of spectra. Calculation of the apparent dissociation constants from the areas under the spectral curves gives values of 2.10 and 4.75 for nicotinic acid, 3.10 for nicotinamide, and 2.10 for trigonelline. Since the spectral effects shown by nicotinamide can be ascribed to the dissociation of the pyridinium ion and the spectral differences exhibited by trigonelline can be accounted for by the dissociation of the carboxyl group, it appears that, within the pH range of stability, the N-substituted nicotinamide grouping of the DPN would make no contribution to the difference spectra of this substance. It should be noted that the effects observed with nicotinic acid and with nicotinamide are of a much smaller magnitude than those found with the other compounds studied.

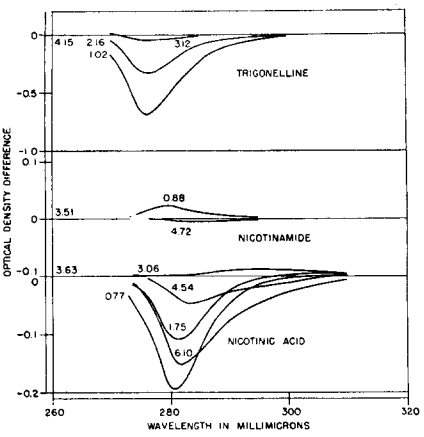

Fig. 4. Representative difference spectra at various pH values for 0.01 *M* solutions of nicotinic acid and of nicotinamide and for 0.001 *M* solutions of trigonelline. The numbers near the curves designate the pH of the solution giving rise to the spectrum shown.

DISCUSSION

In view of the uncertainties attending the estimation of apparent dissociation constants, a comparision of the values found in this study with those determined by conventional methods appears critical. Such a comparison is made in Table I.

Since the values previously reported for the dissociation constants of adenine are in good agreement, it appears that the error of the present method of estimation may be of the order of 0.2 pH unit.

Since the value of pK' for benzoic acid is 4.18[10] while that for pyridine is 5.36[11], it appears that in nicotinic acid, as in other dipolar ions[12], the acidity of both dissociating groups is increased. The first dissociation constant of nicotinic acid appears not to have been previously estimated.

While the present results are mainly elaborations in detail of previously known phenomena, they serve to illustrate the potential usefulness of differential spectroscopy in studying the spectra of resonant structures containing dissociable groups and to indicate the utility of such spectra in approximating the dissociation constants of the ionogenic groups.

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TABLE I

COMPARISON OF APPARENT DISSOCIATION CONSTANTS ESTIMATED FROM DIFFERENCE SPECTRA WITH LITERATURE VALUES

Substance	*pK' from difference spectra*	*Literature value of pK'*	*Reference*
Adenine	4.35	4.12	(5)
		4.15	(6)
		4.1	(7)
	9.72	9.75	(5)
		9.80	(6)
Adenosine-3-phosphoric acid (unresolved)	3.95	—	
α-Adenosine-3-phosphoric acid	—	3.80	(5)
β-Adenosine-3-phosphoric acid	—	3.65	(5)
Nicotinic acid	2.10	—	
	4.75	4.85	(8)
Nicotinamide	3.10	3.10	(9)

SUMMARY

Difference spectra at various pH values have been measured on solutions of adenine, adenosine-3-phosphoric acid, 2,6-diaminopurine, ribonucleic acid, nicotinic acid, nicotinamide, and trigonelline. The nature of the observed difference spectra has been indicated and a method for estimating the dissociation constants of compounds showing pH-dependent difference spectra has been presented.

RÉSUMÉ

Les spectres de différence de solutions d'adénine, d'acide adénosine-3-phosphorique, de 2-6-diamino-purine, d'acide ribonucléique, d'acide nicotinique, de nicotinamide et de trigonelline ont été déterminés à divers pH. La nature des spectres de différence obtenus est décrite et une méthode de détermination des constantes de dissociation fondée sur la relation entre le pH et le spectre de différence est proposée.

ZUSAMMENFASSUNG

Von Adenin, Adenosin-3-Phosphorsäure, 2-6-Diaminopurin, Ribonucleinsäure, Nicotinsäure, Nicotinsäureamid und Trigonellin wurde, bei verschiedenen pH-Werten, das Differentialspektrum gemessen. Die erhaltenen Differentialspektren wurden beschrieben und eine Methode zur Bestimmung der Dissoziationskonstanten von Substanzen, die ein pH-abhängiges Differentialspektrum aufweisen, zum Vorschlag gebracht.

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Received October 8th, 1954