Affinity Labeling of Rabbit Antisaccharide Antibodies*

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ABSTRACT: Rabbit antihapten antibodies against *p*-azophenyl β-lactoside and *p*-azophenyl β-galactoside determinants have been studied with diazoniumphenyl glycosides as affinity labeling reagents. Specific labeling of the combining regions of both antisaccharide antibodies has been demonstrated by the fact that a reversibly binding hapten affords clear-cut protection against the particular labeling reaction. As in earlier studies with several antibenzenoid hapten antibodies, both H and L chains are labeled. Azo spectra of H chains from both labeled antisaccharide antibodies show selective labeling at tyrosine residues; the L chains are characterized by a discrete visible spectrum which is markedly different from that of azotyrosine, azohistidine, or azolysine, the modified residues usually identifiable in azoproteins. While the affinity labeling patterns for both antisaccharide antibodies are identical, they are clearly distinguishable from those obtained in the antibenzenoid hapten systems which reveal specifically labeled tyrosine residues on both H and L chains.

Within the last few years several rabbit antihapten antibodies have been labeled specifically by the method of affinity labeling (Wofsy *et al.*, 1962; Metzger *et al.*, 1963; Fenton and Singer, 1965). This procedure involves the use of specific reagents which are haptens possessing reactive functional groups, that is, haptens which when bound at the antibody active site can react further to form covalent bonds with contact amino acid residues at the site.

Strikingly similar results were obtained with affinity labeling of antibenzene arsonate, antidinitrophenyl, and antitrimethylanilinium antibodies. It was shown by a variety of criteria that highly specific labeling of active sites was obtained in each case. Also, in each case, the predominant product of the reaction with specific diazonium affinity labeling reagents was azotyrosine. Furthermore, when the labeled antibodies were reduced, alkylated, and fractionated into H and L polypeptide chains, both chains showed discrete azotyrosine spectra, indicating that in each of these antibody systems, the H and L chains each contribute one or more tyrosines to the active sites (Metzger *et al.*, 1964; Singer and Doolittle, 1966).

In the present study, we have adapted affinity labeling to the examination of antibodies directed against saccharide determinants. Our primary focus has been the reaction of anti-Lac[1] antibodies with the reagent OD-Lac[1] (Figure 1). The selection of this system for study was based on the fact that it differs markedly in specificity from the antibenzenoid derivative antibodies labeled earlier, in that the contribution of the hapten phenyl ring to the binding interaction with anti-Lac antibodies is minor (Karush, 1957). Thus, one might be able to ascertain if the patterns observed in the earlier labeling studies would be expected to hold generally for all antibody active sites, or whether they might be somewhat restricted to antibodies against determinants with a critical phenyl (or other hydrophobic) component. The *ortho* reagent was chosen so that the diazonium functional group would be in closest proximity to the disaccharide moiety. Parallel to the affinity labeling studies with anti-Lac antibodies, we have examined the reaction of anti-Gal[1] antibodies with OD-Gal.[1]

Materials and Methods

Antigens and Antibodies. Azoprotein conjugates used as immunizing or precipitating antigens were prepared as previously described (Corneil and Wofsy, 1967). In the preparation of anti-Lac or anti-Gal antibodies, the immunizing antigen was the highly coupled azoconjugate of the particular *p*-aminophenyl β-glycoside and keyhole limpet hemocyanin, the latter being isolated and purified according to the procedure of Campbell *et al.* (1963). The carrier protein for the corresponding precipitating azoantigen was human fibrinogen (fraction I, Nutritional Biochemical Corp.), which was succinylated (Habeeb *et al.* 1958) prior to the azo coupling reaction. Antibodies were isolated from pooled, high-titer antisera from rabbits hyperimmunized by standard procedures, which included the use of Freund's complete adjuvant. Specific antibody purification protocols for anti-Lac and anti-Gal were essentially those described for anti-Lac antibodies by Utsumi and Karush (1964)

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[1] Abbreviations used: anti-Lac, anti-Gal, and anti-DNP, antibodies specific for the respective haptenic groups *p*-azophenyl β-lactoside, *p*-azophenyl β-galactoside, and ε-amino-2,4-dinitrophenyl; OD-Lac, the reagent *o*-diazoniumphenyl β-lactoside; OD-Gal, the reagent *o*-diazoniumphenyl β-galactoside; PD-Lac, the reagent *p*-diazoniumphenyl β-lactoside; SAS, saturated ammonium sulfate; SDS, sodium dodecyl sulfate.

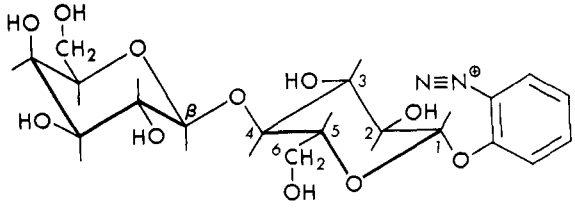

FIGURE 1: Structural formula of OD-Lac reagent.

except for the use of azosuccinylated fibrinogen-precipitating antigens, and the substitution of the corresponding galactose haptens for lactose and *p*-nitrophenyl β-lactoside in the anti-Gal purification. Anti-Gal antibodies were equilibrated with 0.1 M lactose prior to exhaustive dialysis against 0.02 M sodium phosphate–0.17 M sodium chloride buffer (pH 7.2). Antibodies were stored under 40% SAS.[1] Specifically purified anti-DNP antibodies were prepared by the method of Farah *et al.* (1960).

Synthesis of Nitrophenyl and Aminophenyl β-Glycosides. Starting with β-lactose or β-D-galactose (both from Matheson Coleman and Bell), the acetobromo sugar was prepared as described by Bárczai-Martos and Kőrösy (1950). The acetobromo sugar was condensed with *o*- or *p*-nitrophenol (Matheson Coleman and Bell) according to the procedure of Latham *et al.* (1950). Deacetylation was carried out with sodium methylate in methanol (Thompson *et al.*, 1963) to yield the nitrophenyl β-glycoside. The corresponding aminophenyl β-glycoside was obtained by catalytic reduction, 5% palladium on alumina, in aqueous methanol under 45 psi of hydrogen at room temperature.

Melting points and specific rotations on *p*-aminophenyl β-lactoside, the *o*- and *p*-aminophenyl β-galactosides, and on their synthetic precursors corresponded to published literature values (Babers and Goebel, 1934; Seidman and Link, 1950; Beiser *et al.*, 1960). Data are presented below for *o*-aminophenyl β-lactoside and the derivatives prepared at stages in its synthesis.

Heptacetyl-o-nitrophenyl β-lactoside was recrystallized twice from methanol and dried *in vacuo* over P_2O_5, mp 175°.

o-Nitrophenyl β-lactoside was recrystallized twice from methanol and dried *in vacuo* over P_2O_5, mp 196–198° dec; λ_{max}^{KBr} 2.8, 3.4, 6.2, 6.4, 6.7, 7.4, and 8.0 μ; $[\alpha]_D^{23°}$ −51.6° (*c* 0.620, water).

o-Aminophenyl β-lactoside was recrystallized from 80% ethanol and dried *in vacuo* at 40° over KOH, drierite mp 189–190° dec; λ_{max}^{KBr} 2.8, 3.4, 6.1, 6.6, 7.1, 7.9, and 8.2 μ; $[\alpha]_D^{23°}$ −33.3° (*c* 0.900, water).

Anal. Calcd for $C_{18}H_{27}NO_{11}$: C, 49.87; H, 6.29; N, 3.23. Found: C, 49.47; H, 6.52; N, 3.34.

Infrared spectra were determined with a Perkin-Elmer Model 137B Infracord spectrophotometer.

Specific rotations were analyzed with a Bendix automatic polarimeter with 0.1-dm cells.

*Azo Model Compounds. o-(o'-*AZOPHENYL β-LACTOSIDE)-*N*-CHLOROACETYL-L-TYROSINE. The monoazo derivative of OD-Lac and *N*-chloroacetyl-L-tyrosine (Mann CP, mp 195°) was prepared according to procedures used for model monoazotyrosine compounds by Tabachnick and Sobotka (1959). After precipitation at pH 2, the product was washed with ice-cold water, recrystallized three times from 95% ethanol, and dried to constant weight *in vacuo* over KOH, drierite at 45–50°, mp 187–189° dec.

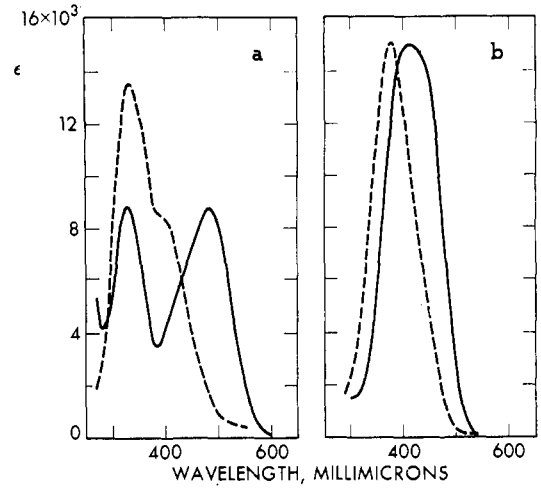

FIGURE 2: Spectra at pH 6.2 (---------) and in 0.15 N (———) of model *o*-azophenyl β-lactosides formed by reaction of OD-Lac with *N*-chloroacetyltyrosine (a) and *N*-acetylhistidine (b). Spectra of the analogous galactoside azo derivatives, formed with OD-Gal, are the same.

Anal. Calcd for $C_{29}H_{36}ClN_3O_{15}\cdot H_2O$: C, 48.40; H, 5.34; N, 5.85. Found: C, 48.34; H, 5.58; N, 5.96.

Descending paper chromatography (S & S orange) in butanol–acetic acid–water (4:1:1, v/v) resulted in a single reddish-brown spot, R_F 0.34; thin layer chromatography (tlc) on silica gel (Eastman Kodak K 310R2) in the same solvent system gave a single colored spot, R_F 0.50. Both chromatograms also showed single coincident spots when subjected to ultraviolet light (long wavelength) and to spraying with α-nitroso-β-naphthol (Archer and Crocker, 1952). Visible spectra at pH 6.2 and in 0.15 N NaOH were recorded on a Cary Model 14 spectrophotometer (Figure 2a).

Other azo model compounds were not isolated, but their spectra were determined by the general procedure of Tabachnick and Sobotka (1959) reacting a limiting amount of diazonium reagent with a 100-fold excess of *N*-acetyl-L-histidine (A grade, Calbiochem) or *N*-chloroacetyl-L-tyrosine. Spectra of the monoazo histidine (Figure 2b) and tyrosine derivatives with OD-Lac were the same as those obtained with OD-Gal. Bis-(*o*-azophenyl β-lactoside)-ε-aminocaproic acid, prepared by reacting ε-aminocaproic acid (grade I, Cyclo) in excess OD-Lac, showed the same spectral maxima as those reported by Tabachnick and Sobotka (1959) for similarly prepared bis-*p*-azophenyl derivatives of ε-aminocaproic

acid, λ_{max} 360–365 mμ at pH 6.2, λ_{max} 380–385 mμ in 0.15 N NaOH; extinctions were not determined.

Diazonium Reagents and Labeling Reaction Procedures. Diazonium reagents were prepared fresh for each labeling experiment as follows. Aminophenyl glycoside (10 μmoles) was dissolved in 0.45 ml of 0.1 N HCl. Maintaining a temperature of 0–5° throughout, 12 μmoles of sodium nitrite was added in 0.05 ml of water; after 10 min, another 4.5 ml of ice water was added to make the stock solution approximately 2×10^{-3} M in reagent. After 45–60 min, desired amounts of the reagent solution were added to the buffered protein solutions. The buffer in which all labeling reactions were performed was 0.12 M sodium borate–0.17 M sodium chloride (pH 8.0).

To check the normality of a reagent stock solution, 0.05-ml aliquots were added to 2.00-ml volumes of 0.01 M *N*-acetylhistidine in 0.1 μ sodium borate buffer (pH 9). After several hours at 5°, the test solutions were allowed to stand at room temperature for 1–2 hr until color development was complete. Absorbance of azo-*N*-acetylhistidine was determined at 370 mμ; at pH 9, ϵ_M is 1.5×10^4 for the OD-Lac and OD-Gal derivatives; ϵ_M is 2.1×10^4 for the PD-Lac derivative. When reagent stock solutions, maintained at 0–5°, were checked in this manner over a 6-hr period, no degradation of the diazoniums was detected except for OD-Gal solutions, in which available reagent diminished by about 8% over 3 hr, 13% over 6 hr. A series of normality checks with each of the diazonium reagents were also performed in the presence and absence of a 10^{-3} M concentration of the corresponding *p*-nitrophenyl glycoside, since the latter conditions were employed in some experiments to test hapten protection against specific labeling of antibodies. No effect by the *p*-nitrophenyl haptens could be observed on either the rate or extent of azo formation in the normality test solutions.

Labeling reactions with whole unreduced proteins were terminated either by making reaction solutions 75% in cold ethanol or 50% in cold SAS. In experiments in which 10^{-3} M *p*-nitrophenyl β-glycoside was used as protector in some solutions, the unprotected samples were also made 10^{-3} M in the protector hapten just prior to reaction termination and work-up. Where ethanol precipitation was used for termination, noncovalently bound reagent and small molecule by-products were removed by repeated alcohol and saline washes as described previously (Wofsy *et al.*, 1962). Where SAS was employed, the precipitate was spun down, then dissolved in a specific hapten solution, 0.02 M *p*-nitrophenyl β-glycoside; precipitation with 50% SAS was repeated three more times, the precipitate being redissolved once again in the 0.02 M hapten solution and then twice in distilled water; samples were then exhaustively dialyzed against distilled water and lyophilized prior to spectral analysis.

Where labeling studies were undertaken with the intention of examining separated H and L chains, antibodies were first reduced in 0.2 M mercaptoethanol and alkylated with iodoacetate according to the procedure of Fleischman *et al.* (1963). After dialysis against pH 8 borate–saline buffer, reduced antibodies were reacted with the affinity labeling reagent and reactions were terminated by dialysis against sodium acetate buffer, 0.1 μ, pH 5.5, 5°. Chains were separated by chromatography on Sephadex G-100 in 1 M propionic acid as described by Metzger and Mannik (1964). Fractions were dialyzed exhaustively against distilled water and lyophilized prior to spectral assay.

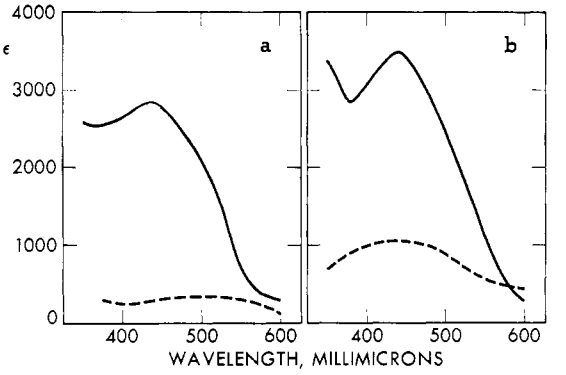

FIGURE 3: Azo spectra in 0.15 N NaOH of antibodies labeled with OD-Lac. The solid lines in a and b represent anti-Lac reacted in absence of protector. The dashed line in a represents anti-DNP. The dashed line in b represents anti-Lac reacted in the presence of protector, *p*-nitrophenyl β-lactoside. Extinction coefficients (ϵ) are based on 150,000 g/l.

Spectral assays on protein samples were performed in 0.02 M sodium phosphate–0.5% SDS (unless otherwise specified). Azo difference spectra were determined by subtracting the background absorbance of unmodified protein from equivalent concentrations of the reacted protein, as previously described (Wofsy *et al.*, 1962). All protein spectral determinations were made with a Zeiss spectrophotometer PMQ II. Protein concentrations were determined from the extinctions at 280 mμ and pH 6.2: for antibody, $\epsilon^{1\%}_{280\ m\mu}$ 14.6; for H chain, $\epsilon^{1\%}_{280\ m\mu}$ 14.5; and for L chain, $\epsilon^{1\%}_{280\ m\mu}$ 13.2 (Utsumi and Karush, 1964).

Other Materials and Chemicals. Rabbit normal γ-globulin was isolated from pooled nonimmune sera by precipitating three times with 33% SAS.

Sodium decyl sulfate was synthesized by the procedure of Dreger *et al.* (1944) and was recrystallized twice from 95% ethanol.

All chemicals not identified as to source were reagent grade.

Results

A. The Reaction of Anti-Lac with OD-Lac. Solutions (1.8×10^{-5} M) of anti-Lac and anti-DNP were reacted separately with 2.2×10^{-5} M concentrations of OD-Lac at pH 8.0, 5°, for 90 min. After purification of the reacted antibodies, their spectra were compared in 0.15 N NaOH–0.5% SDS. As shown in Figure 3a, azo modification of the anti-DNP was very slight; between 375 and 450 mμ, azo absorbance of the anti-Lac is from 8.5 to 10.6 times greater than that of the anti-DNP.

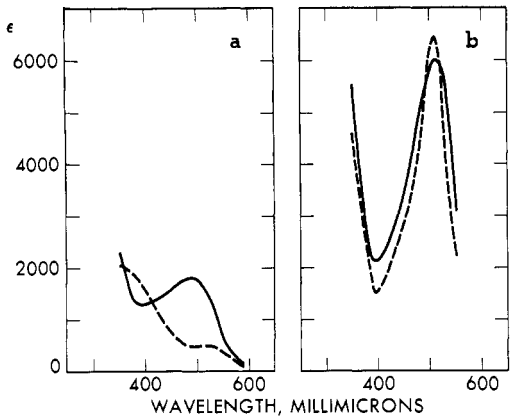

FIGURE 4: Azo spectra at pH 6.2 (---------) and in 0.15 N NaOH(———) of the polypeptide chains of anti-Lac antibodies labeled with OD-Lac: H chains (a) and L chains (b). For purposes of comparison, values for ϵ are based on 150,000 g/l.

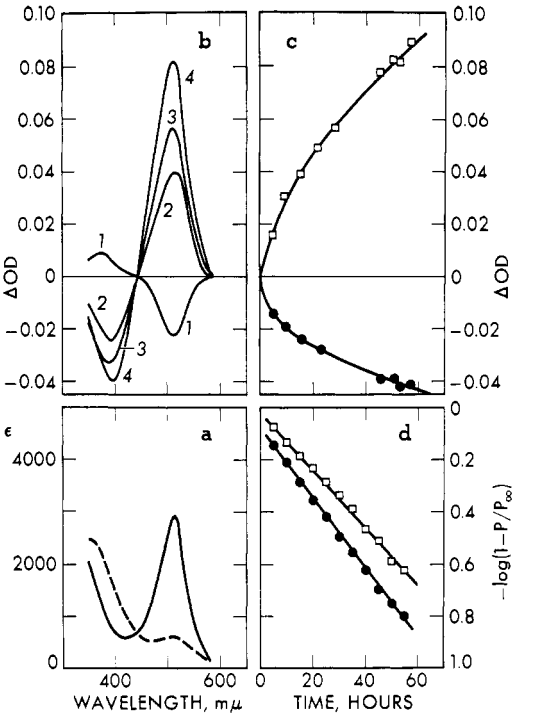

FIGURE 5: Spectral change in a detergent solution (pH 6.2) of affinity labeled anti-Lac L chains. (a) The dashed line represents azo spectrum in water before detergent treatment. The solid line represents azo spectrum after 53 hr in 1% sodium decyl sulfate. ϵ values are based on 150,000 g/l. (b) Azo difference spectra for a 8-mg/ml solution at various times. The base-line time is 3 hr after dissolution of the labeled L chains in detergent, and curve 1 is the difference spectrum determined with an 8-mg/ml aqueous solution prior to detergent treatment. Curves 2, 3, and 4 are the difference spectra 18, 31, and 53 hr, respectively, after dissolution in detergent. (c) ΔOD values for the detergent solution (8 mg/ml) at 510 (open squares) and 390 mμ (closed circles). (d) P/P_∞ represents the fraction of the change in absorbance at 510 (open squares) or 390 mμ (closed circles); P_∞ is estimated as corresponding to $\Delta OD_{510\ m\mu} = 0.11$ and $\Delta OD_{390\ m\mu} = 0.05$.

In order to determine whether anti-Lac antibody combining sites can be specifically protected against reaction with OD-Lac, two 2.8×10^{-5} M solutions of anti-Lac antibodies, one of which was 10^{-3} M in *p*-nitrophenyl β-lactoside, were reacted with 4.1×10^{-5} M concentrations of the reagent at pH 8.0, 25°, for 120 min. The resulting azo spectra of the reacted antibodies in 0.15 N NaOH–0.5% SDS are shown in Figure 3b. Between 375 and 450 mμ, the azo absorbance of the unprotected antibodies is from 3.1 to 3.4 times greater than that of the protected antibodies.

The distribution of label on the H and L polypeptide chains of anti-Lac antibodies was examined with a mildly reduced, alkylated preparation of antibodies, which was reacted at a 2.0×10^{-5} M protein concentration with 2.2×10^{-5} M OD-Lac at pH 8.0, 5°, for 45 min. After fractionation into separate H and L chain pools, dialysis, and lyophilization, azo spectra of samples from each pool were determined in 0.5% SDS, both at pH 6.2 and in 0.15 N NaOH, as shown in Figure 4. The labeled H chains show a typical azotyrosine alkaline shift, with a characteristic alkaline peak around 485 mμ, corresponding very closely to the spectrum of *o*-(azo-*N*-chloroacetyltyrosine)phenyl β-lactoside (Figure 2). The labeled L chains were easily distinguished from the labeled H chains by the pink color which developed on standing overnight in 0.5% SDS–0.002 M phosphate buffer (pH 6.2). As can be seen in Figure 4, the L chain azo spectrum shows no alkaline shift and bears no resemblance to the spectrum of the model compounds of azotyrosine or azohistidine (Figure 2) or bisazo-ε-aminocaproic acid (see Methods).

The chain study was repeated, labeling a larger preparation of mildly reduced and alkylated anti-Lac antibodies. The concentration of the latter was 2.0×10^{-5} M, the OD-Lac concentration was 1.6×10^{-5} M, and the reaction time was shortened to 25 min at pH 8.0, 5°. Whereas in the first chain study the spectra examined were of samples from complete H or L chains pools spectra were now determined of samples from successive fractions as collected off the G-100 Sephadex column. Three approximately equal H chain fractions (early, middle, and late in the order collected) were each characterized by azotyrosine spectra qualitatively the same as that shown for the labeled H chain pool in Figure 4. Two equal L chain fractions, early and late, showed spectra with the same peak at 510 mμ as was observed with the labeled L chain pool of Figure 4.

Unlike labeled H chains, whose azo spectrum underwent no detectable change on standing for 48 hr in 0.5% SDS at pH 6.2, the characteristic 510-mμ peak of the labeled L chains in 0.5% SDS at pH 6.2 became more intense with time. A sample of labeled L chains, which

had not been exposed to detergent, was stored for 2 months under refrigeration after being lyophilized from water. It was then redissolved in water and its azo spectrum was determined. As may be seen in Figure 5a, there is a small absorbance peak at 510 mμ. The spectrum of the aqueous solution was read again after 16 hr at 5°, and no significant change was recorded. The sample was then lyophilized and redissolved in 1% sodium decyl sulfate at pH 6.2; the spectrum was read after 3 hr at room temperature and again at intervals over a period of 60 hr. In Figure 5a, the azo spectrum in water is compared with that after 53 hr in detergent, the latter showing a marked increase in $\epsilon^{max}_{510\ m\mu}$. Figure 5b shows a series of azo difference spectra as a function of time, with 3 hr after dissolution in detergent taken as the base line; there is an isosbestic point at 440 mμ, and a minimum develops at about 390 mμ as $\epsilon^{max}_{510\ m\mu}$ becomes more pronounced. Figure 5c shows the rates of change of optical density at 390 and 510 mμ for the 8-mg/ml solution; at 60 hr, the increase in $OD_{510\ m\mu}$ is about twice the decrease in $OD_{390\ m\mu}$. The initial spectrum in water and the rate data were used to approximate end-point values for the optical density change at 390 and 510 mμ from the time, t_0, of the initial spectral readings in detergent solution to t_∞, and to calculate the fraction, P/P_∞, of the transformation completed at any given time, t. In Figure 5d, the plots of log $(1 - P/P_\infty)$ against time, based either on $\Delta OD_{390\ m\mu}$ or $\Delta OD_{510\ m\mu}$, indicate first-order kinetics with a rate constant of approximately 0.5–1 $\times 10^{-3}$ min^{-1}.

In order to determine whether the label remained attached to the protein after 60 hr in the detergent solution, the same L chain sample was dialyzed extensively against frequent changes of water; the strongly basic ion-exchange resin IR 400 was then added to several volumes of water to dialyze away the decyl sulfate (Utsumi and Karush, 1964). The pink color remained within the dialysis bag, some of it precipitating with protein as the detergent was eliminated. The contents of the bag were lyophilized and redissolved in the 1% decyl sulfate–phosphate buffer (pH 6.2); the spectrum of the resulting solution showed no significant change from that observed after 60 hr in the original detergent solution.

The spectra of 5×10^{-5} M solutions of the mono-*o*-azophenyl β-lactoside derivatives of *N*-chloroacetyltyrosine and *N*-acetylhistidine in the 1% decyl sulfate–phosphate buffer (pH 6.2) were examined and found to be identical with those in pH 6.2 phosphate without detergent shown in Figure 2. Over a 48-hr period at room temperature, at pH 6.2, no spectral change was observed in detergent solutions of these model compounds or of the bisazo derivative of ε-aminocaproic acid.

B. The Reaction of anti-Gal with OD-Gal. Two aliquots of a 1.6×10^{-5} M reduced and alkylated anti-Gal antibody solution, one of which was 1×10^{-3} M in *p*-nitrophenyl β-galactoside, were reacted for 30 min at a concentration of 1.7×10^{-5} M OD-Gal in borate–saline (pH 8.0), 5°. After work-up, one portion of the unprotected and another of the protected anti-Gal samples were each dissolved in 0.5% SDS–phosphate (pH 6.2)

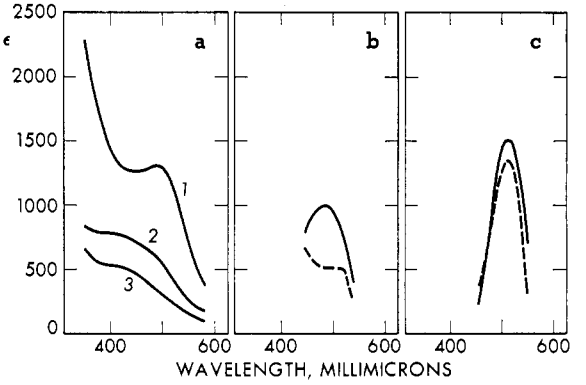

FIGURE 6: Azo spectra of antibodies labeled with OD-Gal. (a) Spectra in 0.15 N NaOH of anti-Gal (curve 1) and anti-Lac (curve 2). The coincident spectra in 0.15 N NaOH of anti-Lac and anti-Gal, each reacted in the presence of its specific *p*-nitrophenyl glycoside protector (curve 3); (b) anti-Gal H chains; (c) anti-Gal L chains at pH 6.2 (---------) and in 0.15 N NaOH (———). ε values are based on 150,000 g/l.

and allowed to stand for 48 hr, after which the azo spectra were compared (Figure 6a). Between 375 and 525 mμ, the alkaline azo extinctions of the unprotected anti-Gal ranged from 2.5 to 4.4 times that of the protected antibodies. The remainder of the unprotected anti-Gal sample was fractionated into H and L chain pools for analysis of azo spectra. The H chain azo spectrum (Figure 6b) is characterized by the alkaline shift and alkaline peak at 480–485 mμ which identifies the model monoazotyrosine derivative formed by reacting OD-Gal with excess *N*-chloroacetyltyrosine (Figure 2). The spectrum of the L chains (Figure 6c) is qualitatively indistinguishable from that of the anti-Lac L chains labeled with OD-Lac (Figure 4b), showing the characteristic pink color development with time, ϵ_{max} occurring at 510 mμ both at pH 6.2 and in 0.15 N NaOH.

C. The Reaction of anti-Lac with OD-Gal. Two 2.2×10^{-5} M solutions of anti-Lac, one of which was 10^{-3} M in *p*-nitrophenyl β-lactoside, were reacted with 2.9×10^{-5} M OD-Gal at pH 8.0, 5°, for 1 hr. Figure 6a shows that the extinctions in 0.15 N NaOH–0.5% SDS of the azo spectrum from 380 to 500 mμ are about 50% higher for the unprotected than for the protected anti-Lac. The spectrum of the protected anti-Lac is indistinguishable from that of the protected anti-Gal reacted with OD-Gal as described in B above.

D. The Reaction of anti-Lac with PD-Lac. Solutions (2.0×10^{-5} M) of rabbit anti-Lac antibodies and rabbit normal γ-globulin were reacted with 2.3×10^{-5} M PD-Lac at pH 8.0, 5°, for 5 hr. The azo spectra of the reacted proteins are shown in Figure 7. The azo absorbance of the labeled anti-Lac is significantly more intense than that observed for the identically treated normal γ-globulin. The azo spectrum of whole anti-Lac antibodies labeled with PD-Lac appears generally similar to that of anti-Lac labeled with OD-Lac (Figure 3). The distribu-

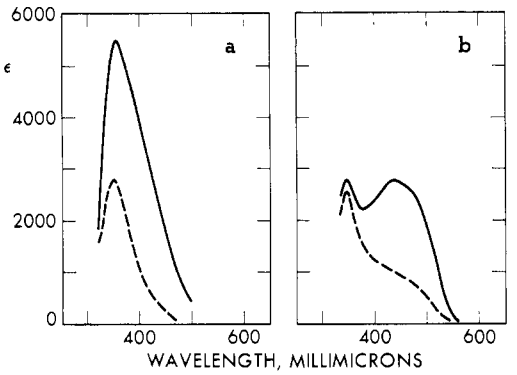

FIGURE 7: Azo spectra of anti-Lac (———) and rabbit γ-globulin (---------) reacted with PD-Lac. (a) pH 6.2; (b) 0.15 N NaOH. ε values based on 150,000 g/l.

tion and chemical identity of *para*-azo label on separated anti-Lac H and L chains were not studied.

Discussion

The results of these affinity labeling studies demonstrate that rabbit anti-Lac and anti-Gal antibodies have been labeled specifically, each with its corresponding *o*-diazoniumphenyl glycoside. Comparing these antibodies of closely similar saccharide specificity, there is clear identity in the particular types of amino acid residues selectively and discretely labeled in both cases. When both are compared with the antibenzenoid antibodies examined previously *via* affinity labeling, there are marked similarities, but also clear-cut distinctions in the labeling patterns.

Two main types of evidence establish the specificity of the affinity labeling reactions reported here: first, the fact that a particular reagent reacts much more rapidly with antibody of complementary specificity than with another specifically purified antihapten antibody or with rabbit normal γ-globulin; second, the fact that a reversibly binding hapten affords specific protection against the corresponding labeling reaction. The significance of such relative rate findings, demonstrating that the affinity labeling reaction proceeds *via* the antibody–hapten complex as an obligatory intermediate, has been discussed in previous papers (Wofsy *et al.*, 1962; Metzger *et al.*, 1963).

Thus, under conditions where rabbit anti-DNP antibody is essentially unmodified, significant labeling of rabbit anti-Lac occurs with OD-Lac (Figure 3a). Furthermore, the enhanced reaction rate of anti-Lac with OD-Lac is largely eliminated in the presence of an excess of protector, *p*-nitrophenyl β-lactoside (Figure 3b). The reagent OD-Gal reacts much more rapidly with anti-Gal than with anti-Lac antibodies, but in both cases there is an affinity labeling reaction which can be specifically retarded by addition of the appropriate hapten protector (Figure 6a). The latter findings are consistent with the observation that rabbit anti-Lac antibodies bind methyl β-D-galactoside with an average association constant which is about 250–300 times smaller than that for the binding of methyl β-D-lactoside (Karush, 1957). The labeling of anti-Lac with PD-Lac is indicated by the enhanced rate of the reaction over that observed when rabbit normal γ-globulin is the protein reacted (Figure 7).

In interpreting the results and comparing them with those obtained in the earlier studies with various antibenzenoid antibodies, consideration should be given to similarities and differences in the character of the labeling reagents employed. The diazonium functional group forms the covalent bond in all cases. In the case of the phenyl glycoside reagents, the diazonium group is not part of, but only adjacent to, the critical portion (ring) of the hapten determinant (Figure 1). However, in contrast to the case where the diazonium group is fixed as a substituent of the critical hapten phenyl ring, the phenyl glycoside reagents permit flexibility in the spacial relationship of the diazonium function to the hapten (saccharide) moiety. Construction of a molecular model of OD-Lac illustrates this flexibility: rotation of the diazoniumphenyl ring about the glycosidic CO bonds sweeps out arcs which permit the *o*-diazonium group to approach, at close to van der Waals distances, the C-1 and C-2 positions and both the pyranosidic and phenyl glycosidic oxygen atoms of the glucoside ring, as well as to intersect portions of the space encompassed by rotation of the hydroxyl substituents at C-2 and C-6. This indicates the potentialities of OD-Lac for the labeling of one or more contact amino acid residues at anti-Lac active sites. The potential of OD-Gal for labeling contact residues in the active sites of anti-Gal and anti-Lac antibodies is more striking, since the galactoside moiety is the terminal portion of these determinants. While the rotational freedom of the diazoniumphenyl glycosides offers an enhanced potential for labeling contact amino acids in the corresponding antibody active sites, this same flexibility enlarges the potential reaction sphere of these affinity labeling reagents and allows the possibility that amino acid residues close to, but not a functional part of, the active sites may be labeled. From the point of view of restricting label formation to contact amino acid residues, the best system for active site labeling is probably represented by the labeling of anti-Lac antibodies with the relatively very small OD-Gal reagent.

The identical labeling patterns achieved when anti-Lac is labeled with OD-Lac and when anti-Gal is labeled with OD-Gal suggest that common features contribute to the specificity of both antisaccharide antibody populations. The H chain azo spectra show that tyrosine has been discretely labeled in both cases (Figures 4a and 6b), while the labeled L chain spectra of both show another distinctive product whose identity is not yet established (Figures 4b and 6c).

These results seem especially significant in defining both antisaccharide specificities. It should be noted that the matching patterns obtain despite the marked difference in size of the lactoside and galactoside moieties; moreover, they are clearly distinguished from the affinity labeling results with several different antibenzenoid antibodies where azotyrosine is the discrete product on

both H and L chains (Singer and Doolittle, 1966).

The unusual L chain modification observed in the labeling of either anti-Lac or anti-Gal antibodies with the corresponding *ortho* reagents is a striking example of the selectivity possible in affinity labeling reactions. Amino acid analyses of rabbit anti-Lac L chains by Koshland *et al.* (1966) show 10.1 tyrosine, 1.4 histidine, and 9.8 lysine residues/mole; yet the labeling reaction on the L chains of both anti-Lac and anti-Gal antibodies does not yield azo derivatives of tyrosine, histidine, or lysine, the usual products of protein azo modification. Spectral assay of the azophenyl glycoside derivatives of *N*-chloroacetyltyrosine and *N*-acetylhistidine (Figure 2) and of ϵ-aminocaproic acid clearly distinguishes these model compounds from the unique L chain label.

The characteristic feature of the labeled L chains is the spectral change which occurs very slowly, but which is readily observed over a period of hours in detergent solution (Figure 5). This spectral change, which is marked by the emergence of an absorption maximum at 510 mμ and which proceeds according to first-order kinetics, is not observed in detergent solutions of either the tyrosine-labeled H chains or the model derivatives of tyrosine, histidine, or ϵ-aminocaproic acid. Whatever the nature of the L chain modification and of the observed spectral transformation, the label remains integrally associated with the protein, since the 510-mμ peak is not altered upon prolonged dialysis and removal of detergent. It is tempting to speculate on two counts that the labeled L chain residue may be tryptophan. (1) While model azotryptophan derivatives have never been isolated as pure compounds, it has been shown that indole acetic acid and other tryptophan derivatives react with diazonium reagents at alkaline pH yielding a yellow reaction solution which turns brown or reddish during efforts to isolate products (Howard and Wild, 1957). (2) Antisaccharide antibody sites may perhaps be somewhat analogous to the oligosaccharide binding site of the enzyme lysozyme, which has been shown by X-ray crystallographic and chemical studies to involve critically several tryptophan residues (Johnson and Phillips, 1965). Efforts to isolate and identify the labeled L chain residue are in progress.

While antibodies of like (saccharide) specificity can be distinguished from antihapten antibodies of grossly different specificities by the characteristic nature of the amino acid residues which are labeled by affinity labeling reagents, there are also striking similarities in the results obtained in all of the rabbit antihapten systems studied to date. Thus, without exception, azotyrosine is the label found on the H chains, emphasizing the high frequency of occurrence of tyrosine at antibody binding sites. Furthermore, the fact that both chains are labeled in the case of the antisaccharide, as well as of the antibenzenoid, antibodies strengthens the conviction that the participation (and close proximity) of both H and L chains in antibody active sites is general.

One may also mention the manifestation of antibody heterogeneity evident in all of the affinity labeling studies. Although in the present work there was no specific quantitative examination of the kinetics of the labeling reaction, it was observed that the rate of labeling, with about equimolar concentrations of anti-Lac and OD-Lac, fell off markedly after the first 0.5 hr and prior to the consumption of a major fraction of the reagent. Our assessment of the degree of chemical relatedness in such a heterogeneous population is, of course, limited by the fact that only a portion of the antibody sites has been labeled. Since a particular objective of these studies was to define and compare azo spectra of specifically labeled antibodies, reactions were performed under conditions calculated to favor maximum specificity (Wofsy *et al.*, 1962), *i.e.*, short reaction times and low reagent and antibody concentrations. Taking into account the molecular weight of H chains (50,000) and employing the extinctions determined for the model azotyrosine derivative (Figure 2), approximately 1 in 14 anti-Lac H chains (Figure 4a) and 1 in 25 anti-Gal H chains (Figure 6b) have been labeled. The percentage of L chains labeled is not readily estimated from our observations. As in earlier affinity labeling studies, however, the intriguing aspect is that the selectivity revealed in the discrete labeling patterns for H and L chains occurs despite heterogeneity within a given antibody population.

The similarities apparent in affinity labeling results have been interpreted as indicating the presence of common features in the binding sites of antibodies of varied specificities (Singer *et al.*, 1964). The present studies support this hypothesis, but point also to particular chemical features shared by antibodies of similar (saccharide) specificity which distinguish them from antibodies of grossly different (benzenoid) specificity.

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Affinity Labeling of Equine Anti-β-lactoside Antibodies*

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ABSTRACT: Equine anti-Lac antibodies of two varieties, γG and γG(T)[2] isolated from a single horse, have been labeled specifically with OD-Lac as affinity labeling reagent. The reaction conditions were the same as those employed in affinity labeling of pooled rabbit anti-Lac antibodies with the same reagent. Azo spectra of H and L chains from the labeled equine antibodies of both varieties are qualitatively indistinguishable. However, these spectra show that the residues selectively modified by affinity labeling of the equine anti-Lac antibodies differ distinctly from those labeled on both H and L chains of the rabbit antibodies. The possible genetic or evolutionary significance of these characteristic distinctions is not yet established.

Affinity labeling studies with rabbit anti-Lac[1] and anti-Gal antibodies point to particular chemical features shared by antibodies of similar (saccharide) specificity which distinguish them from antibodies of grossly different (benzenoid) specificity (Wofsy *et al.*, 1967). The results produced in affinity labeling of antibenzenoid hapten antibodies (namely, the selective modification at active sites of tyrosine residues on both heavy (H) and light (L) chains) have been shown in the case of anti-dinitrophenyl antibodies to hold for all of four species thus far studied (A. H. Good, Z. Ovary, and S. J. Singer, unpublished data). In the present study equine 7S anti-Lac antibodies of two structural varieties, γG and γG(T)[2] (Rockey *et al.*, 1964; Klinman *et al.*, 1964, 1965), have been labeled with the same reagent, OD-Lac, and under the same conditions as were employed in affinity labeling of rabbit anti-Lac antibodies (Wofsy *et al.*, 1967).

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[1] Abbreviations used: anti-Lac and anti-Gal, antibodies specific for the respective haptenic groups *p*-azophenyl β-lactoside and *p*-azophenyl β-galactoside; OD-Lac, the reagent *o*-diazoniumphenyl β-lactoside; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

[2] This was formerly designated γA on the basis of its carbohydrate content and behavior in immunoelectrophoresis. Recent observations have indicated its identity with the T component of equine antiserum as judged by its antigenic identity with similarly prepared diphtheria antitoxin and tetanus antitoxin (R. Genes, N. R. Klinman, R. Hirsh, and F. Karush, unpublished data). In view of the antigenic similarity between γG-immunoglobulin and the T component (Weir and Porter, 1966) and the extensive homology of the C-terminal sequences o their heavy chains (Weir *et al.*, 1966), the designation γG(T), suggested by Weir *et al.* (1966) will be used instead of γA.