Schellman, C. G., and Schellman, J. A. (1958), *Compt. rend. trav. lab. Carlsberg 30*, 463.
Schellman, J. A. (1958), *Compt. rend. trav. lab. Carlsberg 30*, 363.
Schellman, J. A., and Schellman, C. G. (1961), *J. Polymer Sci. 49*, 129.
Scheraga, H. A. (1960), *J. Phys. Chem. 64*, 1917.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Szent-Györgyi, A. G. (1960), *in* The Structure and Function of Muscle, Bourne, G. H., ed., New York, Academic Press, vol. II, p. 1.
Szent-Györgyi, A. G., and Cohen, C. (1957), *Science 126*, 697.
Yang, J. T., and Doty, P. M. (1957), *J. Am. Chem. Soc. 79*, 761.

Anomalous Rotatory Dispersion of Enzyme Complexes. III. Rotatory Dispersion Titration of Liver Alcohol Dehydrogenase with Coenzyme Analogues and *p*-Chloromercuribenzoate*

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The stoichiometry of the interaction of the analogues of DPNH with liver alcohol dehydrogenase has been measured by means of *rotatory dispersion titration*. At pH 7.5 to 9.5—as with DPNH—2 moles of desamino DPNH, 3-acetylpyridine DPNH, and thionicotinamide DPNH bind asymmetrically to each mole of liver alcohol dehydrogenase. Between pH 9.5 and 11, the liver alcohol dehydrogenase–DPNH complex progressively dissociates; the midpoint of the curve is at about pH 10. *p*-Chloromercuribenzoate also dissociates the enzyme-coenzyme complex; a twentyfold molar excess of this agent prevents completely the binding of DPNH and its analogues to liver alcohol dehydrogenase. However, simultaneously, the enzyme undergoes changes in optical rotation indicative of protein denaturation; hence the effect of *p*-chloromercuribenzoate on coenzyme binding and catalytic activity cannot be attributed solely to the interaction of this agent with a specific sulfhydryl group or groups at the active enzymatic site.

Measurement of the binding of coenzymes, substrates, and inhibitors to enzymes is an important experimental problem in elucidating the mechanisms of enzyme action and in ascertaining the nature of active enzymatic sites. In this regard, both the chemistry of the interacting groups of these molecules and their arrangement in three-dimensional space are critical. Thus far, physicochemical methods which jointly examine both of these factors have been lacking (Fraenkel-Conrat, 1960; Koshland, 1960).

Recently we have reported that spectropolarimetry can be employed to study directly the composition, structure, and function of active centers of certain enzymes; the binding of chromophoric molecules at these asymmetric sites induces anomalous optical rotatory dispersion, a Cotton effect, in the absorption bands of the bound chromophores (Ulmer and Vallee, 1961). By means of such Cotton effects, asymmetric enzyme-metal-chelate complexes have been identified qualitatively, and the interactions of coenzymes and inhibitors at the active centers of enzymes have been characterized (Ulmer *et al.*, 1961a). Moreover, it has been learned that the magnitude of such Cotton effects can be used to determine binding quantitatively.

The present work extends previous observations and describes the quantitative measurement of the interactions of liver alcohol dehydrogenase with coenzyme analogues, and the inhibitor, *p*-chloromercuribenzoate, by a procedure which we have termed *rotatory dispersion titration*. The binding to liver alcohol dehydrogenase of each of the coenzyme analogues is identified by the presence of a characteristic Cotton effect centered at the absorption maximum of the enzyme-analogue complex (Ulmer *et al.*, 1961b). The magnitude of the Cotton effect is dependent upon the concentration of this complex, which is, thereby, titrated by rotatory dispersion, but it is independent of the concentration of free analogue and apoenzyme.

MATERIALS AND METHODS

Crystalline alcohol dehydrogenase of horse liver was obtained from C. F. Boehringer and Soehne, Mannheim, West Germany. Prior to use, the enzyme was dialyzed for five days against 0.1 M sodium phosphate buffer, pH 7.5, at 4°, to remove low-molecular-weight impurities which absorb radiation at 280 mμ. In the same buffer at pH 7.5 the enzyme was monodisperse upon sedimentation in the analytical ultracentrifuge (Spinco, Model E). The concentration of protein was determined by

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measurement of the optical density at 280 mμ, based upon an absorbancy index of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). Molar concentrations are based on a molecular weight of 83,300 (Ehrenberg and Dalziel, 1958).

Catalytic activity was measured spectrophotometrically at 23° by observing the rate of DPNH formation as the increase in optical density at 340 mμ as described previously (Ulmer and Vallee, 1961). The 3.0 ml reaction mixture contained 5 μmoles of DPN, 50 μmoles of ethanol, 0.5 ml of 0.1 M pyrophosphate buffer pH 8.8, and 10 μg of enzyme.

DPNH and *p*-chloromercuribenzoate were obtained from Sigma Chemical Company and concentrations were determined by measurement of absorbancy at 340 mμ for DPNH (Kaplan, 1960) and at 232 mμ for *p*-chloromercuribenzoate (Boyer, 1954). 3-Acetylpyridine DPN, pyridine-3-aldehyde DPN, thionicotinamide DPN, desamino DPN, and desamino-3-acetylpyridine DPN were obtained from Pabst Laboratories. These derivatives of DPN were reduced enzymatically with yeast alcohol dehydrogenase and ethanol (Rafter and Colowick, 1957), and the concentrations of the reduced analogues were determined from reported absorption coefficients (Siegel *et al.*, 1959; Anderson and Kaplan, 1959). Sodium dihydrogen phosphate, sodium pyrophosphate, and 95% ethanol were of reagent grade and were used without further purification. Dialyses were carried out in precleaned (Hughes and Klotz, 1956) cellulose casings (Visking Company). The purification of water and glassware has been described (Vallee and Hoch, 1955).

Optical rotation was measured by means of a model 200S–80Q photoelectric spectropolarimeter, with oscillating polarizer prism (O. C. Rudolph and Sons). A high-intensity, high-pressure mercury lamp (A-H6 General Electric Company) was used as light source from 700 mμ to 270 mμ. Excellent stability could be maintained by circulating cooled, deionized water through the quartz-jacketed lamp housing. Maximal intensity of illumination is obtained by adjusting the lamp position until the highest photometer response is reached in the wavelength region under study. All measurements were performed in 5 cm semimicro polarimeter cells with fused quartz end-plates (O. C. Rudolph and Sons). The temperature of the solution was maintained at 10 ± 1° by circulating cooled water through the polarimeter housing from an external bath. Dry nitrogen directed onto the polarimeter tube end-plates prevented fogging. Rotational angles were measured by the method of symmetrical angles (Rudolph, 1955). Titrations of liver alcohol dehydrogenase with DPNH, the coenzyme analogues, and *p*-chloromercuribenzoate were performed by determining the rotatory dispersion of the *complexes* over a limited wavelength range of 300–450 mμ. Enzyme concentrations sufficient to give absolute rotations of 0.05° or more were utilized. The symmetrical angle adjustment of the polarimeter was maintained at 5° throughout, while the monochromator slit width and photometer sensitivity gain were varied to control light intensity. At each wavelength nearly identical slit widths were employed for the sample and its blank, and the slit width was restricted to less than 0.2 mm in most instances. Blank solutions contained no enzyme but otherwise were identical with the experimental sample. Specific rotations were calculated on the basis of enzyme concentrations and are precise to ±1.0°. The dispersion constant, λ_c, was calculated as suggested by Yang and Doty (1957).

p-Chloromercuribenzoate titrations were performed spectrophotometrically by measuring the increase in optical density at 255 mμ, according to the method of Boyer (1954).

Results

Figure 1 illustrates the rotatory dispersion titration of 2×10^{-5} M liver alcohol dehydrogenase with increments of 3-acetylpyridine DPNH at pH 7.5.

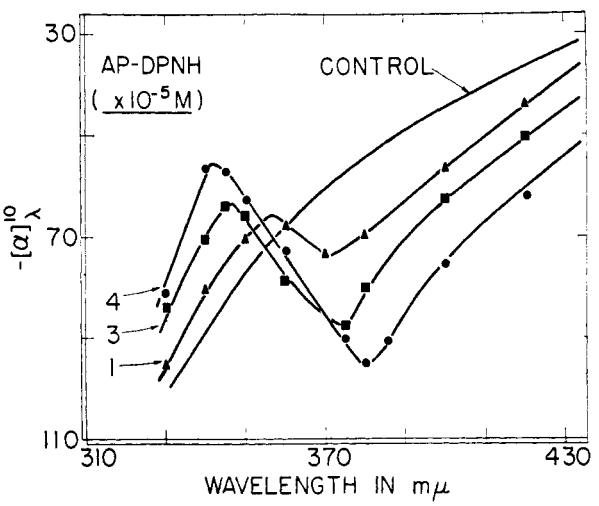

FIG. 1.—The effect of 3-acetylpyridine DPNH concentration on the magnitude of the Cotton effect of the liver alcohol dehydrogenase–3-acetylpyridine DPNH complex. Specific rotation at 10°, $-[\alpha]^{10}_{\lambda}$, is plotted against wavelength. At a fixed enzyme concentration of 2×10^{-5} M, the magnitude of the Cotton effect increases with increasing 3-acetylpyridine DPNH concentration to become maximal at 4×10^{-5} M 3-acetylpyridine DPNH. Conditions: control (———) is 1.66 mg per ml of liver alcohol dehydrogenase in 0.1 M phosphate, pH 7.5, 10°; 1.66 mg per ml of liver alcohol dehydrogenase and 1×10^{-5} M 3-acetylpyridine DPNH (▲—▲), 3×10^{-5} M 3-acetylpyridine DPNH (■—■), 4×10^{-5} M 3-acetylpyridine DPNH (●—●).

Both the amplitude and the breadth[1] of the Cotton effect increase in direct proportion to the amount of coenzyme analogue added. The magnitude of the effect becomes maximal at 4×10^{-5} M 3-acetylpyridine DPNH and is unchanged by further increments of the analogue. At saturation, the Cotton effect has a peak at 340 mμ, a trough at 378 mμ, a breadth of 38 mμ, and an amplitude of 38°.

Similar series of optical rotatory dispersion curves are obtained in titrations of alcohol dehydrogenase with the other coenzyme analogues here employed—desamino DPNH, desamino-3-acetylpyridine DPNH, thionicotinamide DPNH, and pyridine-3-aldehyde DPNH. The charac-

[1] The terminology is in accord with that of Djerassi (1960).

teristics of the Cotton effects obtained by means of these titrations are summarized and compared with those of DPNH itself in Table I.

When the amplitude or the breadth of the Cotton effect recorded for each of the analogues is plotted according to the method of molar proportions, titration curves result which break sharply upon saturation of the enzyme with the analogue (Fig. 2); in each instance precisely 2 moles of coenzyme bind to each mole of liver alcohol dehydrogenase.[2] This stoichiometry is identical to that of DPNH with this enzyme (Theorell and Bonnichsen, 1951), and is in accord with previous spectrophotometric studies for 3-acetylpyridine DPNH (Kaplan *et al.*, 1957).

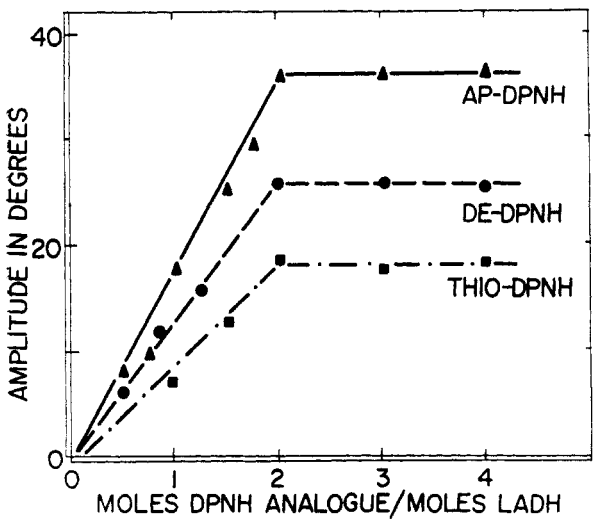

FIG. 2.—Rotatory dispersion titration of liver alcohol dehydrogenase with DPNH analogues. The amplitude of the Cotton effect—the vertical distance in degrees of rotation between the peak and the trough (see Table I)—is plotted against the moles of DPNH analogue added per mole of enzyme. A maximum of 2 moles of each analogue bind to each mole of alcohol dehydrogenase. Conditions: 1.66 mg per ml of enzyme in 0.1 M phosphate, pH 7.5, 10°, titrated with 3-acetylpyridine DPNH (▲—▲), desamino-DPNH (●—●), thionicotinamide DPNH (■···■); the concentrations as indicated in Table I.

It has been shown both by spectrophotometry (Theorell and Bonnichsen, 1951) and by spectropolarimetry (Ulmer *et al.*, 1961b) that at pH 10 only one mole of DPNH binds to each mole of liver alcohol dehydrogenase. By rotatory dispersion titration, only 1 mole of desamino DPNH and 3-acetylpyridine DPNH binds to each mole of the enzyme at pH 10 (Table I). On the basis of such observations a specific group which ionizes below pH 10 has been thought to be involved in binding of DPNH and its analogues to the apoenzyme—perhaps a sulfhydryl group (Theorell and Bonnichsen, 1951). To delineate this possibility better, coenzyme binding by means of rotatory dispersion titration has been determined throughout the pH range 7.5 to 11 (Fig. 3). From pH 7.5 to 9.5, 2 moles of coenzyme bind to each mole of liver alcohol dehydrogenase, while from pH 9.5 to 11, the coenzyme binding capacity of the protein is lost progressively. In the absence of DPNH, exposure of the enzyme to pH 10.5 for 2–3 hours, the period of time required for the titration experiments, does not result in losses of catalytic activity, change in specific rotation, or changes in the dispersion constant. Thus, changes in protein conformation do not seem to be responsible for the loss in coenzyme binding capacity between pH 9.5 and 11.

[2] The magnitude of rotatory power of the Cotton effects of the enzyme with pyridine-3-aldehyde DPNH and desamino-3-acetylpyridine DPNH limits the precision obtainable in determination of the stoichiometry of their interaction by this means.

TABLE I

CHARACTERISTICS OF COTTON EFFECTS OF LIVER ALCOHOL DEHYDROGENASE-DPNH AND LIVER ALCOHOL DEHYDROGENASE DPNH ANALOGUE COMPLEXES[a]

Coenzyme or Coenzyme Analogue	Concentration ($-\times 10^{-5}$ M)	pH	λ_0 (mμ)	Trough λ (mμ)	Trough $-[\alpha]$ (degrees)	Peak λ (mμ)	Peak $-[\alpha]$ (degrees)
3-Acetylpyridine DPNH	6	7.5	359	378	94	340	56
	4	7.5		378	94	340	56
	3	7.5		372	85	346	60
	1	7.5		368	74	358	66
	5	10.0		370	83	352	64
Desamino DPNH	6	7.5	330	350	98	310	73
	4	7.5		349	99	312	74
	2.5	7.5		344	92	317	77
	1.5	7.5		340	89	320	80
	5	10.0		342	92	318	80
Thionicotinamide DPNH	6	7.5	367[b]	384	60	350	42
	4	7.5		385	61	351	43
	3	7.5		377	56	355	43
	2	7.5		373	54	360	47
Desamino-3-acetylpyridine DPNH	6	7.5	363	365	69	355	60
	4	7.5		368	59	359	51
Pyridine-3-aldehyde DNPH DPNH	6	7.5	360	367	55	355	50
	6	7.5	327	357	106	305	50
	4	7.5		355	107	307	50
	3	7.5		350	100	312	60
	1	7.5		342	84	320	73
	5	10.0		347	95	315	65

[a] Liver alcohol dehydrogenase: 1.66 mg per ml in 0.1 M phosphate, 10°. [b] The inflection point, λ_0, of the Cotton effect with thionicotinamide-DPNH occurs at 367 mμ, rather than at 395 mμ, the spectrophotometric absorption maximum. This shift appears to be related to the instability of thionicotinamide-DPNH in the presence of strong ultraviolet illumination required for the measurement.

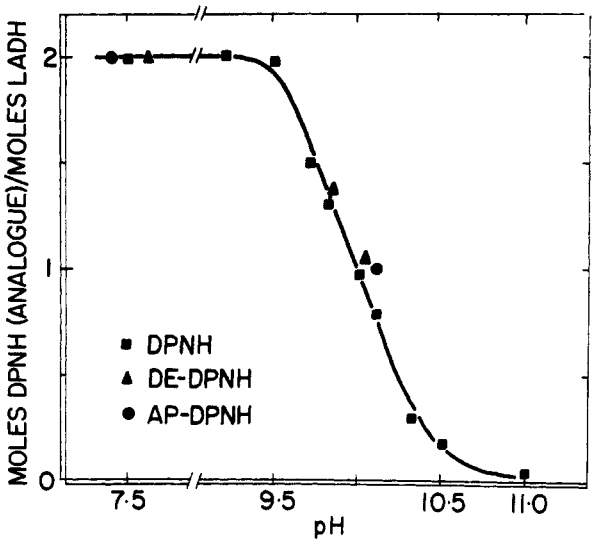

FIG. 3.—The effect of alkaline pH on the binding of DPNH (analogues) to liver alcohol dehydrogenase. The moles of DPNH (analogue) which bind to each mole of enzyme are obtained by rotatory dispersion titration and are plotted against pH. Between pH 9.5 and 11, coenzyme (analogue) binding decreases progressively. Only one mole of DPNH (analogue) binds per mole of enzyme at pH 10, the midpoint of the curve. Conditions: 0.1 M phosphate, 10°, at the indicated pH. 1.66 mg per ml liver alcohol dehydrogenase and DPNH (■—■), desamino-DPNH (▲), 3-acetylpyridine-DPNH (●).

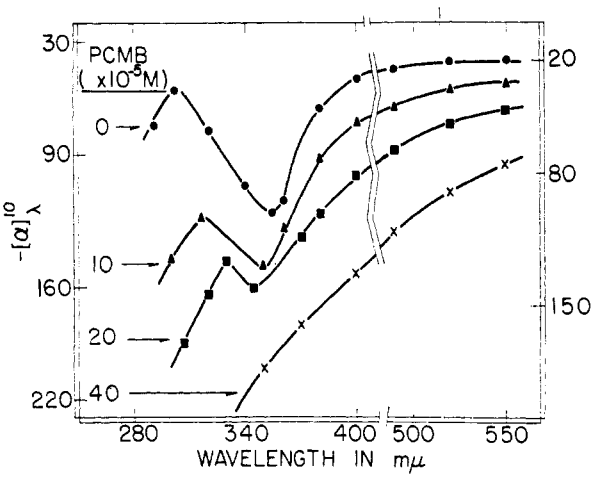

FIG. 4.—The effect of *p*-chloromercuribenzoate on the Cotton effect of the liver alcohol dehydrogenase–DPNH complex. Specific rotation at 10°, $-[\alpha]_{\lambda}^{10}$, is plotted against wavelength. Each increment of *p*-chloromercuribenzoate decreases the magnitude of the Cotton effect until, at 4×10^{-4} M *p*-chloromercuribenzoate, the effect is no longer observed. Concomitantly, the specific rotation of the enzyme and the dispersion constant decrease (see Fig. 5). Conditions: 1.66 mg per ml liver alcohol dehydrogenase and 4×10^{-5} M DPNH in 0.1 M phosphate, pH 7.5, 10° (●—●). 1.66 mg per ml enzyme, 4×10^{-5} M DPNH and 1×10^{-4} M *p*-chloromercuribenzoate (▲—▲), 2×10^{-4} M *p*-chloromercuribenzoate (■—■), 4×10^{-4} M *p*-chloromercuribenzoate (×—×).

The dissociation of the liver alcohol dehydrogenase–DPNH complex by *p*-chloromercuribenzoate has been thought to support the evidence for involvement of a sulfhydryl group in DPNH binding (Theorell and Bonnichsen, 1951). Since *p*-chloromercuribenzoate has recently been found to induce changes in the protein structure of glyceraldehyde-3-phosphate dehydrogenase (Elöidi, 1960) and yeast alcohol dehydrogenase (Snodgrass *et al.*, 1960), the effect of this agent on the binding of DPNH and its analogues to liver alcohol dehydrogenase has been examined by means of rotatory dispersion titration.

In the presence of 4×10^{-5} M DPNH, the titration of 2×10^{-5} M liver alcohol dehydrogenase with *p*-chloromercuribenzoate decreases the magnitude of the Cotton effect of the liver alcohol dehydrogenase–DPNH complex. This decrease is directly proportional to the quantity of added *p*-chloromercuribenzoate (Fig. 4 and 5), and at concentrations of this agent of 4×10^{-4} M and greater, the Cotton effect can no longer be detected. Concomitantly, catalytic activity is inhibited completely. Moreover, after prior exposure of the enzyme to *p*-chloromercuribenzoate, DPNH no longer binds to produce a Cotton effect. Identical results are obtained with coenzyme analogues. These data are in accord, therefore, with previous observations that *p*-chloromercuribenzoate interferes with coenzyme binding by this enzyme.

Measurements of optical rotatory dispersion reveal in addition, however, marked changes in the specific rotation and dispersion constant of the protein. As liver alcohol dehydrogenase is exposed to successively greater *p*-chloromercuri-

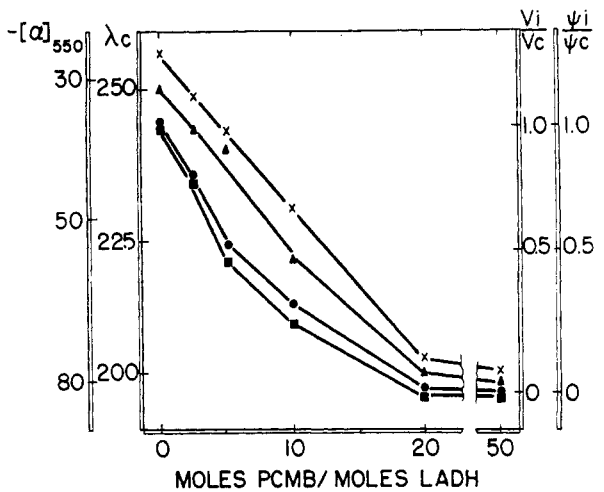

FIG. 5.—The effect of *p*-chloromercuribenzoate concentrations on liver alcohol dehydrogenase and on the liver alcohol dehydrogenase–DPNH complex. The specific rotation, $-[\alpha]_{550}$ (×—×), dispersion constant, λ_c (▲—▲), partial velocity, Vi/Vc (●—●), and partial amplitude of the Cotton effect, $\psi i/\psi c$ (■—■), are plotted against moles *p*-chloromercuribenzoate added per mole enzyme. The specific rotation and dispersion constant of liver alcohol dehydrogenase are obtained in the absence of DPNH. Vc is the activity of the control and Vi, the activity of the enzyme in the presence of *p*-chloromercuribenzoate. ψc is the amplitude of the Cotton effect of the liver alcohol dehydrogenase–DPNH complex in the absence and ψi is the amplitude in the presence of *p*-chloromercuribenzoate. The specific rotation, dispersion constant, partial velocity, and partial amplitude of the Cotton effect all concomitantly decrease in proportion to the moles of *p*-chloromercuribenzoate bound per mole of enzyme. Conditions: 1.66 mg per ml liver alcohol dehydrogenase and 4×10^{-5} M DPNH in 0.1 M phosphate, pH 7.5, 10°.

benzoate concentrations, the specific levorotation increases from approximately −20° to −80° and the dispersion constant progressively decreases from 255 mμ to 210 mμ—changes suggesting alterations in protein conformation.

Both the loss of coenzyme binding capacity, manifest in the loss of catalytic activity and in obliteration of the Cotton effect, and the change in protein structure, reflected by the specific rotation and the dispersion constant, vary in direct proportion to the quantity of *p*-chloromercuribenzoate added (Fig. 5). The end-point of all these alterations, at 20 moles *p*-chloromercuribenzoate/mole alcohol dehydrogenase, is in close agreement with the stoichiometry of the *p*-chloromercuribenzoate–sulfhydryl interaction determined by spectrophotometric titration (Boyer, 1954) of this particular enzyme preparation.

Discussion

Site-specific and selective reagents which *abolish* catalytic activity through the induction of specific, chemical modifications of enzymes have constituted the most widely used methods for the identification of the chemical nature of active enzymatic centers. The studies with analogues of DPNH, pioneered by Kaplan (1960), employ these chemicals as site-specific and selective agents to *induce* enzyme activity: they interact with the active site of apoenzymes, such as liver alcohol dehydrogenase, and the resultant complex is catalytically active. This approach obviates the pitfalls inherent in the interpretation of inhibition experiments, where a variety of mechanisms can lead to the same functional result. The present studies, depending upon the optical activity of the complex at equilibrium, add an additional degree of confidence to deductions possible on the basis of spectral and kinetic data.

The placement of a chromophoric molecule, such as DPNH or its analogues, into an asymmetric environment, here provided by liver alcohol dehydrogenase, renders the absorption band of the chromophore optically active. The resultant Cotton effect is characteristic of the *complex*, which thus has optical properties *qualitatively* distinct from those of its components (Ulmer *et al.*, 1961b). The data here presented confirm that the magnitudes of Cotton effects can be employed to titrate *quantitatively* the total concentration of the complex formed, through rotatory dispersion titration (Li *et al.*, 1961).

The titrations with 3-acetylpyridine DPNH, desamino DPNH, and thionicotinamide DPNH clearly indicate the precision attainable with this method. In all instances, the titration reaches a sharp end-point, precisely at 2 moles of coenzyme analogue per mole enzyme, indicating a stoichiometry of interaction identical to that previously obtained with DPNH (Theorell and Bonnichsen, 1951), and in accord with other spectrophotometric observations (Kaplan *et al.*, 1957).

The capacity of liver alcohol dehydrogenase to induce optical activity in the chromophores of all of the coenzyme analogues studied suggests that all of them are bound asymmetrically. This indicates both that the analogues complement the stereochemistry of the active center and that they possess the groups essential for binding to the active site. Therefore, neither the amino group of the adenine moiety nor the amide group of the nicotinamide side-chain seems to be indispensible for this process (Table I).

As might be expected, these data also reflect the nature of the binding site, as revealed by the pH dependence of the rotatory dispersion titrations. While 2 moles of DPNH/mole of enzyme are bound asymmetrically between pH 7.5 and 9.5, this binding capacity is lost progressively between pH 9.5 and 11. The midpoint of the curve is at about pH 10. Similar pH dependence of the rotatory dispersion titrations has been obtained for desamino DPNH and 3-acetylpyridine DPNH (Fig. 3). Presumably a specific group either of the enzyme or of DPNH (analogues) is being titrated. No groups of DPN have been found which are titratable between pH 5 and 10 (Shifrin and Kaplan, 1960). It is most probable, therefore, that such a group is part of the active site of the apoenzyme.

In this regard, the participation of sulfhydryl groups in coenzyme binding has previously been suggested (Theorell and Bonnichsen, 1951). Their involvement has been deduced from the change in stoichiometry of the liver alcohol dehydrogenase–DPNH complex as a function of pH, from inhibition of the enzyme by *p*-chloromercuribenzoate, and from the shift in the absorption maximum upon addition of *p*-chloromercuribenzoate (from 327 mμ, typical of the liver alcohol dehydrogenase–DPNH complex, to 340 mμ, typical of free DPNH). However, the changes in optical rotation observed on exposure of glyceraldehyde-3-phosphate dehydrogenase to *p*-chloromercuribenzoate suggested to Elöidi (1960) that this agent causes time-dependent changes in the enzyme which he interpreted to denote denaturation. It seemed appropriate, therefore, to reexamine the effect of *p*-chloromercuribenzoate on the liver alcohol dehydrogenase system from this point of view.

The addition of increments of *p*-chloromercuribenzoate to alcohol dehydrogenase results in largely irreversible losses of activity, marked increases in specific levorotation, and concomitant decreases in the dispersion constant—changes of a magnitude and direction ordinarily interpreted as indicative of protein denaturation (Schellman and Schellman, 1958; Blout, 1960). Furthermore, these correlated alterations become maximal at 20 moles *p*-chloromercuribenzoate/mole alcohol dehydrogenase, identical to the number of sulfhydryl groups titrated spectrophotometrically with this agent on this particular enzyme preparation.[3]

These measurements of the interaction of *p*-chlo-

[3] It should be noted that as many as 28 sulfhydryl groups have been found in preparations of liver alcohol dehydrogenase prepared by a different procedure (Witter, 1960). The experimental problems encountered in establishing precise end-points for sulfhydryl titrations and the variable numbers of sulfhydryl groups found in different protein preparations by different investigators may perhaps be due to different degrees of denaturation.

romercuribenzoate with liver alcohol dehydrogenase by optical rotatory dispersion are consistent with Elöidi's observations on glyceraldehyde-3-phosphate dehydrogenase by specific rotation, lending additional support to his suggestion that *p*-chloromercuribenzoate may induce profound changes in protein structure. It would appear that the conformation of this enzyme is maintained, at least in part, by sulfhydryl groups, though the manner in which this is brought about is not elucidated by the present studies.

These circumstances, in fact, complicate the interpretation of the effect of *p*-chloromercuribenzoate on the mechanism of DPNH binding to alcohol dehydrogenase. Theorell and Bonnichsen (1951) established long ago that *p*-chloromercuribenzoate dissociates the liver alcohol dehydrogenase–DPNH complex once formed, a fact which Witter (1960) reconfirmed. The abolition of the DPNH Cotton effect by *p*-chloromercuribenzoate (Fig. 4) is completely consistent with these observations, of course. The measurements of optical rotatory dispersion, however, bear upon the *mechanism* which results in dissociation. The rotational changes suggest that structural alteration of the protein may contribute to the dissociation of the complex, possibly through destruction of the asymmetry of the zinc-containing binding site. A sulfhydryl group or groups may be involved directly in coenzyme binding at the active site; alternately, they may exert indirect effects on coenzyme binding and catalytic activity through changes in protein conformation. It is not possible at present to differentiate between these and perhaps other mechanisms.

These results would appear to be pertinent to the interpretation of interactions of *p*-chloromercuribenzoate with sulfhydryl groups in general. *p*-Chloromercuribenzoate is ordinarily employed as a site-specific and selective reagent, and of all the amino acid side-chains, sulfhydryl groups, because of their highly specific reactivity, have been particularly accessible to study with this and similar reagents (Fraenkel-Conrat, 1960). While a certain degree of discrimination is possible among sulfhydryl groups on the basis of the *rate* of their reactivity with *p*-chloromercuribenzoate (Swenson and Boyer, 1957), a decision as to the *functional* implications of such kinetic differences is not always easy. The present observations by means of optical rotatory dispersion suggest caution in the interpretation of kinetic data when *p*-chloromercuribenzoate is thought to act solely as a site-specific reagent interacting selectively with functional sulfhydryl groups not thought to affect protein structure. It would seem advisable to ascertain the absence of changes in rotatory dispersion prior to attributing modifications in function to alterations of a specific functional group.

References

Anderson, B. M., and Kaplan, N. O. (1959), *J. Biol. Chem. 234*, 1226.

Blout, E. R. (1960), Optical Rotatory Dispersion, Djerassi, C., ed., New York, McGraw-Hill Book Company, p. 238.

Bonnichsen, R. K. (1950), *Acta Chem. Scand. 4*, 715.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 73*, 4331.

Djerassi, C., ed. (1960), Optical Rotatory Dispersion, New York, McGraw-Hill Book Company.

Ehrenberg, A., and Dalziel, K. (1958), *Acta Chem. Scand. 12*, 465.

Elöidi, P. (1960), *Biochim. Biophys. Acta 40*, 272.

Fraenkel-Conrat, H. (1960), *in* The Enzymes, ed. 2, vol. I, Boyer, P. D., Lardy, H., and Myrbäck, K., ed., New York, Academic Press, Inc., p. 589.

Hughes, T. R., and Klotz, I. M. (1956), *in* Methods of Biochemical Analysis, vol. 3, Glick, D., ed., New York, Interscience Publishers, Inc., p. 265.

Kaplan, N. O. (1960), *in* The Enzymes, ed. 2, vol. 3, Boyer, P. D., Lardy, H., and Myrbäck, K., ed., New York, Academic Press, Inc., p. 105.

Kaplan, N. O., Ciotti, M. M., and Stolzenbach, F. E. (1957), *Arch. Biochem. Biophys. 69*, 441.

Koshland, D. E., Jr. (1960), *in* Advances in Enzymology, vol. 22, Nord, F. F., ed., New York, Interscience Publishers, Inc., p. 45.

Li, T. K., Ulmer, D. D., and Vallee, B. L. (1961), Abstracts of Papers, 139th Meeting of the American Chemical Society, p. 22C.

Rafter, G. W., and Colowick, S. P. (1957), *in* Methods in Enzymology, vol. 3, Colowick, S. P., and Kaplan, N. O., ed., New York, Academic Press, Inc., p. 887.

Rudolph, H. (1955), *J. Opt. Soc. Am. 45*, 50.

Schellman, C. G., and Schellman, J. A. (1958), *Compt. rend. trav. lab. Carlsberg. Ser. chim. 30*, 463.

Shifrin, S., and Kaplan, N. O. (1960), *in* Advances in Enzymology, vol. 22, Nord, F. F., ed., New York, Interscience Publishers, Inc., p. 337.

Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochem. Biophys. 82*, 288.

Snodgrass, P. J., Vallee, B. L., and Hoch, F. L. (1960), *J. Biol. Chem. 235*, 504.

Swenson, A. D., and Boyer, P. D. (1957), *J. Am. Chem. Soc. 79*, 2174.

Theorell, H., and Bonnichsen, R. (1951), *Acta Chem. Scand. 5*, 1105.

Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961a), *Fed. Proc. 20*, 222.

Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961b), *Proc. Nat. Acad. Sci. U. S. 47*, 1155.

Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961c), Abstracts of the Vth International Congress of Biochemistry, Moscow, Russia, p. 128 (5.156).

Ulmer, D. D., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 730.

Vallee, B. L., and Hoch, F. L. (1955), *Proc. Nat. Acad. Sci. U. S. 41*, 327.

Witter, A. (1960), *Acta Chem. Scand. 14*, 1717.

Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.