Conformational States of Native and Denatured Phosphoglucose Isomerase. II. Ultraviolet Difference Spectroscopy*

John E. D. Dyson† and Ernst A. Noltmann

ABSTRACT: Ultraviolet difference spectroscopy was applied to rabbit muscle phosphoglucose isomerase to study the relationship between changes in the environment of tyrosine and tryptophan residues and conformational transformations of the enzyme molecule. Perturbations due to the protein matrix have been investigated by exposure of the enzyme to acid or alkaline pH, by addition of denaturing agents and by change of temperature; spectral perturbations arising from addition of 20% ethylene glycol have also been studied with the native and the denatured enzyme. The effects of the various denaturing solvents on phosphoglucose isomerase can be classified in terms of four structural levels resulting from an equal number of increasingly extensive conformational transitions. T_A transition (0.5–1% sodium dodecyl sulfate): dissociation into subunits, exposure of all sulfhydryl groups and of four or five tryptophyls; T_B transition (acid denaturation): exposure of 10–12 tyrosyls, of 15–17 tryptophyls, and of 20 histidyl residues; T_C transition (alkaline denaturation, denaturation in 8 M urea): exposure of all 24 tyrosyls and of up to 20 tryptophyls; T_D transition (pH 2.9 plus 8 M urea): exposure of all tryptophyls. The data are taken to suggest that phosphoglucose isomerase has a relatively rigid core structure which requires severe denaturing conditions for a completely unfolded molecule exposing all of the chromophoric residues.

In the preceding paper (Dyson and Noltmann, 1969), data from electrometric and spectrophotometric titrations were presented relating the number of exposed histidine and tyrosine residues of rabbit muscle phosphoglucose isomerase to various conformational states of the enzyme. This communication is an extension of that work and reports on the use of ultraviolet difference spectroscopy to study spectral perturbations originating from changes in the environment of tyrosine and tryptophan residues. The perturbation arising from the effect of the protein matrix in screening certain of these residues from the solvent has been studied by subjecting the enzyme to acid and alkaline pH, and to urea and sodium dodecyl sulfate as denaturing agents. The degree of exposure to the solvent of tryptophan and tyrosine residues has also been investigated with ethylene glycol as perturbant, both in the native molecule and following disorganization of the native structure by means of the various denaturing solvents. The extent to which tyrosine and tryptophan are accessible to the medium under these altered conditions compared with the native state has been taken as a measure of conformational changes of phosphoglucose isomerase occurring during the process of denaturation.

Materials and Methods

Phosphoglucose isomerase was isolated from rabbit skeletal muscle (Noltmann, 1964, 1966) and solutions of the enzyme were prepared for spectral analysis as described in the preceding paper (Dyson and Noltmann, 1969). For measurement of acid or alkaline difference spectra, the enzyme was dialyzed against CO_2-free 0.2 M KCl (except where indicated otherwise). For the solvent perturbation studies with ethylene glycol, the dialysis medium was Tris-Cl of the concentration and pH specified in the legends to the respective figures. Perturbation spectra were obtained as the absorbance difference between a cuvet containing the protein solution plus the added perturbant and a cuvet containing the identical protein solution without perturbing agent plus an amount of KCl solution or buffer equal in volume to that occupied by the perturbant in the sample cuvet. Spectral changes were calculated as changes in molar absorptivity, $\Delta\epsilon$, based on a molecular weight of 132,000 (N. G. Pon, M. N. Blackburn, G. C. Chatterjee, and E. A. Noltmann, unpublished experiments).

The difference spectra were measured in a Cary Model 15 recording spectrophotometer equipped with thermostatted cuvet holder. Dynode voltages of 2 or 3 were used for total absorbances between 1 and 2 (at 280 mμ); the slit width never exceeded 0.2 mm. Temperature control was maintained within ±0.1° by means of a constant-temperature circulating-water bath.

Cuvet and methods for determination of protein concentration, pH measurements, and preparation of urea solutions were as described in the preceding paper (Dyson and Noltmann, 1969), except that a number of the measurements described here were made with 1.0-cm path-length tandem cells (Pyrocell Manufacturing Co., New York). Where these were not used, solvent base lines were determined separately and subtracted. *N*-Acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, DL-phenylalanine, and Tris (A grade) were products of Calbiochem. Ethylene glycol was Fisher Certified reagent and sodium

* From the Department of Biochemistry, University of California, Riverside, California 92502. *Received December 19, 1968.* This is paper 12 in a series dealing with studies on phosphohexose isomerases. The work was supported in part by U. S. Public Health Service Research Grant AM 07203 and by Cancer Research Funds of the University of California.

† Present address: Department of Biochemistry, University of North Dakota School of Medicine, Grand Forks, N. D. 58201.

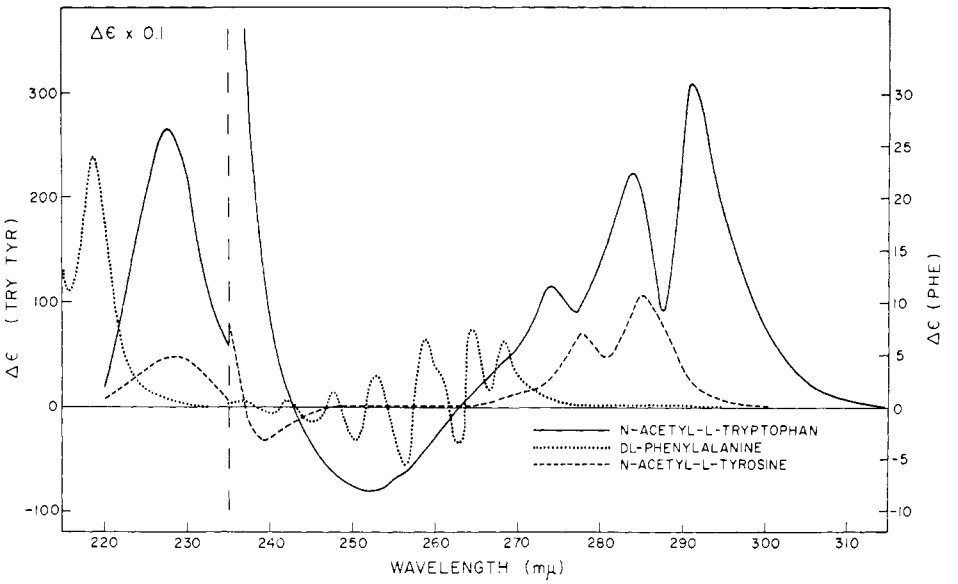

FIGURE 1: Perturbation difference spectra of *N*-acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, and DL-phenylalanine in 20% ethylene glycol. Spectra were obtained at 30° from solutions of the following concentrations of the compounds in 5 mM Tris-Cl (pH 7.0): acetyltryptophan, 0.131 and 0.0131 mM; acetyltyrosine, 0.358 and 0.0358 mM; phenylalanine, 9.67 mM. The sample cuvet contained ethylene glycol at a final concentration of 20%; to maintain identical concentrations of the respective amino acid in both cuvets, buffer was added to the reference cuvet equal in volume to that of the ethylene glycol in the sample cuvet. The left-hand ordinate represents the differences in the molar absorptivities of tryptophan and tyrosine, the right-hand ordinate those of phenylalanine. To permit presentation of the complete spectra in a single figure, the absorptivity differences for the range between 215 and 235 mμ have been decreased by a factor of 10.

dodecyl sulfate was from Matheson Coleman and Bell. All other reagents were of analytical quality.

Results

Solvent Perturbation Difference Spectra of Model Compounds. In order to interpret perturbation difference spectra of the enzyme, it was first necessary to establish reference spectra[1] with known concentrations of the chromophores. Figure 1 represents the change occurring in the spectra of *N*-acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, and DL-phenylalanine in the presence of 20% ethylene glycol. These spectra were used to quantitate the number of exposed tryptophan and tyrosine residues in the enzyme protein, under the various experimental conditions, by trial fitting of composite spectra calculated for various amounts and ratios of these two amino acids to the experimentally obtained perturbation spectra, until best agreement was reached (Donovan, 1964). The very small contribution by phenylalanine has been ignored in this fitting process but some of the fine structure in the protein spectra between 240 and 270 mμ is undoubtedly due to that amino acid. The spectra of the individual chromophores presented in Figure 1 make it apparent that the peak at 291–292 mμ, found in protein perturbation spectra, originates largely from tryptophan, whereas the peak found with proteins at 283–286 mμ contains contributions from both tryptophan and tyrosine, the ratio of the molar absorptivities at the latter wavelength corresponding to approximately 2.0. The same ratio had also been found by Donovan (1964), and by Herskovits and Sorensen (1968).

Perturbation Difference Spectra of Phosphoglucose Isomerase in 20% Ethylene Glycol. Difference spectra[2] resulting from the addition of 20% ethylene glycol to the enzyme solution are shown in Figure 2 for both the native and the acid-denatured enzyme. The enzyme difference spectra can best be fitted by composite spectra for 10 tryptophyl residues for the native, and for 11 tyrosyl and 10 tryptophyl residues for the acid-denatured enzyme.

Acid and Alkaline Difference Spectra. Perturbation spectra[2] produced on acid denaturation of phosphoglucose isomerase are shown in Figures 3 and 4A. It is evident on comparison of the acid difference spectrum (Figures 3 and 4A) with the difference spectrum produced by addition of 20% ethylene glycol to the acid-denatured enzyme (Figure 2) that a discrepancy exists between the results of the two measurements. The ethylene glycol difference spectra of Figure 2 indicate that no further exposure of tryptophyl residues takes place on acid denaturation, whereas the acid difference spectrum (Figure

[1] After this manuscript was completed, a paper was published by Herskovits and Sorensen (1968) in which detailed tables of molar absorptivity differences for *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tryptophan ethyl ester in various perturbants are presented. The data for ethylene glycol agree closely with the spectra presented in our Figure 1, the small differences perhaps attributable to the fact that in our work the N-acetylated amino acids with free carboxyl groups served as model compounds, whereas Herskovits and Sorensen used the ethyl esters.

[2] For the purpose of showing all difference spectra with a positive denotation, the following convention for positioning a cuvet as "sample" or "reference" had been adopted for the work described in this paper: for the solvent perturbation spectra, ethylene glycol was added to the sample cuvet (*i.e.*, the cuvet located in the *sample compartment* of the spectrophotometer); for the acid or alkaline difference spectra, the sample cuvet was kept at pH 6.7 and acid or base was added to the cuvet located in the reference compartment of the spectrophotometer; sodium dodecyl sulfate was added to the reference cuvet and measured against the sample without detergent.

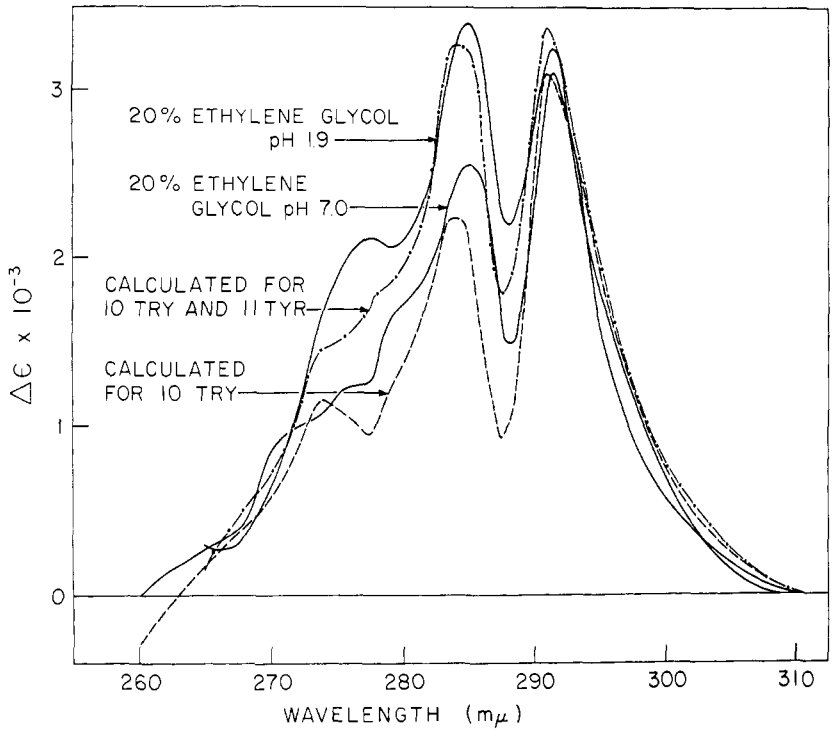

FIGURE 2: Perturbation difference spectra of rabbit muscle phosphoglucose isomerase in 20% ethylene glycol. The spectra were obtained at 30° and at a protein concentration of 6.4×10^{-6} M, either in 5 mM Tris-Cl at pH 7.0 or, for the denatured enzyme, with the pH of the solution adjusted to pH 1.9 with 5 N HCl, prior to pipetting equal aliquots into both the sample and the reference cuvet; no KCl was added. Ethylene glycol addition was made as described in the legend to Figure 1. The theoretical curves representing the closest fit to the protein difference spectra were calculated on the basis of the difference spectra of the model compounds shown in Figure 1.

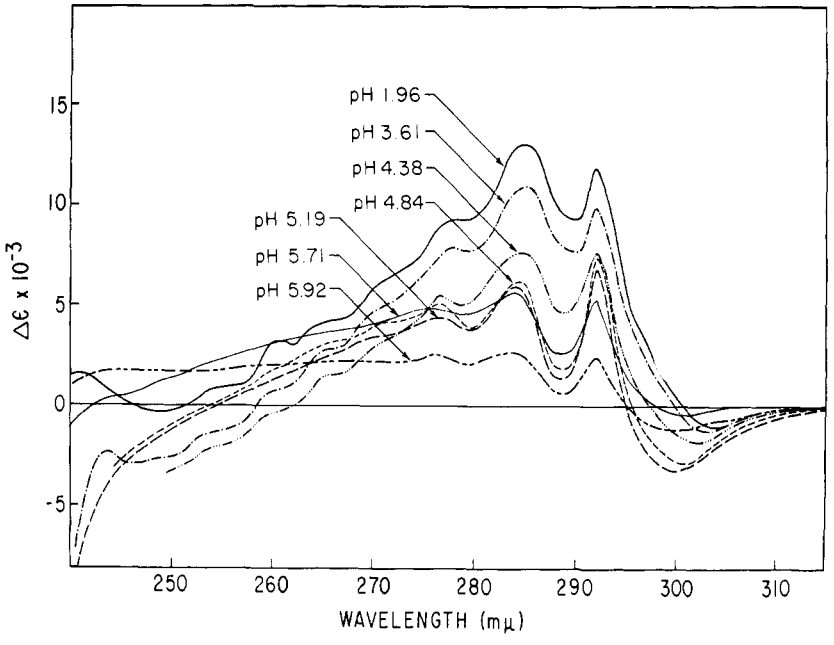

FIGURE 3: Acid difference spectra of rabbit muscle phosphoglucose isomerase. Measurements were made at 30° and at protein concentrations of 0.8–4.15 $\times$ 10^{-6} M. Original enzyme solutions were prepared in 5 mM Tris-Cl (pH 6.7) and the ionic strength was adjusted to 0.2 with KCl with the exception indicated. The pH of the *reference* solution was varied as indicated by addition of 5 N HCl and the ensuing absorbance change was measured against the sample solution kept at pH 6.7. The figure demonstrates the gradual development of the peaks at 284–285 and 291–292 mμ on increasing the extent of acid denaturation.

4A) shows that a $\Delta\epsilon_{291}$ of 15,000 is produced on acid denaturation at pH 1.96 (without added KCl). Since the peak at 291 mμ is largely due to tryptophan (to an extent of about 92%), the acid difference spectrum would appear to indicate that a relatively extensive exposure of tryptophyl residues takes place on acid denaturation. A possible explanation for this discrepancy may be that acid denaturation results in a conformation change sufficient to bring a number of tryptophyl residues into contact with the solvent and thus to produce the acid difference spectrum of Figure 3. These tryptophyl residues, however, although exposed to the solvent, still lie in clefts of the enzyme surface which are too narrow to admit an ethylene glycol molecule (mean diameter, 4.3 Å; Herskovits, 1967). Similar discrepancies between the results obtained for acid difference spectra and the difference spectra due to added perturbants have been noted for α-lactalbumin (Kronman *et al.*, 1965; Kronman and Holmes, 1965) and for serum albumin (Herskovits and Laskowski, 1962). The latter authors found that reduction of the disulfide bridges allowed access by the perturbant molecules to the chromophoric residues. However, as no disulfide linkages have been detected in phosphoglucose isomerase (K. D. Schnackerz and E. A. Nolt-

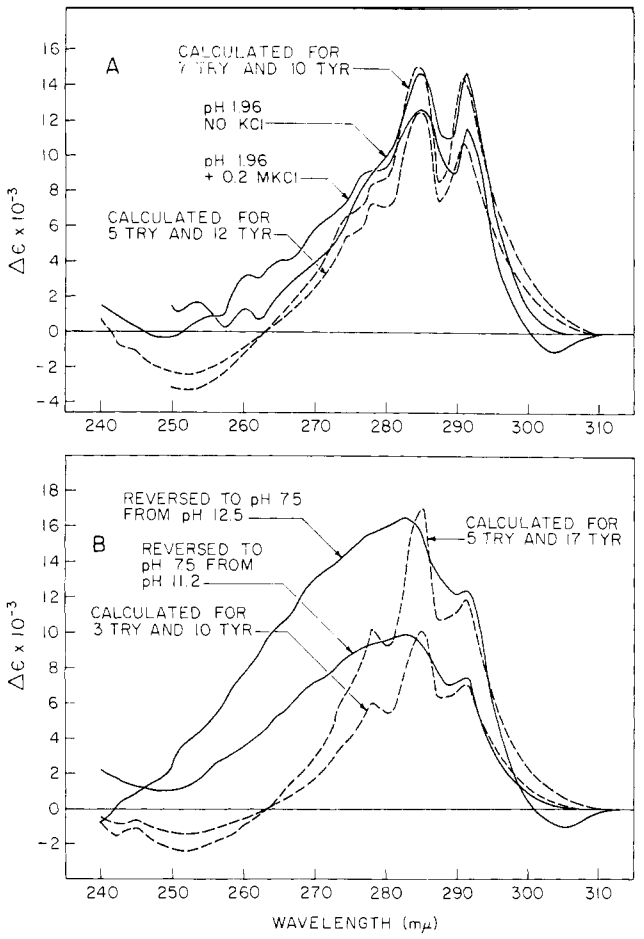

FIGURE 4: (A) Acid difference spectra of rabbit muscle phosphoglucose isomerase at pH 1.96, with 0.2 M KCl added (protein concentration 2.5×10^{-6} M), and in the absence of KCl (protein concentration 4.2×10^{-6} M). Other experimental details as for Figure 3. The theoretical, fitted curves were calculated from the difference spectra of the model compounds shown in Figure 1, multiplied by 6. (B) Alkaline difference spectra of rabbit muscle phosphoglucose isomerase resulting from partial (at pH 11.2) and complete (at pH 12.5) alkaline denaturation and subsequent neutralization. Temperature, 30°; 0.2 M KCl; protein concentration, 1.8×10^{-6} M. Theoretical curves were calculated as described for part A.

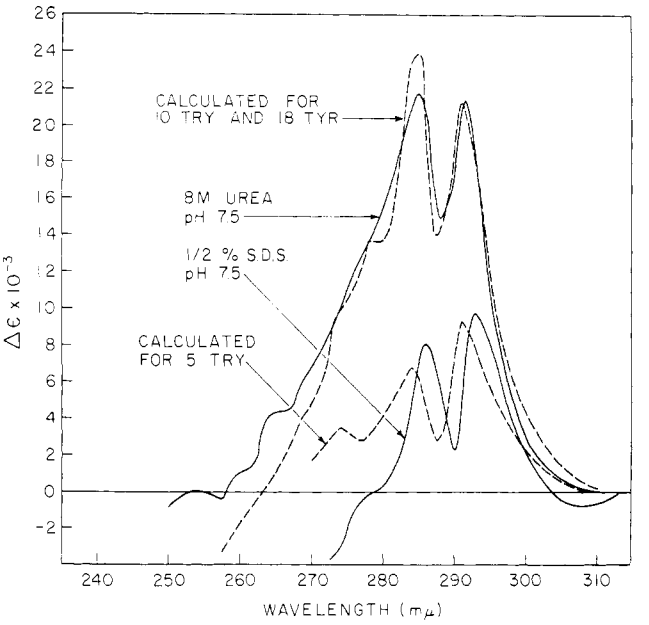

FIGURE 5: Difference spectra resulting from denaturation of rabbit muscle phosphoglucose isomerase in either 8 M urea or 0.5% sodium dodecyl sulfate. Measurements were made against a cuvet containing the identical concentration of the native enzyme. Temperature, 30°; 5 mM Tris-Cl (pH 7.5); no KCl added; protein concentration, 2.5×10^{-6} M. Theoretical curves were fitted as described for Figure 4A.

mann, unpublished experiments), this cannot be the cause of the steric hindrance to the perturbant, in this protein. An alternative explanation for the lack of an effect of acid pH on the ethylene glycol perturbation spectra might be that addition of 20% ethylene glycol tends to cause partial renaturation of the enzyme molecule, and thus rescreening of the tryptophyl residues (Simpson and Kauzmann, 1953). There are, however, two objections to such an interpretation: firstly, Herskovits and Laskowski (1962) found that this renaturation effect was absent at acid pH; secondly, Kronman and Holmes (1965) observed that ethylene glycol had essentially no effect on the acid difference spectrum of α-lactalbumin.

Because of the different results obtained with the acid difference and ethylene glycol difference spectra, a value for the ratio of the perturbation caused by the protein matrix to that caused by 20% ethylene glycol, cannot be obtained directly in the manner used by Donovan (1964). However, a value for this ratio can be arrived at as follows. The spectrum for the native enzyme in the presence of 20% ethylene glycol (Figure 2) shows that 10 tryptophyl residues are available to the solvent; thus, 14 tryptophyls and 24 tyrosyls are buried in the native molecule. The ratio $\Delta\epsilon_{Tyr}/\Delta\epsilon_{Trp}$ is 0.081 at 291 mμ. If the 24 tyrosyls are multiplied by 0.081 and the result added to the 14 tryptophyls, an equivalent number of 15.94 residues is obtained. If it is assumed that 8 M urea plus a pH of 2.93 are sufficiently rigorous conditions to expose all of the tryptophyl and tyrosyl residues, then a sum total $\Delta\epsilon_{291}$ of 29,800 is obtained for exposure of these residues to the solvent (21,700 from Figure 5 plus 8100 from Figure 7). Division of 29,800 by 15.94 gives a value for $\Delta\epsilon_{291}$ of 1870/tryptophyl residue, due to the protein matrix. This value may be compared with the value of 309, obtained for the $\Delta\epsilon$ per tryptophyl due to 20% ethylene glycol (Figure 1), resulting in a ratio of 1870/309 = 6.05, in excellent agreement with the value of 6, found by Donovan (1964). The spectra of Figure 1 for tyrosine and tryptophan, multiplied by 6, have thus been used to quantitate the difference spectra resulting from disorganization of the protein molecule (Figures 4A,B, 5, and 7). This method assumes that the ratio $\Delta\epsilon_{Tyr}/\Delta\epsilon_{Trp}$ remains the same in 8 M urea as in aqueous medium at acid pH. This seems to be reasonable since the perturbation originates from the protein matrix, even though in one case the denaturation is produced by 8 M urea, and in the other case by acid or alkaline pH. Moreover, the measurements of Herskovits and Sorensen (1968) on the effect of 20% ethylene glycol in the presence of 8 M urea show that, if 8 M urea does affect this ratio, it could do so only to the extent of a few per cent, and would thus not seriously change the values given in the figures.

The difference spectra presented in Figures 4A,B, 5, and 7

TABLE I: Effect of Various Methods of Denaturation on the Number of Exposed Amino Acid Residues in Rabbit Muscle Phosphoglucose Isomerase.[a]

Denaturant	Method of Measurement[b]	% Tyrosine	% Tryptophan	% Cysteine	% Histidine
		Exposed Residues/Enzyme Molecule[c,d]			
None (native enzyme)		0 (0)[d]	10 (42)	4 (33)[e]	27 (58)
Acid (pH 2, no KCl)	Ethylene glycol	11 (46)	10 (42)		
Acid (pH 2, no KCl)	Acid difference	10 (42)	17 (71)		
Acid (pH 2, 0.2 M KCl)[f]	Acid difference	12 (50)	15 (63)		47 (100)[f]
Alkali (pH 12.5 and neutralized)	Alkaline difference	17 (71)	15 (63)		
Alkali (pH 12.5–13)[f]		24 (100)[f]	17 (71)[f]		
8 M urea (pH 7)	Urea difference	18 (75)	20 (83)		
8 M urea (pH 7)	Ethylene glycol	18 (75)	18 (75)		
8 M urea (pH 2.9)	Acid difference	21 (88)	24 (100)		
0.5–1.0% sodium dodecyl sulfate	Sodium dodecyl sulfate difference	0 (0)	15 (63)	12 (100)[f]	
0.5% sodium dodecyl sulfate	Ethylene glycol	0 (0)	14 (58)		

[a] Includes data from the preceding paper (Dyson and Noltmann, 1969). [b] Where ethylene glycol is shown as not present, measurement was made with an enzyme solution in the reference cuvet with denaturant added as indicated, against an enzyme solution of identical concentration in the sample cuvet, but without added denaturant. Where ethylene glycol is shown to be present, it was added to the sample cuvet to a concentration of 20% and measured against a reference of identical protein concentration, without added ethylene glycol. [c] Based on a molecular weight of 132,000 (N. G. Pon, M. N. Blackburn, G. C. Chatterjee, and E. A. Noltmann, unpublished experiments). [d] Figures in parentheses refer to the exposed amino acid residues in per cent of their total content in rabbit muscle phosphoglucose isomerase. [e] From Chatterjee and Noltmann (1967). [f] Values partially derived from data of the preceding paper (Dyson and Noltmann, 1969), *i.e.*, value for exposure of histidyl from the electrometric titration curve; value for exposure of cysteine from spectrophotometric titration of sulfhydryl groups, also from Chatterjee and Noltmann (1967); values for tryptophyl and tyrosyl residues after alkaline denaturation at pH 12.5–13 (refer to the discussion in the text).

have been fitted by calculated curves representing total exposure of the number of tryptophyl and tyrosyl residues shown in the figures. However, it is understood that this is an oversimplification, required for fitting the curves calculated from the model compounds to the experimental spectra. The experimental spectra undoubtedly contain contributions from both totally and partially exposed tryptophyl and tyrosyl residues, as shown by the consistently higher values for residues available to the aqueous medium, in contrast to the numbers accessible to ethylene glycol. In Table I, therefore, the results obtained from the various spectra, as to the number of residues exposed by the different methods of denaturation, are presented both in terms of the number of residues which could be totally exposed, and as percentage exposure of the total tyrosine or tryptophan content of the enzyme.

Acid difference spectra obtained at pH 1.96 and quantitated in the manner described above, are shown in Figure 4A; they are best fitted by 5–7 tryptophyls (a total of 15–17 exposed tryptophyls) and by 10–12 tyrosyls, depending upon the ionic strength. The degree of exposure of the tryptophyl residues increases with decreasing ionic strength, suggesting that increased electrostatic interaction, at the lower ionic strength, causes the partially disorganized enzyme molecule to assume a more open configuration. The number of tyrosyl residues, shown by the acid difference spectra to be exposed to the solvent, is in good agreement with the value derived from the spectrum obtained for the acid-denatured enzyme in the presence of 20% ethylene glycol. These tyrosyls appear to be located in relatively highly disorganized regions of the enzyme molecule allowing unhindered access of the ethylene glycol. With regard to the tryptophyl residues, however, the different values obtained from the 20% ethylene glycol perturbation spectrum of the acid-denatured enzyme (10 tryptophyls exposed, as in the native molecule), suggest as discussed above, that access to the 5–7 tryptophyls exposed by acid denaturation is still to some degree hindered. This interpretation is supported by the ionic strength effect, *viz.*, access of the solvent to the tryptophyls is increased at low ionic strength without markedly affecting the tyrosyl residues.

Alkaline denaturation at pH 12.5 followed by neutralization to pH 7.5 results in the exposure of 5 tryptophyl and 17 tyrosyl residues (Figure 4B). Alkaline denaturation thus leads to a greater exposure of tyrosyl residues than acid denaturation. Data of the preceding paper (Dyson and Noltmann, 1969), however, suggest that alkaline denaturation actually causes a more extensive disorganization of the enzyme molecule than Figure 4B would indicate. It thus appears that on neutralization from pH 12.5 to 7.6 some refolding of the protein

occurs, excluding the solvent from some of the previously exposed tryptophyl and tyrosyl residues. This conclusion may be drawn from a comparison of the $\Delta\epsilon$ value at 285 mμ for the alkaline-denatured and then neutralized enzyme, *i.e.*, 16,800, with the value obtained directly by subtracting from the total $\Delta\epsilon$, measured at pH 12.5–13, the absorbance contribution stemming from the ionization of tyrosine,[3] *i.e.*, 24,000. The difference in absorptivity of approximately 7200 corresponds to about 2 tryptophyl and 6 or 7 tyrosyl residues that become shielded again from the solvent in the process of neutralization. This would bring the number of residues exposed by alkaline denaturation to 17 tryptophyls and 24 tyrosyls. Such a conclusion would also resolve the difference in the value for tyrosyl residues exposed by denaturation at pH 12.5 given in Figure 4B (17 residues), with that obtained from titration of the tyrosyl residues, and given in the preceding paper (Dyson and Noltmann, 1969). The data from the latter study indicate that at pH 12.5 all 24 tyrosyl residues are exposed and ionized.

It was usually quite difficult to obtain, on neutralization from pH 12.5 or above, the enzyme in soluble form at pH values below 9.5. Apparently, phosphoglucose isomerase maintains a rigid core structure which is only abolished at highly alkaline pH. When the denaturation process is severe enough to affect the integrity of this core region, a nucleus for refolding will not be available and the protein will aggregate and precipitate on neutralization. If, on the other hand, the central region has retained its structure, refolding is possible and the protein will remain soluble at neutral pH.

Denaturation in 8 M *Urea.* In Figure 5, the difference spectrum depicted is obtained when an enzyme solution in 8 M urea is measured against a solution of identical concentration of the native enzyme (at neutral pH). The spectrum is best fitted by a curve calculated for 10 tryptophyl (a total of 20 tryptophyl exposed, *cf.* Table I) and 18 tyrosyl residues. In Figure 6, the difference spectrum is shown which results from addition of 20% ethylene glycol to the enzyme in 8 M urea, measured against a solution without ethylene glycol, but otherwise identical. A calculated curve,[4] which closely resembles the experimental spectrum, is obtained for 18 tryptophyl and 18 tyrosyl residues. It appears from the near agreement of the urea difference spectrum and the ethylene glycol perturbation spectrum in 8 M urea, that in this denaturant the aqueous medium and ethylene glycol have access to approximately equal numbers of tryptophyl and tyrosyl residues. The action of 8 M urea must, therefore, result in a considerably greater degree of disorganization of the enzyme molecule, than acid denaturation.

Denaturation in Sodium Dodecyl Sulfate. It was shown in the preceding paper (Dyson and Noltmann, 1969) that the action of sodium dodecyl sulfate apparently did not result in

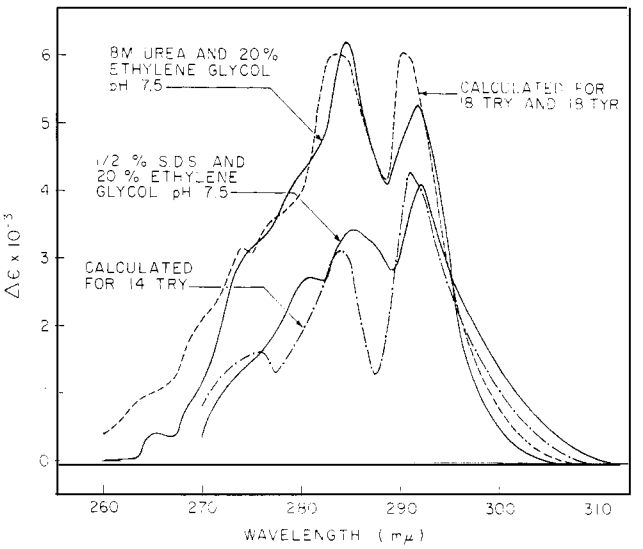

FIGURE 6: Perturbation difference spectra induced by addition of 20% ethylene glycol to rabbit muscle phosphoglucose isomerase denatured by either 8 M urea or 0.5% sodium dodecyl sulfate. Temperature, 30°; 5 mM Tris-Cl (pH 7.5); no KCl added; protein concentration, 6.5×10^{-6} M. Both sample and reference cuvets contained either 8 M urea or 0.5% sodium dodecyl sulfate, as indicated. Ethylene glycol was added as described in the legend to Figure 1. The theoretical curve for the sodium dodecyl sulfate spectrum was calculated as described for Figure 2. The theoretical curve fitted to the urea spectrum was calculated with use of the model spectra determined in 8 M urea by Herskovits and Sorensen (1968).

the exposure of any tyrosyl residues in phosphoglucose isomerase. Sodium dodecyl sulfate does have some effect on the structure of the enzyme molecule, however, since it causes the exposure of a number of previously screened sulfhydryl groups (Chatterjee and Noltmann, 1967; Dyson and Noltmann, 1969). The difference spectra resulting from the action of sodium dodecyl sulfate were therefore investigated. In Figure 5, the difference spectrum is shown which results from addition of sodium dodecyl sulfate (final concentration, 0.5%) to the reference cuvet. This spectrum is best fitted by a curve calculated for five tryptophyls. Addition of 20% ethylene glycol to the sodium dodecyl sulfate denatured enzyme, and measurement against an otherwise identical solution without ethylene glycol, results in the spectrum shown in Figure 6. A curve calculated for four tryptophyl residues corresponds closely to this experimental spectrum. These results thus agree in a qualitative manner with the data obtained by titration of tyrosyl residues in the presence of sodium dodecyl sulfate (Dyson and Noltmann, 1969). This calculation of the number of tryptophyl residues must be considered only approximate, however, since the curve fitting was carried out with spectra of model compounds obtained in the absence of sodium dodecyl sulfate. Differences in the spectral changes induced by acid denaturation and by denaturation in sodium dodecyl sulfate have been also attributed to masking of the exposed tyrosyl residues by the detergent (Leonard and Foster, 1961) and this could be an alternative explanation for the effects referred to above; but it is difficult to understand why the masking effect should apply to tyrosyl and not to tryptophyl residues. Moreover, Polet and Steinhardt (1968) have shown that sodium dodecyl sulfate interacts with both tyrosyl and tyrptophyl res-

[3] The $\Delta\epsilon$ at 285 mμ, measured on alkali-induced denaturation, contains contributions from both the ionization of tyrosine and from environmental changes of tyrosine and tryptophan. The $\Delta\epsilon$ due solely to the environmental changes can be arrived at by subtracting the contribution due to the ionization of tyrosine, as described in the preceding paper (Dyson and Noltmann, 1969).

[4] In fitting the calculated curve to the experimental spectrum, obtained by addition of 20% ethylene glycol to the enzyme denatured in 8 M urea, the reference spectra of Herskovits and Sorensen (1968) for *N*-acetyl-L-tyrosine and *N*-acetyl-L-tryptophan, determined by these authors in the same environment, were used.

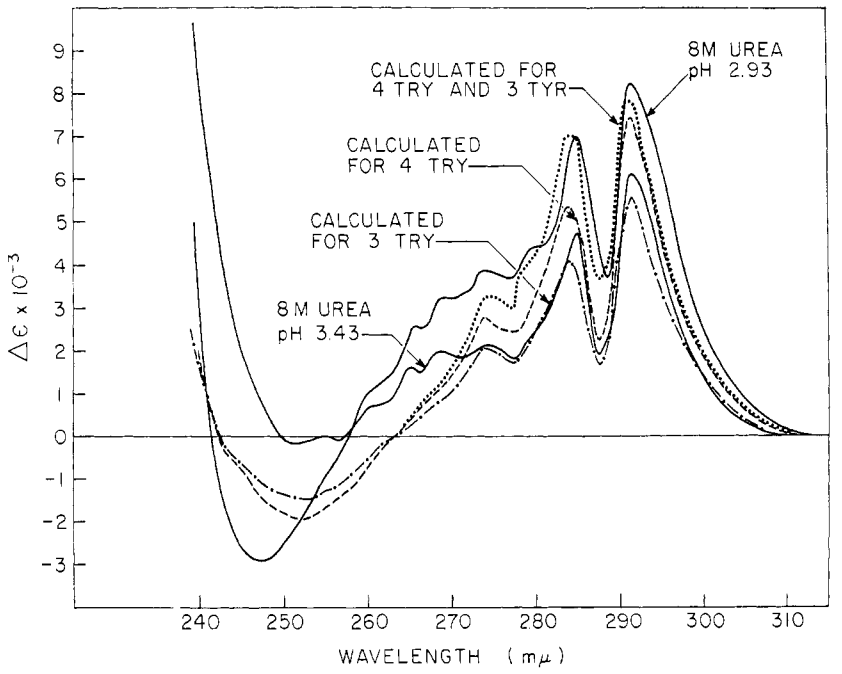

FIGURE 7: Acid difference spectra of rabbit muscle phosphoglucose isomerase in 8 M urea. The enzyme solution was prepared as described in the legend to Figure 3; the spectra were taken at 30° and at a protein concentration of 2.5×10^{-6} M. Both the sample and the reference cuvet contained 8 M urea; 5 N HCl was added to the reference cuvet to adjust the pH as indicated. The theoretical curves were calculated from the data shown in Figure 1, multiplied by 6.

idues in bovine serum albumin, and also that the detergent *exposes* the tyrosyl residues in that protein.

Acid Difference Spectra in 8 M Urea. Since the ethylene glycol perturbation spectrum for the enzyme denatured in 8 M urea indicated that, at neutral pH, about four to six tryptophyl and five tyrosyl residues were still shielded from the solvent, it was of interest to investigate whether the increased electrostatic repulsion resulting from acid conditions would cause further disruption of the enzyme molecule. In Figure 7, two protein difference spectra are depicted, each measured in 8 M urea at the indicated pH against the identical enzyme solution in 8 M urea, but at neutral pH. Best agreement was obtained with curves calculated for the exposure of an additional three tryptophyl residues at pH 3.4, and an additional four tryptophyl residues at pH 2.9. A somewhat better agreement is obtained with the curve fitted to the latter spectrum when three tyrosyl residues are included in the calculated curve. This again suggests, as do the results above, that 8 M urea does not completely expose all the tyrosyl residues (*cf.* also Discussion).

Discussion

It has been suggested in the preceding paper (Dyson and Noltmann, 1969) that phosphoglucose isomerase has several levels of structural integrity which have different degrees of resistance to various denaturing reagents. The data presented here are consistent with this proposal and permit further characterization of the structural entities. In Table I, the types and number of amino acid residues exposed by the various methods of denaturation are summarized. It is apparent that the exposure of initially shielded groups follows a definite pattern, which allows the distinction of three levels of structure, or of four, if dissociation of the enzyme into subunits by sodium dodecyl sulfate[5] is considered disruption of a first level. For the purpose of this discussion, these levels will be designated as A, B, C, and D, with A pertaining to the structure of the native phosphoglucose isomerase molecule; transitions to lower levels will be called T_A, T_B, etc., T_A designating the initial conversion of the oligomeric enzyme molecule into its subunits. In Table II, these structural levels are presented schematically, according to the transitions that occur on treatment with various denaturing solvents. Also shown are the number of tryptophyl and tyrosyl residues exposed on disruption of the different levels of structure.

T_A Transition. Loss of the A structure occurs in the presence of 0.5–1% sodium dodecyl sulfate and is characterized by dissociation into subunits[5] and by exposure of all the sulfhydryl groups (Chatterjee and Noltmann, 1967; Dyson and Noltmann 1969) and of four to five tryptophyl residues. The T_A transition, however, does not involve exposure of any tyrosyl residues. These data thus lead to the conclusion that in the native state certain of the sulfhydryl groups and of the tryptophyl residues are situated between the subunits of the molecule and, furthermore, that acid denaturation initially does not cause dissociation into subunits, since it is unable to cause sufficient disruption of the enzyme molecule to bring any tryptophyl residues into contact with added ethylene glycol.

T_B Transition. Disruption beyond the B structure at alkaline pH begins to occur at about pH 10, with the time-dependent exposure of 6 or 7 tyrosyl residues (see Figure 5 of the preceding paper (Dyson and Noltmann, 1969)). At a pH of about 11.1, the 6 or 7 tyrosyl residues are exposed immediately; with time or at higher pH values the denaturation extends to the C level (see below). At acid pH values disruption of the B structure commences at about pH 6, and increases with decrease of pH until a plateau is reached at about pH 2 (*cf.* Figure 10 of the preceding paper (Dyson and Noltmann, 1969)). At this point, about 10–12 tyrosyl and 15–17 tryptophyl residues are exposed (Figures 3 and 4A), and acid denaturation, in contrast to alkaline denaturation, is

[5] M. N. Blackburn and E. A. Noltmann, unpublished experiments.

TABLE II: Structural Levels of Rabbit Muscle Phosphoglucose Isomerase.

Structural Level	Denaturant	Groups Exposed
Native A		10 tryptophyls, 4 cysteinyls, 27 histidyls
	Sodium dodecyl sulfate (0.5%)	
B	Acid (pH 2, no KCl)	15 tryptophyls, 12 cysteinyls, ? histidyls
C	Alkali (pH 13), 8 M urea (pH 7)	15–17 tryptophyls, 10–12 tyrosyls, 47 histidyls
D	8 M urea (pH 2.9)	17–20 tryptophyls, 18–24 tyrosyls
		24 tryptophyls, 21–24 tyrosyls

apparently unable to cause further disorganization of the phosphoglucose isomerase molecule.

T_C Transition. Alkaline denaturation at high pH (~12.5–13) or 8 M urea apparently disrupts the native structure of the phosphoglucose isomerase molecule to approximately the same extent, although some differences may exist. The limit of the C structure may be defined by the degree of disorganization of the enzyme molecule produced as a consequence of denaturation by either method, resulting in the exposure of 17–20 tryptophyl and of 18–24 tyrosyl residues. There is a slight uncertainty in the number of residues exposed on complete disruption of the C level of structure. The data for the titration of tyrosyl residues, as discussed above, strongly suggest that all tyrosyl residues are exposed at high pH, since the number of exposed residues, calculated with a $\Delta\epsilon_{295}$ of 2330 (Beaven and Holiday, 1952), is in excellent agreement with the value obtained by direct amino acid analysis.[6] The data for the titration of tyrosyl residues in the presence of 8 M urea also indicate that in this medium the solvent has access to all 24 tyrosyl residues (Dyson and Noltmann, 1969). It is, however, possible that in 8 M urea the last 3–5 tyrosyl residues become available to the solvent only at strongly alkaline pH (this conclusion is supported by the observation (see below) that acidification of the enzyme in 8 M urea results in the exposure of a further 3 tryptophyl residues). If this were the case, the titration curve would not differ appreciably from a curve in which all 24 residues were equally exposed throughout the pH range.

[6] Spectrophotometric titration and other analytical methods have established that rabbit muscle phosphoglucose isomerase contains 24 tyrosine residues per molecule of 132,000 molecular weight (Dyson and Noltmann, 1969; K. D. Schnackerz and E. A. Noltmann, unpublished experiments, 1967).

T_D Transition. It will be recalled that the maximum $\Delta\epsilon$, at 285 mμ, achieved by alkaline denaturation is 24,000 (*cf.* Figure 10 of the preceding paper (Dyson and Noltmann, 1969)). The total number of buried tryptophyl and tyrosyl residues is 14 and 24, respectively, from which it may be calculated (see Results), that a total $\Delta\epsilon$, at 285 mμ, of 32,300 would be obtained for complete exposure of all of these residues (after subtracting the contribution from the ionization of tyrosine[3]). The difference between the calculated and observed values is 8300, which suggests that 6 or 7 tryptophyl residues are still not accessible to the solvent after alkaline denaturation ($\Delta\epsilon_{285}$ per tryptophyl residue = 6 × 205 (from Figure 1) = 1230 for protein matrix perturbation). This number is in good agreement with the conclusion reached above (*cf.* Results) that alkaline denaturation results in a total of 17 tryptophyl residues being exposed to the solvent and 6 or 7 residues remaining shielded.

Total exposure of all 24 tryptophyl residues cannot even be accomplished by the addition of 8 M urea. If, however, in addition, the pH is decreased to 2.9 (Figure 7), further disruption of the enzyme structure seems to occur, since a difference spectrum is obtained that can be fitted by 4 tryptophyl and 3 tyrosyl residues. The observation, that the fit of the calculated curve to the pH 2.9 difference spectrum in 8 M urea is improved by the inclusion of 3 tyrosyl residues, supports the above-mentioned possibility that at neutral pH and in 8 M urea, 3–5 tyrosyl residues are still not accessible to the solvent. It therefore appears that an increase in the net charge of the protein molecule, *i.e.*, acid or alkaline pH which add electrostatic repulsion to the action of the 8 M urea, is required for final disruption of the D structure of phosphoglucose isomerase. It could perhaps be argued that at neutral pH some aggregation of the denatured protein may occur in 8 M urea (Frensdorff *et al.*, 1953; Herskovits and Laskowski, 1962)

and that, on addition of acid or base, exposure takes place of those tryptophyl or tyrosyl residues that are screened in the aggregate. The observation, however, that strongly alkaline conditions are unable to achieve complete exposure of all of the tryptophyl residues, is more in line with the first explanation.

It should be emphasized that these considerations are obviously dependent upon the validity of the assumption that the molar absorptivities derived from difference spectra of model compounds are also applicable for chromophoric residues in the protein perturbed by the various agents employed. If this were not the case, the conclusions would still be qualitatively correct, although the numerical values for the exposed residues might differ somewhat from those presented here. The very rigorous conditions, required to bring all the tryptophyl residues into contact with the surrounding medium, exemplify the high stability of this last level of organized structure in phosphoglucose isomerase. On the basis of presently available data, conclusions regarding the forces responsible for this stability cannot be arrived at. It is hoped that future work on the subunit structure will provide further insight into the complex molecular architecture of this enzyme.

Acknowledgment

We wish to express our gratitude to Dr. J. B. Mudd for generously providing access to his Cary 15 spectrophotometer.

References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Chatterjee, G. C., and Noltmann, E. A. (1967), *J. Biol. Chem. 242*, 3440.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Dyson, J. E. D., and Noltmann, E. A. (1969), *Biochemistry 8*, 3533 (this issue; preceding paper).
Frensdorff, H. K., Watson, M. T., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5167.
Herskovits, T. T. (1967), *Methods Enzymol. 11*, 748.
Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Sorensen, Sr. M. (1968), *Biochemistry 7*, 2523.
Kronman, M. J., Cerankowski, L., and Holmes, L. G. (1965), *Biochemistry 4*, 518.
Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 526.
Leonard, W. J., Jr., and Foster, J. F. (1961), *J. Biol. Chem. 236*, PC73.
Noltmann, E. A. (1964), *J. Biol. Chem. 239*, 1545.
Noltmann, E. A. (1966), *Methods Enzymol. 9*, 557.
Polet, H., and Steinhardt, J. (1968), *Biochemistry 7*, 1348.
Simpson, R. B., and Kauzmann, W. (1953), *J. Am. Chem. Soc. 75*, 5139.

The Glycoprotein Structure of Yeast Invertase*

Norbert P. Neumann and J. Oliver Lampen

ABSTRACT: Invertase in yeast appears to exist in two forms. One is intracellular and devoid of carbohydrate. The other is localized externally to the cell membrane in the cell wall and, unlike the internal enzyme, is a glycoprotein which contains approximately 50% carbohydrate. An estimate of both the number and sizes of the carbohydrate units in the enzyme has been obtained by an examination of glycopeptides obtained after proteolytic digestion of the enzyme by *Streptomyce griseus* protease.

The results indicate that approximately 30 chains of polysaccharide of varying size are present per molecule of enzyme. The nature of the carbohydrate–protein linkage has been examined and appears to involve a glucosylaminyl–asparagine bond.

There is evidence that yeast invertase exists in at least two forms which are related to its location in the cell (Gascón and Ottolenghi, 1967; Sutton and Lampen, 1962; Islam and Lampen, 1962; Lampen *et al.*, 1967). The properties of the internal enzyme have been described recently (Gascón and Lampen, 1968; Gascón *et al.*, 1968). The internal enzyme is devoid of detectable carbohydrate. In contrast, the external enzyme which is localized in the cell wall is a glycoprotein containing about 50% carbohydrate (predominantly mannan with a small percentage of glucosamine) (Neumann and Lampen, 1967).

During the past few years the chemical properties of glycoproteins from a variety of sources have been examined. The glycosylamine linkage of the carbohydrate to the amide group of asparagine has been demonstrated or postulated to occur in ovalbumin (Fletcher *et al.*, 1963), γ-globulin (Rosevear and Smith, 1961), α_1-acid glycoprotein (Kamiyama and Schmid, 1962), soybean hemagglutinin (Lis *et al.*, 1966), ovomucoid

* From Rutgers Medical School and the Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey. *Received April 17, 1969.* This investigation was supported by a U. S. Public Health Service Grant A1-04572. Some of the findings were presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 1966.