samples. The maximum variation in Soret absorption was 7%, and there was no correlation between the small differences in absorption and the differences in activity.

As a final complication, we should note that there was a difference in the distribution of *visible* light absorption between the three peaks of Lots 5457 and 5458, as observed in the sedimentation diagrams. In Lot 5458 absorption was associated with the leading peak only, whereas all three species absorbed light in Lot 5457.

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Spectropolarimetric Studies on Proteins. Bovine Plasma Albumin and Insulin

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Optical rotatory dispersion curves from which the contributions of the sulfur-containing amino-acids were subtracted have been obtained for insulin and bovine plasma albumin. Drude equation and Moffitt equation constants derived from these curves have been calculated. Similar data on these proteins after cleavage of disulfide bonds provide a comparison of effects of the cleavage itself on optical rotatory properties of the proteins; application of two different methods of cleavage provides a comparison of effects of specific procedures, as well. Measurements on all materials were made in two different solvent systems. The selected solvents represent different extremes in terms of the conformational behavior of protein molecules. The presence of zinc in the insulin molecule is shown to have an appreciable effect on its optical rotatory properties.

Considerable emphasis is presently being placed on the measurement and interpretation of optical rotatory dispersion curves of proteins as an aid in the evaluation of the secondary and tertiary structural features of their configurations. In particular, the work of Moffitt,[1-3] Doty,[4-6] Yang[2,4,5], Blout,[7-9] and others involving optical rotatory dispersion measurements has shown interesting correlations with the apparent helical nature of polypeptides and proteins in solution.

A complicating feature of the analysis of optical rotatory dispersion curves is the necessity for separation of the contributions to the optical rotatory dispersion of the ordered secondary structure itself from those of the amino acid constituents of the protein. A working concept of these contributions is one which consists of a consideration of the anomalous character of the dispersion curve as being due to the ordered structure while that of the intrinsic rotatory contributions of the amino acid residues is thought of as being simple dispersion. A difficulty in utilizing this concept was pointed out by Turner, Bottle and Haurowitz,[10] who demonstrated the effect of oxidation of cystine bridges in albumin on the optical rotation values at the D-line (5890 Å.). In this case the anomalous rotatory behavior of certain of the amino acid constituents themselves namely cystine, can be interpreted as contributing to the over-all anomalous rotatory characteristic of this protein.

Since the studies mentioned above were carried out in a solvent (88% formic acid) in which helix formation is *not* believed to be favored, the results might be misleading in the sense that the implication is that the oxidation of cystine bridges does not alter the helical content of the protein. Of course, one expects the helical content to be virtually zero either before or after oxidation of *albumin* in 88% HCOOH; however, a more rigid structure such as that found in insulin might exhibit significant changes in secondary order on cleavage of –S–S– bonds even in formic acid. The problem also arises regarding results of such oxidative procedures in solvents wherein the formation of a helix *is* favored.

It is also possible that the *manner* in which the disulfide bonds are cleaved could have some effect on the optical rotatory dispersion, particularly if the reaction conditions could permit interaction of some groups other than –S–S–; if the hypothesis of Turner, *et al.*, is supported, however, the manner of cleavage should not cause significant alteration of rotatory properties *if the disulfide bond contributions and those of their conversion products are first subtracted* (assuming no other changes in the molecule).

In order to get a more complete picture of the optical rotatory changes which take place, the proteins in this study—bovine plasma albumin and insulin—were examined spectropolarimetrically; thus the continuous optical rotatory dispersion curves from 300 to 589 mμ were obtained, rather than $[\alpha]_D$ values alone. Likewise to make some interpretation of the effect of the manner of cleavage on the rotatory changes seen, the –S–S– bonds of these proteins were cleaved reductively with sulfite as well as oxidatively with performic acid in separate experiments.

The oxidation of –S–S– bonds with performic acid

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(10) J. E. Turner, R. T. Bottle and F. Haurowitz, *ibid.*, **80**, 4117 (1958).

is an irreversible procedure, but the sulfite cleavage is more or less reversible (depending on the environment of the bridge).[11] For this reason, the possibility of re-formed protein or newly-formed bridges in the isolated reaction product could not be excluded. Amperometric titration, however, of the parent proteins and cleavage products after they had been isolated, lyophilized, and stored several months under refrigeration indicated disulfide contents of only 0.051 mole per mole of sulfite-cleaved insulin and no measurable residual disulfide content of sulfite-cleaved bovine plasma albumin. The same procedure[12] yielded values of 3.10 moles of disulfide per mole of insulin and 17.1 moles per mole of bovine plasma albumin while their performic acid-oxidized counterparts both exhibited zero measurable disulfide contents.

As a basis for the comparison of optical rotatory changes which have occurred, the *residual* optical rotatory dispersion curves of the native proteins and their oxidized and sulfite-cleaved counterparts have been obtained. These values were arrived at by selecting specific rotation values from the observed dispersion curves at each of twelve wave lengths and subtracting the apparent rotatory contribution of the S-containing amino acid at that same wave length. The result was a 12-point o.r.d. curve for that protein *minus* the contribution for any S-containing amino acid. It may be somewhat naive to presume that the o.r.d. contribution of the amino acid residue *in the protein* is the same as it is in the free amino acid state, but Würz and Haurowitz,[13] have shown this to be approximately true for cystine and its conversion to cysteic acid or S-sulfocysteine in a variety of proteins under varying conditions when rotation measurements were made at the D-line only.

Experimental

To help answer the question of solvent effects on the optical rotatory properties of the materials examined, two different extremes of conditions with respect to the hydrogen bonding relationships of solvent and solute were examined. On the one hand, 88% formic acid, in which hydrogen bonding of solvent molecules to solute would be expected to be favored was used; on the other, a solvent composed mainly of 2-chloroethanol—known to favor a more ordered configuration and, presumably, intramolecular hydrogen-bonding of polypeptide solutes[14]—was employed. It was found necessary to modify the latter solvent by addition of small amounts of trifluoroacetic acid and water in order to obtain sufficient solubility of some of the materials examined. The final composition of the solvent was 90% $ClCH_2CH_2OH$, 5% CF_3COOH and 5% H_2O. In this case, a compromise in conditions had to be accepted since greater order in the protein configuration appears to be associated with decreased solubility—at least in solvents which appear to favor intramolecular hydrogen bonding.

It is probable that some interaction between solvent and solute occurs in these solvent systems. In any event, the comparison of parent protein and its oxidized and reduced forms is made under exactly the same solvent conditions for all three materials. The effect of such alteration of optical rotatory properties as may be attributed to the reaction of the protein with the solvent should thus be minimized.

A. Materials. 1. Proteins.—(a) Zinc-insulin [I(Zn)], lot no. 708850 (Eli Lilly and Co.), was used for nearly all studies. A few comparisons with other manufactured lots were made to assure consistency of optical rotatory dispersion data from lot to lot. The zinc content of lot 708850 was 0.56% on an anhydrous basis; moisture content was 3%. Cleavage experiments were conducted with this material. (b) Zinc-free insulin [I(Zn-free)], lot no. 683300 (Eli Lilly and Co.), was used as a reference material only. Moisture content was 4.7% and Zn content was 0.0061%. (c) Oxidized insulin [OI] was prepared by performic acid oxidation according to Sanger.[15] Zinc content of the lyophilized product was 0.48%. (d) Sulfite-cleaved insulin [SO_3-I]: the method of Bailey and Cole[16] was used to prepare this material. The lyophilized product contained only 0.0016% Zn. (e) Bovine plasma albumin [BPA]: Armour lot no. U68712 and T68204 were used interchangeably after satisfactory comparison of the optical rotatory dispersion curves of the original samples. Oxidized [OBPA] and sulfite-cleaved [SO_3-BPA] albumin preparations were made using essentially the same techniques as were employed for insulin; purification of OBPA involved repeated precipitation with 5 *M* NaCl rather than acetone, however.

2. Amino Acids.—(a) L-Cystine, Merck reagent; (b) cysteic acid, Eastman Kodak Co. (E93–18 spec. list); (c) S-sulfocysteine, gift of Prof. F. Haurowitz, synthesized (as Na Salt) by method of Clarke.[17]

3. Solvents.—(a) Formic acid 88–90%, Baker and Adamson, reagent; (b) 2-chloroethanol, Eastman Kodak Co., redistilled; (c) trifluoroacetic acid, Eastman Kodak Co.

B. Apparatus.—Optical rotatory dispersion data were obtained using a model 260 Rudolph recording spectropolarimeter at the "medium" sensitivity range. The reproducibility of the observed rotations is approximately ±0.01° on this position. Samples of the various amino acids and proteins were measured in their respective solvents at either 0.5 or 1.0% concentration in 10-cm. or 1-cm. cells. The shorter path lengths were used in the shorter wave length regions of rotation when the observed rotatory values were sufficiently high to avoid gross error factors. The light absorption of the proteins and of the solvents prevented accurate measurements below 300 mμ in most cases. As the studies described here progressed, a great many solvents and solvent mixtures were examined and subsequently discarded; the criterion for acceptance was the fact that the parent proteins, their oxidation products, their sulfite-cleavage products, and the individual S-containing amino acids involved had to be soluble therein.

Calculations.—As a method for evaluating the optical rotatory changes which have occurred under the different solvent conditions and independent of the S-amino acid contributions, both single-term Drude[18] equations and Moffitt[3] equations of the "reduced" o.r.d. curves have been evaluated. The one-term Drude equations $[\alpha] = k/(\lambda^2-\lambda_c^2)$ were evaluated graphically for k and λ_c (intercept and slope, respectively) from plots of $[\alpha]$ *vs.* λ^2 $[\alpha]$. The more complex Moffitt equation

$$[m'] = \frac{3}{n^2+2} \times \frac{M_0}{100} \times [\alpha] = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

(where n = refractive index, M_0 = average residue wt., $[m']$ = effective residue rotation) was treated empirically as suggested by Doty.[19] The evaluation was made first in the conventional manner using λ_0 = 2100 Å. for all the data. This was done through a modification of the graphical approach used by Moffitt and Yang[2]; in the present study, however, some of the tedious aspects of the graphical evaluation were avoided by using a computer program. Thus it was possible automatically to convert the twelve pairs of $[\alpha]$ *vs.* λ data to values for $1/(\lambda^2-\lambda_0^2)$ and $[m'](\lambda^2-\lambda_0^2)$ and to compute the slope and intercept for the best straight line through the data points. When the slope and intercept terms, $b_0\lambda_0^4$ and $a_0\lambda_0^2$, were evaluated, values for a_0 and b_0—constants believed to be related to secondary structural characteristics and/or solvent–solute interaction effects—were obtained.

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TABLE I

OPTICAL ROTATORY DISPERSION DATA ([α]) IN Å.

Sample	5890 FA[a]	5890 2C[a]	5500 FA	5500 2C	5000 FA	5000 2C	4500 FA	4500 2C	4250 FA	4250 2C	4000 FA	4000 2C
BPA	− 82°	− 28°	− 94°	− 34°	−118°	− 48°	−159°	− 68°	−189°	− 85°	−219°	−112°
OBPA	− 68	− 16	− 82	− 18	−106	− 24	−136	− 34	−159	− 44	−191	− 62
SO_3–BPA	− 62	− 13	− 84	− 16	−110	− 26	−144	− 40	−168	− 53	−200	− 74
I (Zn)	− 80	− 41	− 94	− 51	−117	− 71	−155	− 96	−180	−113	−212	−138
I (Zn-free)	− 70	− 38	− 85	− 47	−113	− 63	−145	− 85	−167	− 99	−201	−121
O–I	− 50	− 19	− 63	− 24	− 83	− 33	−107	− 44	−125	− 54	−147	− 69
SO_3–I	− 58	− 24	− 72	− 22	− 93	− 30	−121	− 46	−140	− 56	−166	− 72
L-Cystine	−259	−256	−316	−300	−409	−386	−513	−486	−589	−556	−692	−652
L-Cysteic acid	+ 13	+ 64	+ 14	+ 70	+ 17	+ 78	+ 24	+ 90	+ 29	+ 98	+ 37	+104
S-Sulfocysteine	− 84	− 81	− 92	−100	−123	−126	−162	−156	−184	−178	−206	−209

Sample	3800 FA	3800 2C	3600 FA	3600 2C	3400 FA	3400 2C	3200 FA	3200 2C	3100 FA	3100 2C	3000 FA	3000 2C
BPA	−255°	−139°	−304°	−181°	−366°	−245°	−451°	−343°	−499°	−420°	−590°	−540°
OBPA	−225	− 82	−290	−108	−330	−140	−390	−220	−460	−280	−550	−340
SO_3–BPA	−232	− 98	−276	−133	−342	−189	−426	−280	−488	−342	−520	−435
I (Zn)	−245	−167	−292	−208	−349	−266	−427	−370	−457	−430	−500	−510
I (Zn-free)	−232	−145	−277	−182	−336	−234	−414	−320	−450	−380	−540	−470
O–I	−174	− 85	−209	−106	−251	−132	−310	−190	−360	−230	−410	−290
SO_3–I	−195	− 92	−232	−118	−279	−162	−337	−226	−400	−270	−460	−324
L-Cystine	−785	−744	−912	−868	−1126	−1026	−1277	−1198	−1410	−1320	−1600	−1520
L-Cysteic acid	+ 44	+118	+ 58	+138	+ 75	+164	+101	+206	+121	+240	+148	+278
S-Sulfocysteine	−232	−237	−269	−276	−326	−318	−391	−368	−399	−420	−470	−470

[a] Solvent FA = 88% HCOOH; solvent 2C = 90% $ClCH_2CH_2OH$, 5% CF_3COOH, 5% H_2O.

The computer program further afforded the opportunity to verify easily the most effective linearization of the data in terms of λ_0. The Moffitt equations for a particular set of o.r.d. data were calculated for λ_0 values from 1800 to 2900 Å. at 100-Å. intervals; the interaction standard deviation of the x and y parameters ($x = 1/\lambda^2 - \lambda_0^2$) and $y = [m'](\lambda^2 - \lambda_0^2$) was also automatically deduced and expressed as $S^2_{x,y}$; the best linear fit of the data was taken as that value of λ_0 for which $S^2_{x,y}$ was a minimum. It was found that for many of the "reduced" o.r.d. curves as well as for some of the uncorrected ones, λ_0 for the most linear plots of the parameters was not 2100 Å. When the equations containing $\lambda_0 \neq 2100$ Å. were solved for their constants, values of $A\lambda_0$ and $B\lambda_0$ were obtained (to distinguish them from a_0 and b_0 since the identity of the latter with $\lambda_0 = 2100$ Å. has already been established). As may be noted from the data listed in Table I, the selection of wave lengths has been weighted to favor the ultraviolet region. In general, the precision of these measurements is greater than the longer wave length values; they more clearly reflect the approach to the region of anomalous rotatory behavior. The procedure is much more analogous to the graphical method than would appear to be the case if equal wave length intervals had been utilized.

The observation that Moffitt equation data may sometimes to be more appropriately fitted to a linear plot with values of λ_0 other than 2120 Å. has been made by others[20,21]; this fact has a significant bearing on the utilization of the b_0 term for expressing degree of order among helical molecules. Under these circumstances, $b_0(\lambda_0 = 2100$ Å.) is not a constant since the Moffitt plot is not truly linear. On the other hand, while $B\lambda_0$ values derived from equations in which $\lambda_0 \neq 2100$ Å. are constants, they cannot be simply related to each other unless the coefficients being compared were obtained using the same λ_0; thus for a given set of λ vs. $[m']$ values, the magnitude of $B\lambda_0$ is some function of λ_0. In qualitative terms, the results suggest that some chromophoric contributions are made by the secondary order or spatial asymmetry of the molecule itself; changes in this secondary order are seen as changes in the frequency terms of linear Moffitt and Drude equations. It is fairly obvious from recent discussions by Schellman and Schellmann[22] that the optical rotatory dispersion of proteins such as albumin and insulin can be expected to be a composite of the contribution of several structural types; the fact that evaluation of equations representing only one type of secondary structure yields inconsistencies is not surprising.

Since the comparison of optical rotatory constants in these experiments is relative to the constants of the parent protein, it is not necessary to assign quantitative significance to them. Thus, although b_0 values near −630 are indicative of essentially helical structures in synthetic polypeptides and in some proteins, the numerical values obtained here do not necessarily indicate a "per cent. helix" based on the fraction obtained by dividing the b_0 for the particular protein by −630. The interpretation of results depends only on the order of magnitude and direction of change of the constants a_0, b_0, λ_0, $A\lambda_0$ and $B\lambda_0$. It must be recognized that comparisons of different b_0, λ_0 and $B\lambda_0$ terms is made only with regard to the similarity or lack of similarity of behavior of these terms as a result of the transformation of the protein through the two different cleavage paths.

Results and Conclusions

Table II and III report constants for the Moffitt equation evaluations, both uncorrected and with the S-containing amino acid contribution subtracted; the former contains a_0 and b_0 evaluations in the conventional manner while the latter shows results of selection of λ_0 to give the best linear fit. The values of $A\lambda_0$ and $B\lambda_0$ in Table III correspond to a_0 and b_0 when the λ_0 selected is 2100 Å. Table IV contains constants derived for one-term Drude equations of the curves of the same proteins.

There are certain general conclusions which can be drawn from a comparison of these values: 1. The differences between the optical rotatory dispersion curves of bovine plasma albumin and its oxidatively and reductively cleaved counterparts do not appear to be completely accounted for by the differences in S-containing amino acid contribution—even in a solvent favoring the random configuration of the peptide secondary structure. The $B\lambda_0$ values in Table III show excellent agreement for BPA and OBPA, but are obtained from two different values of λ_0. Since both λ_0's are quite different from 2100 Å., the b_0's are probably not too useful for comparison purposes, either. The Drude equation constants also indicate some differences among the preparations not accounted for by subtracting the S-containing amino acid contributions.

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TABLE II

MOFFITT EQUATION EVALUATION (λ_0 = 2100 Å.)

Difference curves (S-amino acids subtracted)						Uncorrected curves					
Bovine plasma albumin			Insulin			Bovine plasma albumin			Insulin		
A. Formic acid	a_0	b_0	A. Formic acid	a_0	b_0	A. Formic acid	a_0	b_0	A. Formic acid	a_0	b_0
BPA	−392	− 66	I (Zn)	−288	−12	BPA	−492	−54	I (Zn)	−500	+15
OBPA	−419	−100	I (Zn-free)	−229	−85	OBPA	−417	−92	I (Zn-free)	−445	−58
SO_3–BPA	−372	−108	O–I	−329	−88	SO_3–BPA	−422	−98	O–I	−320	−69
			SO_3–I	−263	−81				SO_3–I	−365	−62
B. Chloroethanol	a_0	b_0	B. Chloroethanol	a_0	b_0	B. Chloroethanol	a_0	b_0	B. Chloroethanol	a_0	b_0
BPA	−13	−430	I (Zn)	− 29	−296	BPA	−110	−396	I (Zn)	−226	−266
OBPA	−67	−277	I (Zn-free)	− 4	−264	OBPA	− 39	−288	I (Zn-free)	−201	−234
SO_3–BPA	+32	−416	O–I	−149	−155	SO_3–BPA	− 20	−404	O–I	−93	−175
			SO_3–I	+ 14	−246				SO_3–I	− 87	−230

TABLE III

MOFFITT EQUATION EVALUATION

Difference curves (S-amino acids subtracted)									Uncorrected curves							
Bovine plasma albumin				Insulin					Bovine plasma albumin				Insulin			
A. Formic acid	λ_0,Å.	$A\lambda_0$	$B\lambda_0$	A. Formic acid	λ_0,Å.	$A\lambda_0$	$B\lambda_0$		A. Formic acid	λ_0,Å.	$A\lambda_0$	$B\lambda_0$	A. Formic acid	λ_0,Å.	$A\lambda_0$	$B\lambda_0$
BPA	2500	−272	+36	I (Zn)	2600	−175	+29		BPA	2400	−375	+46	I (Zn)	2500	−338	+62
O–BPA	2600	−266	+36	I (Zn-free)	2600	−152	+12		O–BPA	2600	−263	+36	I (Zn-free)	2500	−306	+44
SO_3–BPA	2700	−213	+27	O–I	2600	−209	+27		SO_3–BPA	2700	−238	+32	O–I	2600	−201	+34
				SO_3–I	2600	−169	+21						SO_3–I	2500	−254	+34
B. 2-Chloroethanol	λ_0,Å.	$A\lambda_0$	$B\lambda_0$	B. 2-Chloroethanol	λ_0,Å.	$A\lambda_0$	$B\lambda_0$		B. 2-Chloroethanol	λ_0,Å.	$A\lambda_0$	$B\lambda_0$	B. 2-Chloroethanol	λ_0,Å.	$A\lambda_0$	$B\lambda_0$
BPA	2200	−27	−275	I (Zn)	2100	− 29	−295		BPA	2200	−115	−257	I (Zn)	2100	−226	−266
O–BPA	2400	−75	− 66	I (Zn-free)	2300	− 21	−114		OBPA	2300	− 52	−120	I (Zn-free)	2300	−182	− 79
SO_3–BPA	2100	+32	−416	O–I	2400	−125	− 23		SO_3–BPA	2100	− 20	−404	O–I	2200	− 92	−111
				SO_3–I	2100	+ 14	−246						SO_3–I	2200	− 87	−147

TABLE IV

DRUDE EQUATION EVALUATION

Difference curves (S-amino acids subtracted)						Uncorrected curves					
Bovine plasma albumin			Insulin			Bovine plasma albumin			Insulin		
A. Formic acid	k	λ_c, mμ	A. Formic acid	k	λ_c, mμ	A. Formic acid	k	λ_c, mμ	A. Formic acid	k	λ_c, mμ
BPA	−0.98	224	I (Zn)	−0.68	219	BPA	−1.19	221	I Zn)	−1.04	218
O–BPA	−1.07	227	I (Zn-free)	− .65	232	O–BPA	−1.08	229	I (Zn-free)	−1.07	219
SO_3–BPA	−1.01	228	O–I	− .86	225	SO_3–BPA	−1.12	229	O–I	−0.82	225
			SO_3–I	− .72	231				SO_3–I	−0.91	225
B. 2-Chloroethanol	k	λ_c, mμ	B. 2-Chloroethanol	k	λ_c, mμ	B. 2-Chloroethanol	k	λ_c, mμ	B. 2-Chloroethanol	k	λ_c, mμ
BPA	−0.41	289	I (Zn)	−0.38	281	BPA	−0.58	276	I (Zn)	−0.86	260
O–BPA	− .47	279	I (Zn-free)	− .25	288	O–BPA	− .36	284	I (Zn-free)	− .75	258
SO_3–BPA	− .31	292	O–I	− .40	257	SO_3–BPA	− .43	286	O–I	− .41	265
			SO_3–I	− .20	292				SO_3–I	− .45	270

2. If one considers the corrected curves in the chloroethanol solvent it is again apparent that the behavior of the solute molecules involves the production of optical rotatory changes (asymmetric environment) beyond those which may be accounted for in terms of the cleavage of disulfide bonds. The importance of solvent effects on the secondary structure of proteins in solution has been vividly presented by others, particularly Yang and Doty[5] for such substances as silk fibroin and ribonuclease.

3. An important consideration in the case of insulin is the presence or absence of zinc in the molecule. Some alteration in secondary structure as measured by o.r.d. seems evident when one compares the constants derived for the Moffitt equation or the Drude equation for zinc-insulin and for zinc-free insulin. Possible implications are that Zn-binding takes place with some distortion of the polypeptide "backbone" or that the chelate chromophore effect on a nearby asymmetric center is quite marked. The suggested binding of Zn intermolecularly through the histidine residues to form the 12000 mol. wt. unit would not appear to be favored by the solvent conditions existing in these experiments.

4. Cleavage of disulfide bonds of bovine plasma albumin might be expected to produce changes in secondary order which are of the same magnitude and direction without regard to the method of cleavage. Thus the conversions BPA → OBPA and BPA → SO_3-BPA should both presumably result in reaction products having the same type of alteration of secondary structure. The assumption is made that the contributions of the S-containing amino acids to the optical rotatory dispersion curves can, in fact, be subtracted from the observed curves and a valid "corrected" o.r.d. curve obtained. One would then expect the optical rotatory constants obtained from the equations for these corrected curves to reflect a similarity in magnitude and direction of change for the values of the constants which are related to the degree of order in the secondary structure. The experimental

evidence indicates, however, that both the Drude equation constant (λ_c) and the Moffitt equation constant (b_0) are not altered in a manner which would suggest close similarity of secondary structural change for the two cleavage products with respect to the parent protein. The alterations of the λ_0 value producing the best linear fit of the data as noted in Table III for these transformations are also different for the two types of cleavage.

5. Similarly, expectation of structural changes ensuing upon cleavage of the disulfide bonds of insulin are not fulfilled experimentally. The conversions I(Zn) → OI and I(Zn-free) → SO_3-I produce changes in λ_c, b_0 and λ_0 for the corrected curves which are not similar with respect to the native molecule (it is necessary to use the Zn free insulin values in the one case since SO_3-I does not contain appreciable Zn). The cleavage of the disulfide bridges in insulin results in a dissociation of the A and B chains, of course; this is properly an alteration in tertiary structure, but could conceivably have an unaccounted effect on the optical rotatory dispersion of the system.

6. Basically, there does not appear to be any real over-all improvement in correlation of constants for the *corrected* curves over that for the *uncorrected* ones. One must conclude that if a common secondary structural characteristic does exist exclusive of the disulfide bond contribution, it is not manifest in a common set of optical rotatory constants derived from the Moffitt or one-term Drude equations.

7. It is further evident that one cannot *a priori* account for optical rotatory changes observed when the disulfide bonds of a protein are cleaved solely in terms of the rotatory changes occurring in the S-containing amino acid residues. The observed o.r.d. changes are a function both of the solvent and of the manner of cleavage. The changes in secondary structural order which must occur when these bonds are cleaved obviously *do* have a significant effect on the optical rotatory dispersion. A more satisfactory interpretation of this relationship awaits a more detailed evaluation of the contributions of the various segments of a protein molecule.

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The Reaction of *p*-Nitrophenyl Acetate with Thiols[1,2]

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Cysteine, and other thiols studied, hydrolyze *p*-nitrophenyl acetate at a rate dependent upon the concentration of anion, RS^-, present. The initial products of the reaction are *p*-nitrophenol and thiol ester as determined spectrophotometrically. E_a, ΔH^*, ΔF^* and ΔS^* for the reaction of cysteine with *p*-nitrophenyl acetate are 8.0, 7.4, 16.7 kcal./mole and −30.7 e.u., respectively.

Introduction

During investigation of hydrolysis of *p*-nitrophenyl acetate (NPA) by the proteolytic enzyme, ficin, it was found that cysteine, added as an activator of ficin, could also hydrolyze this ester. Previously, reduced glutathione had been found to hydrolyze *p*-nitrophenyl benzoate in essentially neutral solution.[3] Dirks and Boyer[4] reported that cysteine splits NPA by a reductive process. While the present investigation was in progress Schonbaum and Bender[5] reported the results of a study of the catalyzed hydrolysis of NPA by *o*-mercaptobenzoic acid.

The present study was undertaken to determine the mechanism by which cysteine hydrolyzes NPA with the hope that it might aid in understanding the mechanism by which such sulfhydryl enzymes as ficin and papain catalyze the hydrolysis of susceptible substrates.

Experimental

Materials.—*p*-Nitrophenyl acetate was prepared by the method of Chattaway[6] and had a melting point of 82.4–83.1° (melting point block). Reagent grade *p*-nitrophenol (NP) (Fisher) was dried at 72° in a vacuum oven for 18 hours for standard curve preparation. *n*-Propyl mercaptan was Eastman Kodak Co. white label product. The other compounds tested were obtained from Nutritional Biochemicals Corporation. Ficin was a three-times acetone-precipitated (80% v./v.) vacuum-dried powder prepared from Kadota fig latex in this Laboratory. Deionized water (Barnstead mixed-bed resin) was used throughout this work.

Methods.—Reactions were followed spectrophotometrically in a DU spectrophotometer equipped with a thermostated cell compartment. Temperatures were determined inside the reaction cuvette with thermocouples. In most cases, the rate of appearance of *p*-nitrophenolate at 402 mμ was followed; the rate of disappearance of NPA measured at 272 mμ gave identical results. The rate of formation of thiol ester was followed at 240.7 mμ, the isosbestic point of NPA and NP. The reactions were carried out in the presence of 0.064 *M* phosphate buffers and 5.0% (v./v.)

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