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Preparation of Protein Conjugates via Intermolecular Hydrazone Linkage†

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ABSTRACT: Proteins can be modified at their amino groups under gentle conditions to contain an average of three to six aryl aldehyde or acyl hydrazide groups. These two types of modified proteins at about 10 μM concentration condense with each other at pH ~5 to form conjugates linked by hydrazone bonds. Under proper conditions conjugates mainly of dimers and trimers in size or, if desired, higher oligomers can be obtained. The conjugates can be dissociated to their individual protein components by an exchange reaction with an excess of acetyl hydrazide. The reversible hydrazone bonds of conjugates can be reduced with $NaCNBH_3$ to give stable hydrazide bonds. The stability of protein-hydrazone conjugates was found to be significantly greater than that of the model compound, the *N*-acetylhydrazone of *p*-carboxybenzaldehyde. This difference is believed to result from the presence of multiple hydrazone linkages in protein conjugates.

For various biochemical studies it will be useful to have simple methods for preparing conjugates of one protein with another protein or peptide. The most widely used method for this purpose involves the coupling of two components by reaction with glutaraldehyde (Reichlin, 1980) to give a mixture of conjugates of like and unlike components. To avoid formation of conjugates of like components, one uses two components with different reactive groups for the coupling reaction. Three reported methods utilizing this principle involve the reaction of the sulfhydryl group of one protein with the haloacetyl group (Rector et al., 1978; Eberle et al., 1977), the maleimido group (Kitagawa & Akikawa, 1976), or the 4-dithiopyridyl group (Carlsson et al., 1978; King et al., 1978) of another protein or peptide.

Hydrazone formation has been used to prepare conjugates of proteins with various ligands (Heitzmann & Richards, 1974; Itaya et al., 1975; Rando et al., 1979). In those studies, glycoproteins were either chemically or enzymatically oxidized to generate aldehyde groups and then coupled to low molecular weight ligands containing hydrazide groups.

In this work, methods were devised so that proteins can be readily modified to contain aryl aldehyde or acyl hydrazide groups. Studies were then made to establish optimal conditions for coupling of aldehyde- and hydrazide-containing proteins to form conjugates by hydrazone bond formation and for reduction of hydrazone linkages with $NaCNBH_3$. The chemical reactions involved are given in eq 1-5 for Figure 1. Model experiments were also made on the formation and reduction of the hydrazone of *p*-carboxybenzaldehyde with acetyl hydrazide.

Materials and Methods

Unless noted otherwise, all organic chemicals were obtained from Aldrich Chemical Co., and all protein samples were obtained from Worthington Biochemical Corp. or Sigma Chemical Co. *N*-Hydroxysuccinimide esters of *N*-(bromoacetyl)-β-alanine (Santi & Cunnion, 1974), *p*-carboxybenzaldehyde (Kraehenbuhl et al., 1974), 2-nitro-5-thiobenzoic acid (Degani & Patchornik, 1971), and *N*-acetylhomocysteinyl hydrazide (Taylor & Wu, 1980) were prepared according to published procedures.

The proteins, which contain free sulfhydryl groups, were treated at a concentration of about 5 mg/mL with 1.5 mM iodoacetamide in 0.1 M phosphate of pH 7.8 for 1 h at room temperature and dialyzed to remove excess reagent prior to modifications.

Synthesis of N-Acetylhydrazones of Different Aldehydes. These were prepared by bringing to boil a 0.1-0.5 M solution of aldehyde and acetyl hydrazide (10% molar excess) in ethanol for a minute or less. On cooling, the crystalline product was collected by filtration and then recrystallized from ethanol. The hydrazones of acetaldehyde, benzaldehyde, and *p*-nitrobenaldehyde had the reported values for their melting points (Lindegren & Nieman, 1949; Tisler, 1957; Gutman et al., 1961). The hydrazone of *p*-carboxybenzaldehyde melted

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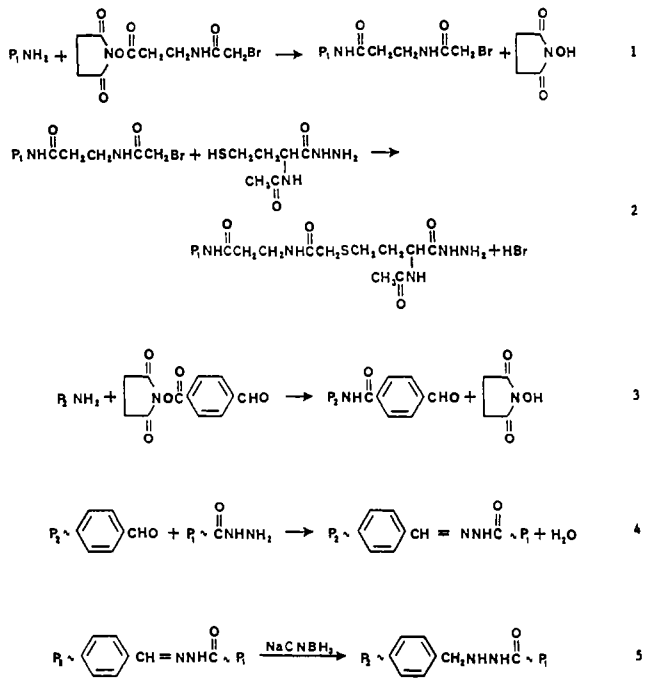

FIGURE 1: Chemical reactions for preparation of protein conjugates via intermolecular hydrazone linkage. P_1 and P_2 are abbreviations for proteins 1 and 2; the symbol (~) is an abbreviation for the atoms joining the aldehyde or hydrazide group to P_1 or P_2.

at 284–286 °C and it had the expected elemental composition.

Synthesis of 1-Acetyl-2-(p-carboxybenzyl)hydrazine. The compound was prepared by the reduction of 22 mM *N*-acetylhydrazone of *p*-carboxybenzaldehyde with 460 mM $NaCNBH_3$ in 0.14 N sodium acetate buffer (pH 4.7) containing 9.1% dimethyl sulfoxide. After 20 h at 25 ± 1 °C, the reaction mixture (11 mL) was diluted with 30 mL of 0.01 N ammonium acetate buffer (pH 4.7) for application to a 5 × 2.2 cm column of reversed-phase C18 adsorbent (Polygosil; Rainin). The column was eluted at 60 mL/h with a 2-propanol gradient of 2.5% per 100 mL of 0.01 N ammonium acetate buffer (pH 4.7). The product was detected by its absorbance at 240 nm and it was eluted at about 3.5% 2-propanol concentration. After rechromatography under the same conditions to remove contaminants, the product gave a single peak by analytical HPLC, and it gave the expected M + 1 ion on chemical ionization mass spectrometry. Its ultraviolet spectrum is shown in Figure 2. Unsuccessful attempts were made to crystallize the product. The yield of isolated product was 63%.

Analytical HPLC analysis of hydrazide was carried out on a 25 × 0.45 cm column of Micropak C18 (Varian). The column was eluted with an 2-propanol gradient from 2.0% to 7.2% in 0.01 N ammonium acetate buffer of pH 4.7 for the first 12 min and another gradient from 7.2% to 12.0% for the following 5 min.

Introduction of Hydrazide Groups into Proteins. To 5 mL of a 4 mg/mL protein solution, corresponding to 0.09 mM for ovalbumin of 44 000 Da, in 0.1 M sodium phosphate buffer of pH 7.8, was added, with rapid mixing, 0.10–0.40 mL of freshly prepared 30 mM *N*-(bromoacetyl)-β-alanyl *N*-hydroxysuccinimide ester in acetonitrile. After 30 min at 25 ± 1 °C, 0.50 mL of 0.44 M *N*-acetylhomocysteinyl hydrazide in pH 7.8 phosphate buffer was added to the reaction mixture. After 5 h, the mixture was dialyzed against 0.1 M sodium acetate buffer of pH 5.2.

The number of bromoacetyl groups introduced was measured from the absorbance decrease at 412 nm of a solution containing about 10 μM modified protein and 0.2 mM 2-nitro-5-thiobenzoic acid in 0.05 M phosphate buffer of pH 7.5 containing 1 mM EDTA after overnight at 25 ± 1 °C (Yun & Suelter, 1970). The ε value at 412 nm used for 2-nitro-5-thiobenzoic acid was 1.36×10^4.

Table I: Ultraviolet Spectral Properties of Some Aldehydes and Their Hydrazones with Acetyl Hydrazide[a]

compound	λ_{max} (nm)	ε
benzaldehyde	249	1.16×10^4
its hydrazone	282	2.33×10^4
p-carboxybenzaldehyde	258	1.52×10^4
its hydrazone	296	2.78×10^4
p-nitrobenzaldehyde	268	1.44×10^4
its hydrazone	318	2.02×10^4
acetaldehyde		
its hydrazone	226	1.18×10^4

[a] All spectra were taken with freshly prepared solutions in 0.1 M sodium acetate buffer of pH 4.73 containing 3.3% ethanol.

The number of hydrazide groups introduced was measured from the absorbance change at 340 nm after reaction of the modified protein (1–6 μM) with 0.5 mM *p*-nitrobenzaldehyde in pH 4.73 acetate buffer containing 2.5% acetonitrile for 5 h at 25 ± 1 °C to form hydrazone. An ε value of 1.35×10^4 at 340 nm was used to calculate the concentration of hydrazone of *p*-nitrobenzaldehyde.

Introduction of Aldehyde Groups into Proteins. To 5 mL of a 4 mg/mL protein solution in 50 mM sodium phosphate buffer of pH 7.8 was added 0.10–0.40 mL of freshly prepared 30 mM *N*-hydroxysuccinimide ester of *p*-carboxybenzaldehyde in acetonitrile with rapid mixing. After 30 min at 25 ± 1 °C the mixture was dialyzed against 0.1 M sodium acetate buffer of pH 5.2. The number of *p*-formylbenzoyl groups introduced was estimated from the absorbance values at 258 and 280 nm of the modified protein. The ε values at these two wavelengths of the modified protein were assumed to be the sum of those of *p*-carboxybenzaldehyde and the native protein. The ε values at 258 and 280 nm for ovalbumin were 1.50×10^4 and 2.99×10^4, respectively, and the corresponding values for *p*-carboxybenzaldehyde were 1.48×10^4 and 4.58×10^3.

Coupling of Aldehyde-Containing Protein with Hydrazide-Containing Protein. This reaction was carried out with the two modified proteins each at about 10 μM concentration in the appropriate buffer at 25 ± 1 °C. At time intervals, aliquots were removed for measurement of hydrazone formation by its absorbance increase at 310 nm; an ε value of 1.98×10^4 for N-acetylhydrazone of *p*-carboxybenzaldehyde was used. Also 20-μL aliquots were used for measurement of the sizes of conjugates formed by gel filtration chromatography on a Du Pont GF-250 column. The column was eluted with 0.2 M phosphate buffer of pH 7.0 at 1 mL/min, and protein was detected by absorbance at 220 nm.

RESULTS

Hydrazone Formation with Model Compounds. The hydrazones of acetyl hydrazide with benzaldehyde, *p*-carboxybenzaldehyde, or *p*-nitrobenzaldehyde were each found to differ in their ultraviolet spectra from those of the corresponding aldehydes. In each case there was a red-shift of the absorption maximum by 30–50 nm as summarized in Table I. This spectral difference of *p*-carboxybenzaldehyde and its hydrazone is shown in Figure 2 and it can be used to monitor changes in their concentrations.

In Figure 3 are summarized the yields of the hydrazone formed from acetyl hydrazide and *p*-carboxybenzaldehyde at 25 ± 1 °C under different conditions. The pH dependence of hydrazone formation is shown in experiments 1–4 of Figure

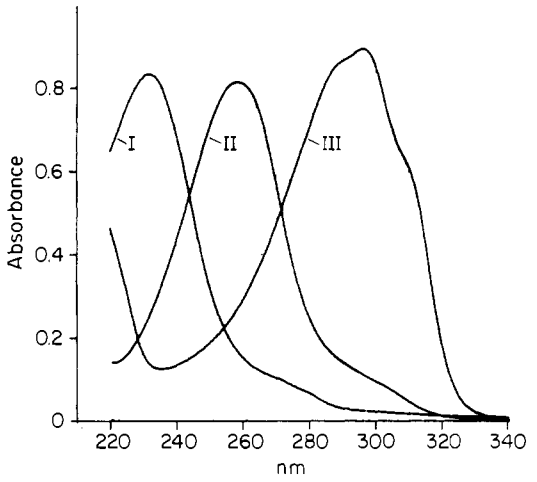

FIGURE 2: Ultraviolet spectra of 1-acetyl-2-(*p*-carboxybenzyl)-hydrazine (I), *p*-carboxybenzaldehyde (II), and *N*-acetylhydrazone of *p*-carboxybenzaldehyde (III). Their concentrations were 97, 47, and 32 μM, respectively, in sodium acetate buffer of pH 4.7. The light path of the cuvette was 10 mm.

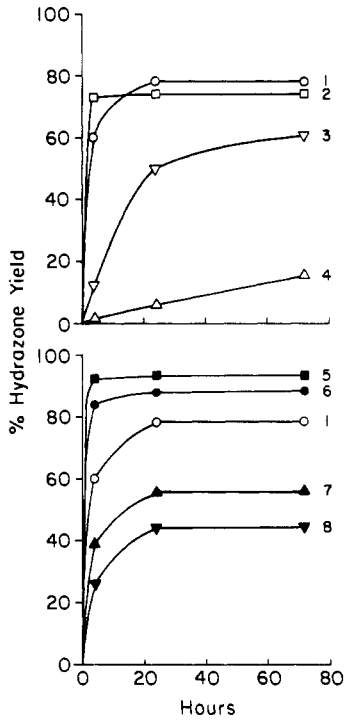

FIGURE 3: Hydrazone formation of acetyl hydrazide with *p*-carboxybenzaldehyde under different conditions. For experiments 1–4, identical initial concentrations of 40 μM aldehyde and 100 μM hydrazide were used and the pH values of the reaction mixtures were 4.73, 4.20, 6.00 and 7.95, respectively. For experiments 5–7, a constant pH of 4.73 and a constant initial concentration of 40 μM aldehyde were used, and the initial concentrations of hydrazide were 500, 250, and 50 μM, respectively. For experiment 8, the reaction mixture at pH 4.73 contained initially 23 μM aldehyde and 25 μM hydrazide. All experiments were carried out at 25 ± 1 °C. The yields of the hydrazones were calculated from absorbance changes at 258 and 296 nm. The compositions of the buffers were pH 7.95, 0.05 M Tris-HCl, pH 6.00, 0.10 M sodium phosphate, and pH 4.73 and 4.20, 0.1 M sodium acetate.

3. The best yield of hydrazone was obtained at pH 4.73 (experiment 1) and the yield decreased at pH 4.2 (experiment 2) or at pH 6.0 and 7.95 (experiments 3 and 4). The results also show that hydrazone formation was rapid at pH 4.73 and 4.20 and the reaction was nearly at equilibrium after about 20 h. However, the reaction was slow at pH 6.00 and 7.95. In experiments 5–8 the influence of reagent concentration on

Table II: Hydrolysis of *N*-Acetylhydrazone of *p*-Carboxybenzaldehyde[a]

pH	% remaining after 18 h	% remaining after 72 h
4.2	59	59
5.0	62	57
6.0	90	64
8.0	99	98

[a] The decrease in hydrazone concentration at 25 °C was followed by spectral changes at 258 and 296 nm. The initial hydrazone concentration was 59.7 μM.

Table III: $NaCNBH_3$ Reduction of *N*-Acetylhydrazone of *p*-Carboxybenzaldehyde[a]

	% yield					
	0.1 M $NaCNBH_3$		0.2 M $NaCNBH_3$		0.4 M $NaCNBH_3$	
	4 h	20 h	4 h	20 h	4 h	20 h
alcohol	5	14	5	8	3	4
aldehyde	3	0	1	0	0	0
hydrazide	26	76	45	92	74	96
hydrazone	66	10	49	0	24	0

[a] Reduction of 0.215 mM hydrazone with $NaCNBH_3$ of different concentrations was carried out in 0.15 N sodium acetate buffer of pH 4.73 at ambient temperature. The mixture was analyzed by HPLC (see Materials and Methods) with 98 ± 8% recovery of starting material and products; the alcohol, aldehyde, hydrazide, and hydrazone were eluted at 9.1, 9.7, 12.4, and 17.0 min, respectively.

hydrazone formation at pH 4.73 was studied. Increasing the molar ratios of the two reagents resulted in increasing yields of hydrazone as shown in experiments 1, 5, 6, and 7, in which the molar ratio of hydrazide to aldehyde varied from 1.2 to 12. Decreasing the concentrations of reagents resulted in lesser yield of hydrazone as shown by comparing experiments 7 and 8, which differ in their reagent concentration by twofold. These results are in accord with the fact that hydrazone formation is a general acid catalyzed reversible reaction (Hammett, 1940).

We next studied the susceptibility of hydrazones to undergo hydrolysis. In Table II are summarized the results obtained with the *N*-acetylhydrazone of *p*-carboxybenzaldehyde in the pH region 4–8. The hydrazone was susceptible to hydrolysis at pH values less than 7. The rate of hydrolysis was much greater at pH 5 than at pH 6. From the data of Figure 3 and Table II, one can calculate that the equilibrium constant for formation of hydrazone is about 4.2×10^4 M^{-1} at pH 4–5. Similar studies for the hydrazones of acetylhydrazide with benzaldehyde, *p*-nitrobenzaldehyde, or acetaldehyde showed their equilibrium constants to be about 2.2×10^4, 1.8×10^5, and 5×10^3, respectively. The results indicate that hydrazones formed from aldehydes with electron-withdrawing groups are more stable than those of aldehydes with electron-donating groups.

N-Acetylhydrazone of *p*-carboxybenzaldehyde was reduced very slowly if at all by $NaBH_4$ or $NaCNBH_3$ at pH 9. It was reduced by $NaCNBH_3$ at pH < 6 and 25 ± 1 °C to give the corresponding hydrazide. The reduction was faster at pH 3.6 than that at pH 4.73, but there was a greater extent of hydrolysis of hydrazone at the more acidic pH. The results in Table III show that greater than 90% yield of the hydrazide was obtained by carrying out the reduction at pH 4.73 in the presence of a large excess of $NaCNBH_3$.

Modification of Proteins To Contain Acyl Hydrazide or Aryl Aldehyde Groups. Hydrazide groups were introduced upon acylation of the amino groups of 0.1 mM proteins at pH 7.8 with 0.5–2.2 mM *N*-(bromoacetyl)-β-alanyl *N*-hydroxy-

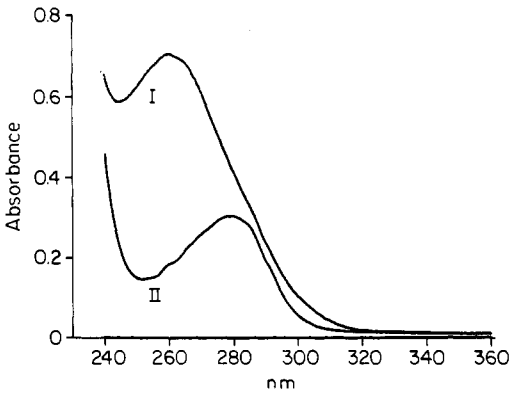

FIGURE 4: Ultraviolet spectra of ovalbumin (I) modified with 1.1 mM *p*-carboxybenzaldehyde *N*-hydroxysuccinimide ester and of ovalbumin (II) modified with 1.1 mM *N*-(bromoacetyl)-β-alanyl *N*-hydroxysuccinimide ester followed by reaction with *N*-acetylhomocysteinyl hydrazide. The protein concentrations were 7.8 and 9.8 μM, respectively, in sodium acetate buffer of pH 5.2. The light path of the cuvette was 10 mm.

succinimide ester, followed by reaction with 38 mM *N*-acetylhomocysteinyl hydrazide (eq 1 and 2 of Figure 1). It was not necessary to isolate the intermediate product since there was quantitative conversion of the bromoacetyl groups to the hydrazide-containing groups. The average number of bromoacetyl groups of the modified protein was determined spectrophotometrically on reaction with 1-nitro-4-thiobenzoic acid, and the average number of hydrazide groups was determined also spectrophotometrically after reaction with *p*-nitrobenzaldehyde to form hydrazone groups.

Aldehyde groups were introduced upon acylation of the amino groups of 0.1 mM proteins at pH 7.8 with 0.5–2.2 mM *N*-hydroxysuccinimide ester of *p*-carboxybenzaldehyde (eq 3 of Figure 1). Ovalbumin modified with aryl aldehyde groups has an ultraviolet spectrum different from that of ovalabumin modified with hydrazide groups, as shown in Figure 4. The average number of aldehyde groups of the modified protein was determined from the absorbance values at 256 and 280 nm on the assumption that the molar extinction coefficient at each of these two wavelengths of the modified protein is the sum of the corresponding values of *p*-carboxybenzaldehyde and the native protein. Alternatively, the average number of aldehyde groups was determined from the absorbance values at 280 and 296 nm following reaction of the modified protein with an excess of acetyl hydrazide. Again the assumption was made that the molar extinction coefficients of the modified protein at each of these two wavelengths is the sum of the corresponding values of the *N*-acetylhydrazone of *p*-carboxybenzaldehyde and the native protein. There was good agreement (±15%) of the number of aldehyde groups determined by these two methods.

As would be expected, the average number of aldehyde or hydrazide groups introduced per mole of protein varied with the concentration of *N*-hydroxysuccinimide ester used for the modification. For the model protein ovalbumin, the average numbers of aldehyde groups introduced were 3.3, 4.7, and 6.4 when the active ester concentrations were 0.55, 1.1, and 2.2 mM, respectively. The average numbers of hydrazide groups introduced into ovalabumin were 3.1, 6.4, and 8.5 when the active ester concentrations were similarly varied. Modification studies were also made with bovine plasma albumin, glucose oxidase, lysozyme, and sheep γ-globulin with similar results (results not given).

Conjugate Formation of Aldehyde- and Hydrazide-Containing Proteins. The reaction (eq 4 of Figure 1) was studied at pH 4.6–5.9 and 25 ± 1 °C with ovalbumins of different

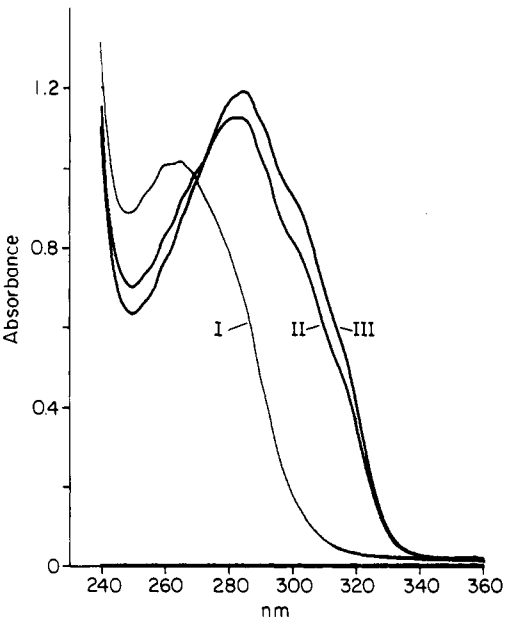

FIGURE 5: Ultraviolet spectra of a mixture of 8.3 μM ovalbumin with 4.7 aldehyde groups and 10.5 μM ovalbumin with 6.4 hydrazide groups in 0.1 M sodium acetate buffer of pH 5.2 at 0, 20, and 48 h (I, II and III, respectively). The light path of the cuvette was 10 mm.

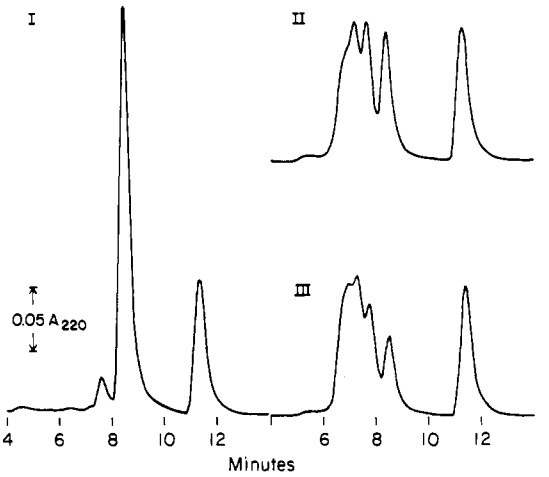

FIGURE 6: Gel filtration analysis of a mixture of 8.3 μM ovalbumin with 4.7 aldehyde groups and 10.5 μM ovalbumin with 6.4 hydrazide groups in 0.1 M sodium acetate buffer of pH 5.2 at 0, 20, and 48 h (I, II, and III, respectively). The peaks eluted in the region of 6–10 min represent ovalbumins of different sizes and that at 11.3 min is due to acetate buffer present in the reaction mixture. The small dimer peak, eluted at 7.7 min in chromatogram I, was present in the ovalubmin sample prior to modification.

degrees of substitution. Hydrazone formation was followed spectroscopically, and the extent and the size of conjugates formed were followed by gel filtration chromatography. In Figure 5 are shown the ultraviolet spectral changes of the reaction at pH 5.2 of ovalbumins substituted with about five aldehyde and hydrazide groups. There was a decrease of the aldehyde peak at 260 nm with time accompanied by an increase of the hydrazone peak at 296 nm. The hydrazone formed could be estimated from the increased in absorbance at 310 nm, a spectral region where ovalbumin has negligible absorption. The hydrazone yields were 58% and 61% at 20 and 48 h, respectively, based on the concentration of aldehyde-containing ovalbumin used.

In Figure 6 are shown the gel filtration chromatograms of the reaction mixtures of Figure 5. The monomeric ovalbumin peak at 8.5 min decreased with time while the dimer, trimer,

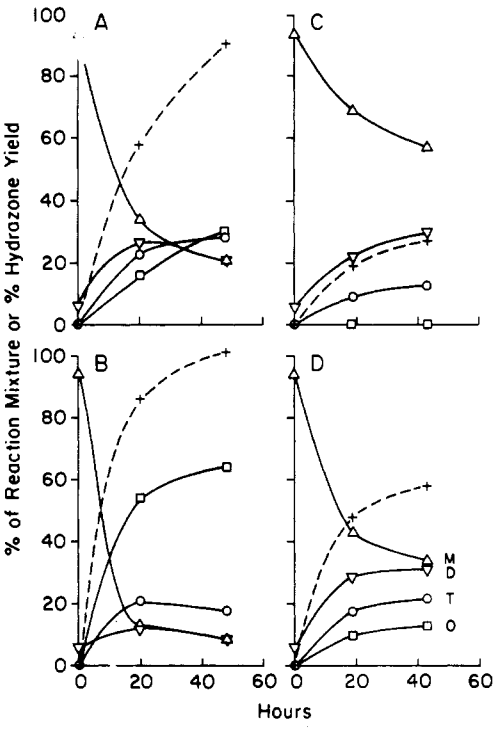

FIGURE 7: Conjugate formation of modified albumins at pH 5.2 (A and C) and pH 4.6 (B and D). A mixture of 8.3 μM ovalbumin with 4.7 aldehyde groups and 10 μM ovalbumin with 6.4 hydrazide groups was used in A and B. A mixture of 10 μM ovalbumin with 3.3 aldehyde groups and 6.4 μM ovalbumin with 3.1 hydrazide groups was used in C and D. Each reaction was analyzed for its hydrazone yield (+) and for it monomer (△), dimer (▽), trimer (○), and oligomer (□) contents.

and tetramer together with higher oligomer peaks at 7.7, 7.2, and 6.9 min, respectively, increased in size. Since the tetramer and higher oligomer peaks were not well resolved in the chromatograms, these two peaks were grouped together for assessing the yields of conjugates of different sizes, as was done in Figure 7 together with the results of three other experiments.

The experiments in Figure 7 were carried out at 25 ± 1 °C with 6–10 μM concentrations of the two modified ovalbumins. The four experiments differ in the modified ovalbumins with about three or five substituents per mole and they differ in the pH of the reaction, 4.6 or 5.2. The hydrazone yield and the size distribution of conjugates for each experiment are given in Figure 7.

The extent of hydrazone formation was found to depend on the pH as well as on the degree of substitution of the modified proteins. The pH dependence is seen by comparing the experiments at pH 5.2 and 4.6 (Figure 7, A and B or C and D). Conjugate formation was also studied at pH 5.90 and it was less extensive than that at pH 5.2 (results not shown). These results are accord with those of model compounds in Figure 3 that hydrazone formation proceeded to equilibrium more rapidly at acidic pH than at neutral pH. The increasing yield of hydrazone with increasing degree of substitution of ovalbumin is illustrated by comparing experiments in Figure 7, A and C, or in Figure 7, B and D.

The hydrazone yields of the experiments in Figure 7 ranged from 30% to 100% and they were calculated on the basis of the limiting aldehyde or hydrazide component used. As indicated earlier, the equilibrium constant for hydrazone formation of acetyl hydrazide and *p*-carboxybenzaldehyde is 4.2 × 10^4 M^{-1} at pH 4–5. With this value the expected hydrazone yield is 24% on mixing two 10 μM solutions of ovalbumins, each containing one aldehyde or hydrazide group. For equilibrium constants of 4.2 × 10^6 and 4.2 × 10^8 M^{-1}, the calculated hydrazone yields are 86% and 99%, respectively.

The rate of formation of conjugates of different sizes and their yields depends also both on the pH of the reaction mixture and on the degree of substitution of ovalbumins. The decrease in monomer content of the reaction mixtures shows that conjugate formation was more rapid at pH 4.6 (Figure 7, B and D) than that at pH 5.2 (Figure 7, A and C).

Dimer, trimer, and oligomer conjugates of ovalbumin were isolated from a reaction mixture corresponding to that of Figure 7A at 20 h. The isolation was done on a preparative scale by gel filtration on a 200 × 0.9 cm column of Sephadex G-100. The separated conjugates were found to be stable for at least 1 month on storage as approximately 10 μM solutions in 0.1 M phosphate buffer of pH 7.0, as no significant change of their gel filtration pattern was observed. These results are not shown.

Conjugate formation was also observed with modified bovine plasma albumins, bovine plasma albumin + ovalbumin, lysozyme + ovalbumin, and sheep γ-globulin + glucose oxidase. These results are not give.

Reduction of Hydrazone Linkages of Conjugate Proteins. This was tested with conjugate proteins that were obtained on coupling of ovalbumins substituted with about six aldehyde and hydrazide groups. Following addition of 180 μM $NaCNBH_3$ to 5–10 μM conjugate proteins at pH 4.9, the absorbance of the mixture in the region 250–340 nm decreased steadily and the spectral changes were in close accord with those observed on reduction of the model compound *N*-acetylhydrazone of *p*-carboxybenzaldehyde. The concentration of the remaining hydrazone linkages could be determined from the absorbance at 310 nm. In this way we estimated the yield of reduction to be 66% and 79% respectively at 24 and 48 h for a sample of conjugate proteins initially containing 24 μM hydrazone linkages. The reduction also proceeded at pH 5.2 but the reaction was slower.

In the presence of $NaCNBH_3$, any remaining aldehyde groups of the conjugate proteins can in principle undergo inter- or intramolecular reductive arylation of the amino groups of conjugate proteins. This arylation reaction is not believed to occur, as no changes in the distribution of oligomer, trimer, dimer, and monomer were detected for the conjugate proteins of ovalbumin following reduction. Furthermore, the model compound *p*-carboxybenzaldehyde was rapidly reduced to its alcohol under the reducing conditions used (Table III).

Sensitivity of Hydrazone Linkage in Conjugate Proteins to Exchange with Acetyl Hydrazide. Treatment of conjugate proteins of ovalbumin with an excess of acetyl hydrazide led to a decrease of its oligomer contents with a concomitant increase of its monomer content. This is shown in Figure 8 for a conjugate mixture that initially contained 11% ovalbumin monomer, and its monomer content rose to 90% following reaction with acetyl hydrazide. The reaction was rapid at pH 4.7 and 5.2 but slow at pH 7.0. Reduction of conjugates with 100 mM $NaCNBH_3$ at pH 4.9 or 5.2 for 24 h prior to treatment with acetyl hydrazide prevented the decrease in oligomer and polymer contents.

Discussion

The above results show that aldehyde- and hydrazide-containing proteins can condense with each other to form oligomeric conjugates that are bound by hydrazone linkages. The size of the conjugates formed and their yields are shown to depend on the pH of the reaction and on the degree of substitution of modified proteins. The yields of protein conjugates are greater than expected from the known equilibrium con-

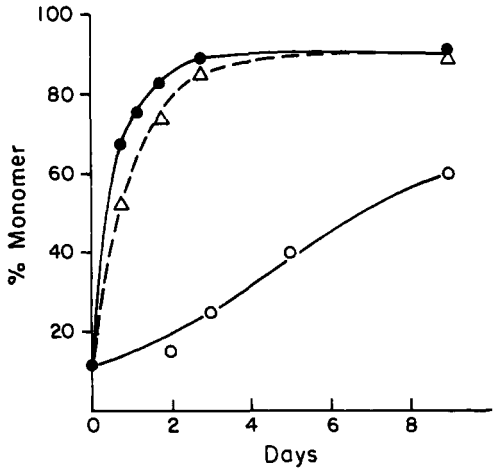

FIGURE 8: Reaction of 18 mM acetyl hydrazide with 0.14 mM ovalubmin conjugates at pH 4.7 (●), 5.2 (△), and 7.0 (○). The reaction was carried out at 25 ± 1 °C with a mixture of conjugates of different sizes. The course of reaction was followed by gel filtration chromatography.

stants for hydrazone formation of the model compounds acetyl hydrazide and *p*-carboxybenzaldehyde. This difference is probably a consequence of the presence of multiple hydrazone linkages in each conjugate. For example, it is known that multisite interactions of antigen and antibody molecules enhance the stability of the resulting complex (Thompson & Jackson, 1984).

Hydrazide groups can, in principle, be introduced into proteins by carbodiimide-catalyzed condensation of the carboxyl groups of proteins with hydrazine. We have used a two-step procedure for this purpose, by acylation of the amino groups of proteins with an active ester of *N*-(bromoacetyl)-β-alanine followed by reaction with *N*-acetylhomocysteinyl hydrazide. This two-step procedure was chosen as it is possible to vary the length of the spacer between the protein and the hydrazide group. We have in fact made modifications using active esters of bromoacetic acid and *N*-(bromoacetyl)-6-aminohexanoic acid in place of the active ester of *N*-(bromoacetyl-β-alanine. When these hydrazide-containing proteins with longer or shorter spacer arms were tested for conjugate formation, no significant difference was observed (results not given).

The procedure for introduction of aryl aldehyde groups into proteins had been developed for preparing a peptide–antibody conjugate via Schiff base formation at pH 9 followed with reduction to yield a stable benzylamine linkage (Kraehenbuhl et al., 1974). Schiff base formation also occurs at neutral pH but the equilibrium is in favor of the starting materials; only in the presence of $NaCNBH_3$ can the reaction be driven to completion upon reductive formation of stable alkylamine linkages (Jentoft & Dearborn, 1983). In neutral and acidic pH the hydrazone linkage is more stable than the Schiff base linkage is, as it is possible to isolate protein hydrazone conjugates. Hydrolysis of the protein hydrazone conjugate takes place very slowly at neutral pH, if at all, and it can be prevented following reduction with $NaCHBH_3$ to give stable hydrazide linkages. The reversible nature of hydrazone linkages can be useful for certain special applications as the conjugates are dissociated readily by an exchange reaction with an excess of acetyl hydrazide.

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Registry No. PhCHO, 100-52-7; PhCH=NNHAc, 940-48-7; *p*-$HO_2CC_6H_4CH$=NNHAc, 103851-19-0; *p*-$HO_2CC_6H_4CHO$, 619-66-9; *p*-$O_2NC_6H_4CHO$, 555-16-8; *p*-$O_2NC_6H_4CH$=NNHAc, 25996-47-8; AcH, 75-07-0; MeCH=NNHAc, 20156-05-2; *p*-$AcNHNHCH_2C_6H_4CO_2H$, 103883-54-1; $AcNHNH_2$, 1068-57-1.

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