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Energetics and Spectral Changes in Ligand Binding by Homogeneous Rabbit Anti-Lactose Antibody†

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ABSTRACT: Homogeneous isoelectric fractions of rabbit anti-lactose antibodies induced with a killed vaccine of *Streptococcus faecalis*, strain N, have been characterized with respect to their interaction with a lactose-containing ligand. This has been done by measurement of the association constants as a function of temperature, using equilibrium dialysis, to obtain the thermodynamic parameters of the binding reaction. In addition, structural differences among the combining sites have been recognized by optical analyses of the antibody–ligand complex. All of the isoelectric fractions were similar with respect to the energetics of the binding reaction, namely, the predominance of the ΔH term and the minor contribution of the ΔS_u term. It was inferred, therefore, that the stability of the complex arises primarily from multiple hydrogen bonding between the lactosyl group and donor and acceptor groups of the contact amino acids. In contrast, the difference spectra associated with the binding of the chromophoric ligand provided a unique, phenotypic characterization of each homogeneous antibody population although all fractions exhibited hyperchromicity. This common spectral feature may be the result of the enhanced stabilization of the planar structure of the resonance system. Induced circular dichroism of the bound ligand has also been found, exhibiting differences among antibody populations from different animals. The common feature has been a positive circular dichroism (CD) band reflecting, presumably, a generally similar asymmetric environment. The identity of the CD band for three fractions derived from a single animal and the close similarity of their thermodynamic properties have provided the basis for the suggestion of identical V_L and V_H germ-line genes in the emergence of the clones producing these fractions. Finally, it was found that the binding of lactose to the antibody site could lead either to enhancement or quenching of the tryptophan fluorescence. It is evident that optical probes can provide a highly characteristic identification of the monoclonal product and serve as tools for the recognition of the corresponding clone.

In a recent publication (Ghose and Karush, 1973) we have utilized a bacterial vaccine of *Streptococcus faecalis*, strain N (Pazur *et al.*, 1971, 1973), to induce in rabbits the formation of IgG anti-lactose antibody of restricted heterogeneity. The specifically purified antibody was further fractionated by preparative isoelectric focusing (Freedman and Painter, 1971) into functionally homogeneous populations, presumably monoclonal in origin. The specific binding properties of these fractions were evaluated and the temporal variation of their distribution over the period of 1 year was studied in relation to the maturation of the immune response.

The affinity of the isoelectric fractions spanned a range of 100-fold in association constant although in some animals the range was much more restricted. The availability of these functionally homogeneous populations of common specificity but different affinity has made feasible the examination of the microenvironment of the combining sites of the antibodies. This has already been done to a limited extent in our previous investigation (Ghose and Karush, 1973) by the measurement of the association constants for a lactose-containing ligand. A finer analysis of the combining sites appeared desirable because of the possibility that a more detailed phenotypic characterization would emerge than that provided by the affinity values. It was anticipated that this further characterization of the isoelectric fractions would provide clues to the origins of the variable region genes (V_L and V_H) selected for the expression of anti-lactose specificity.

The present study describes the examination of the combining sites of homogeneous antibody fractions by a thermodynamic analysis of their interaction with a lactose-containing ligand (Lac dye)[1] and by the use of optical probes. The latter involved the measurement of difference spectra arising from changes in the absorption spectrum of the Lac dye when complexed with antibody and the measurement of circular dichroism (CD) of the Lac dye induced by complex formation. In addition changes in the tryptophan fluorescence of the antibody arising from the occupancy of the combining site by lactose were also evaluated. It has become apparent from the results of these analyses that the optical probes are particularly

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[1] Abbreviations used are: Lac dye, *p*-(*p*-dimethylaminophenylazo)-phenyl β-lactoside; PBS, 0.15 M NaCl–0.02 M phosphate (pH 7.4).

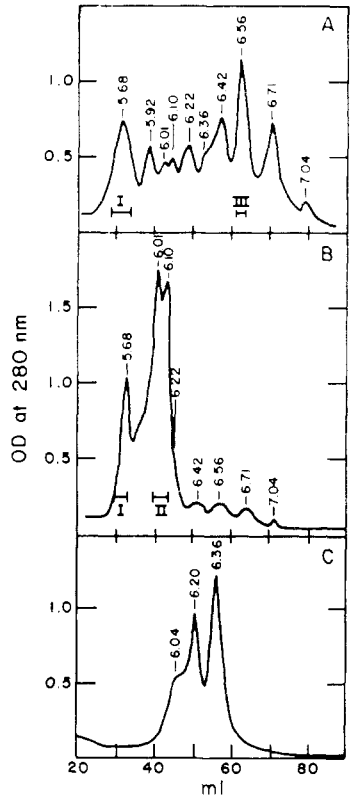

FIGURE 1: The liquid isoelectric focusing elution profiles of purified anti-lactose antibodies in the pH range 5–8. Profiles A and B were obtained from rabbit 1472 after one and four courses of immunization, respectively. Profile C was obtained from rabbit 1480 after four courses of immunization. The different isoelectric bands and shoulders are characterized by average isoelectric pH values shown at the top. The individual variation of the pH values was within ±0.01 pH unit of the average value. For rabbit 1472 fractions under the same isoelectric bands, marked in the figure as I, II, and III, were pooled and used for all experiments. Anode is at the left.

useful in the detection of subtle structural differences in the combining sites of the homogeneous fractions. These probes can, therefore, provide a highly characteristic identification of a monoclonal product and thereby permit the recognition of the corresponding clone. This capability should prove useful in studies concerned with the emergence and decline of clones in the course of the immune response and with the inheritance of V_L and V_H genes.

Materials and Methods

Antibody Preparations. Anti-lactose antibodies were obtained from rabbits by immunization with a bacterial vaccine of *Streptococcus faecalis* (strain N) whose cell wall contains the lactosyl moiety which serves as an immunodominant group in rabbits (Pazur *et al.*, 1971, 1973). The details of immunization, bleeding schedule, and antibody assay have been described earlier (Ghose and Karush, 1973). The antibody was specifically purified with the use of an immunoadsorbent column and further fractionated by preparative isoelectric focusing as described previously (Ghose and Karush, 1973). The isoelectric fractions employed in this study have been characterized in the earlier work. A rabbit anti-Lac antibody was also utilized in this study. Its preparation and properties are detailed elsewhere (Utsumi and Karush, 1964). Antibody concentrations were determined spectrophotometrically using a value for $E_{1\,cm}^{1\%}$ of 14.4 at 280 nm (Utsumi and Karush, 1964) and a molecular weight of 150,000 for rabbit IgG.

Equilibrium Dialysis. The association constants for the binding of Lac dye were determined with microdialysis cells using a tritiated preparation of the Lac dye (Karush and Sela, 1967). The experimental details of this procedure and the analysis of the data have already been documented (Ghose and Karush, 1973). The binding measurements were done at the specified temperatures controlled to ±0.05° in PBS (pH 7.4). The calculation of the values of the association constant (K) and the heterogeneity index (a) was based on the linear extrapolation of the binding data to an intercept value of $r = 2$.

Difference Spectra. The spectral changes accompanying the binding of Lac dye by anti-lactose antibody were measured as difference spectra in a Cary spectrophotometer, Model 15 (Applied Physics Corp., Monrovia, Calif.), equipped with a temperature-controlled cell holder. The antibody–hapten mixture was contained in the sample cell and hapten in the reference cell. A blank run was made with the protein in the sample cell and solvent in the reference cell. The results were corrected for the free hapten in the antibody–hapten mixture on the basis of the binding experiments.

Circular Dichroism. Measurements of circular dichroism were conducted with a Durrum-Jasco J-10 circular dichrometer at 25° in a cell with a 1-cm path length. Runs were made with the hapten and protein separately as well as mixtures of them. The base line was obtained with the solvent alone. The results are expressed in terms of molar ellipticity, $[\theta]_\lambda$, in units of (deg cm²)/dmol, where $[\theta]_\lambda = \theta_\lambda M/10\,cl$, in which θ_λ is the observed ellipticity in degrees at wavelength λ, M is the molecular weight of the hapten, l is the path length in cm, and c is the concentration of the bound hapten in g/cm³. The fraction of the total hapten bound was calculated from the binding curves.

Antibody Fluorescence. Fluorescence measurements were made with an Aminco-Bowman fluorometer equipped with a thermostated cell holder and operated at 25°. For this purpose the antibody was digested with pepsin to yield the $F(ab')_2$ fragment as described previously (Utsumi and Karush, 1965). A reference solution of L-tryptophan was used to check the stability of the fluorometer at frequent intervals. Fluorescence spectra were obtained with the $F(ab')_2$ fragments alone and in solutions containing 2×10^{-3} M lactose. This concentration of lactose was sufficient to saturate the antibody combining sites (Ghose and Karush, 1973).

Results

The homogeneous anti-lactose antibody preparations used in these studies were mainly obtained from the sera of two rabbits, 1472 and 1480. Three of these are the isoelectric fractions I, II, and III from bleedings of rabbit 1472 after one and four courses, as shown in Figures 1A and B. The other protein was obtained from the sera of rabbit 1480 which were high in antibody content (*e.g.*, 25 mg/ml) and indicated only two major and one minor isoelectric bands (Figure 1C) during a limited period after four courses of immunization (Ghose and Karush, 1973). All of these preparations exhibited functional homogeneity over the temperature range 4–37° as shown by the linearity of the Scatchard plots (Scatchard, 1949) and by the Sips heterogeneity index values (Karush, 1962) which are close to unity (Table I). In Figure 2, the Scatchard plots of the binding data of rabbit 1480 anti-lactose antibody obtained at 4, 20, and 37° are shown as a representative example. The association constants (K) presented in Table I are the average of two determinations. It can be seen

TABLE I: Temperature Dependence of the Association Constants Obtained by Equilibrium Dialysis for the Binding of [^{3}H]Lac Dye by Rabbit Anti-Lactose Antibody Fractions.

Antibody Fraction	Temp (°C)	Association Constant K (M^{-1})	Heterogeneity Index a
1472 (I)	4	$3.54 \pm 0.18 \times 10^6$	0.99
	20	$1.36 \pm 0.07 \times 10^6$	1.05
	37	$4.25 \pm 0.22 \times 10^5$	0.97
1472 (II)	4	$5.40 \pm 0.26 \times 10^6$	0.99
	20	$1.88 \pm 0.16 \times 10^6$	1.06
	37	$5.71 \pm 0.13 \times 10^5$	1.00
1472 (III)	4	$1.49 \pm 0.09 \times 10^6$	1.02
	20	$5.49 \pm 0.16 \times 10^5$	1.02
	37	$1.70 \pm 0.12 \times 10^5$	0.97
1480	4	$6.67 \pm 0.18 \times 10^6$	1.06
	20	$2.88 \pm 0.20 \times 10^6$	1.03
	37	$1.04 \pm 0.06 \times 10^6$	1.01

that the values of K increase with decrease of temperature for all the fractions. The enthalpy change for the formation of the hapten–antibody complex was calculated from the van't Hoff plot (log K *vs.* $1/T$) of the values in Table I. Although each fraction showed a slight curvature in its van't Hoff plot, the curvature was not of statistical significance when compared with the spread of the K values presented in Table I. Therefore, the best linear fit was used to obtain the slopes and the ΔH values for the hapten–antibody association reaction (Table II). There is also shown in Table II the corresponding changes in the unitary free energy (ΔF_u) (Karush, 1962). For comparison, values of ΔF_u, ΔH, and ΔS_u obtained with rabbit anti-Lac antibodies (Karush, 1962) and equine IgM anti-lactose antibody (Kim and Karush, 1973) are also presented in the table. The results show that with all of these antibodies the hapten–antibody association reaction is accompanied by a substantial decrease in the enthalpy of the system whereas the ΔS_u values are quite small.

The absorption spectra of free Lac dye in PBS shows a maximum at 454–455 nm and a shoulder at about 400 nm. When bound to heterogeneous rabbit anti-Lac antibody there is an increase in the extinction coefficient at the absorption maximum (hyperchromicity) as well as a slight shift of the maximum toward the red (Karush, 1957). In Figure 3, the difference spectra of free and bound Lac dye for different anti-lactose antibodies are shown. It can be seen that the general feature with all of the antibodies is the hyperchromicity of the bound dye spectra. However, each population of anti-

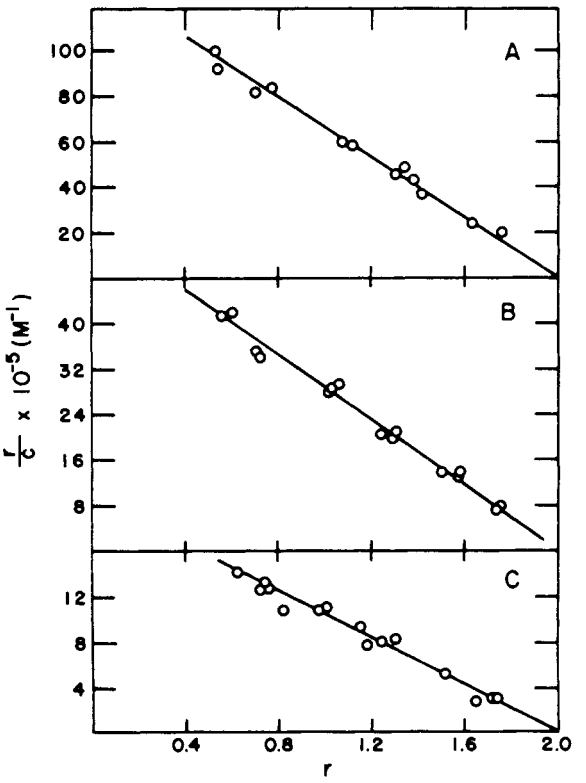

FIGURE 2: Scatchard plots of equilibrium dialysis data for rabbit 1480 anti-lactose antibody binding with [^{3}H]Lac dye at (A) 4°, (B) 20°, and (C) 37° where r represents the moles of Lac dye bound per mole of antibody at the equilibrium free dye concentration c. The antibody concentration was about 3×10^{-6} M and the initial ligand concentrations ranged from 2×10^{-5} to 1×10^{-6} M.

bodies exhibits a distinctive difference spectrum which is readily distinguishable from the others. Fractions I and II of rabbit 1472 show a difference spectral band at 470 nm and shoulders near 400 nm whereas fraction III of rabbit 1472 does not show a distinct difference spectral maximum but exhibits a rather broad spectral band which can be resolved into two bands of similar magnitude at about 460 and 390 nm. In contrast the antibody of rabbit 1480 exhibits two distinct difference spectral bands, one at 458 nm and the other in the 395–400-nm region. When Lac dye is bound to rabbit anti-Lac antibody the difference spectrum also shows two distinct bands, one at 475 nm and the other at 410 nm.

The induced circular dichroism of Lac dye when bound to different anti-lactose antibody preparations is shown in Figure 4. It should be noted that free Lac dye exhibits a broad and very weak negative CD band largely in the 380–480-nm

TABLE II: Thermodynamic Parameters for the Binding Reaction between [^{3}H]Lac Dye and Rabbit Anti-Lactose Antibody Fractions.[a]

Antibody	ΔF_u (kcal/mol)	ΔH (kcal/mol)	ΔS_u (cal mol^{-1} deg^{-1})
1472 I	-10.5 ± 0.0	-10.9 ± 0.0	-1.4 ± 0.0
1472 II	-10.7 ± 0.1	-11.3 ± 0.2	-2.0 ± 1.0
1472 III	-10.0 ± 0.0	-11.0 ± 0.1	-3.4 ± 0.3
1480	-11.0 ± 0.0	-9.5 ± 0.2	$+5.1 \pm 0.7$
Rabbit anti-Lac antibody[b]	-9.47[c]	-9.7	-0.8
Equine anti-lactose IgM[d]	-9.7 ± 0.1[c]	-11.0 ± 0.2	-4.7 ± 1.1

[a] The unitary free energy, ΔF_u, is given for 20° and the thermodynamic values refer to the binding of 1 mole of hapten (see Karush (1962)). [b] Data taken from Karush (1962). [c] Values refer to 25°. [d] Data taken from Kim and Karush (1973).

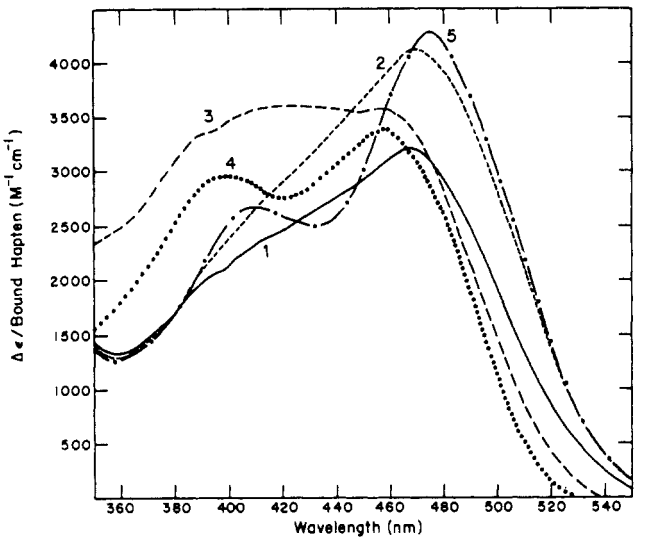

FIGURE 3: Difference spectra of antibody-bound and free Lac dye measured at 25° using rabbit anti-lactose antibodies 1472 I (1), 1472 II (2), 1472 III (3), 1480 (4) and rabbit anti-Lac antibody (5). Protein concentrations varied between 0.7×10^{-5} and 1.5×10^{-5} M and hapten concentrations ranged from 1×10^{-5} to 2.3×10^{-5} M. Solvent was PBS buffer. The values of $\Delta\epsilon$ are given per mole of bound dye.

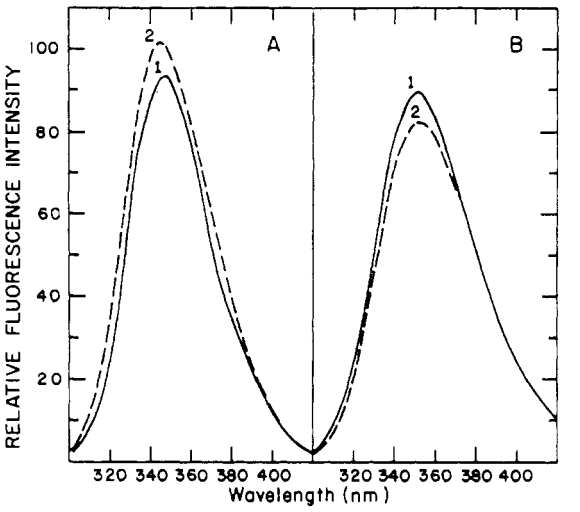

FIGURE 5: Fluorescence spectra of $F(ab')_2$ fragments of anti-lactose antibody: (A) rabbit 1472 (II) antibody alone (1) and in presence of 2×10^{-3} M lactose (2); (B) rabbit 1480 antibody alone (1) and in presence of 2×10^{-3} M lactose (2). Excitation was at 290 nm and the emission spectra were recorded at 25°. The solvent was 0.01 M phosphate in 0.2 M NaCl, pH 6.0. Optical density of the protein solutions at 280 nm was 0.15 and 0.20 for 1472 (II) and 1480 antibodies, respectively.

range. When bound to the combining site, the same dye generates positive CD bands with all of the antibody preparations studied. However, these induced CD bands are weak except with rabbit 1480 antibody in which case there is a rather strong CD band centering around 450–460 nm. With the 1472 antibody fraction II, the hapten shows a weak positive band in the 390–400-nm region which is similar to bands obtained with the other two fractions (I and III) of rabbit 1472 antibody (not shown) and with rabbit anti-Lac antibody. For comparison the CD spectrum of the bound hapten obtained with another rabbit (1479) anti-lactose antibody of restricted heterogeneity is shown in Figure 4. This spectrum is similar to that of rabbit 1480 in that it shows a positive band in the 450–460-nm region.

The fluorescence spectra of two anti-lactose antibodies were measured in the presence and absence of lactose (Figure 5).

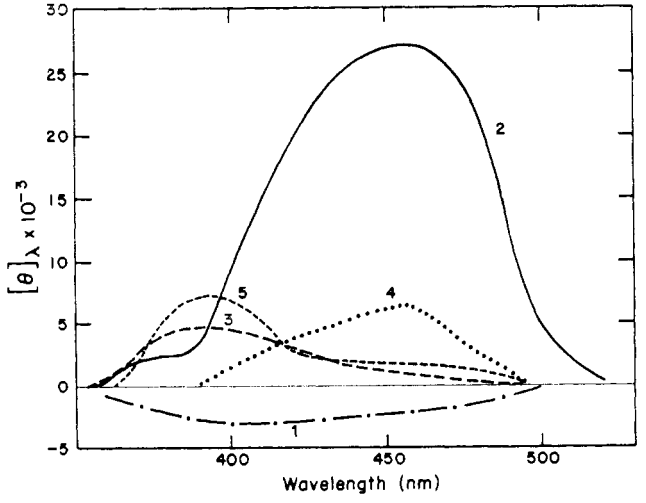

FIGURE 4: Circular dichroic spectra of Lac dye in free solution (1), Lac dye bound to rabbit anti-lactose antibodies 1480 (2), 1472 II (3), 1479 (4), and rabbit anti-Lac antibody (5). CD spectra (2–5) are corrected for the CD contribution of free Lac dye. Protein concentration varied between 1×10^{-5} and 1.5×10^{-5} M and the hapten concentration ranged from 2.5×10^{-5} to 4.5×10^{-5} M.

In order to maximize the difference in the emission spectra between free antibody and lactose-bound antibody, $F(ab')_2$ was used instead of whole IgG. It can be seen that on excitation at 290 nm the $F(ab')_2$ fragment of rabbit 1472 (II) antibody shows an emission maximum at 346–348 nm whereas for rabbit 1480 $F(ab')_2$ the maximum is at 350–352 nm. Both of these are characteristic of tryptophan emission as free tryptophan and *N*-acetyl-L-tryptophanamide show a fluorescence maximum at 353 nm in aqueous media (Edelhoch *et al.*, 1968). When the fluorescence spectra of these fractions were measured in the presence of lactose (2×10^{-3} M), there was no detectable shift in the emission maximum of either protein. However, a small enhancement of fluorescence could be detected in the case of $F(ab')_2$ fragment of rabbit 1472 (Figure 5A). On the other hand, the $F(ab')_2$ fragment of rabbit 1480 exhibited a small quenching of fluorescence (Figure 5B). The emission maximum of normal rabbit $F(ab')_2$ was at 348–350 nm and in the presence of lactose there was neither any change in the fluorescence intensity nor any wavelength shift of its fluorescence maximum.

Discussion

The thermodynamic parameters for the binding of Lac dye by isoelectric fractions of rabbit anti-lactose antibody exhibit a distinctive pattern (Table II). It is evident that the free energy change for complex formation is almost entirely dependent on the enthalpy term (ΔH) which ranges from −9.5 kcal/mol of hapten bound to −11.3 kcal. This narrow range of values of ΔH suggests that the antibody–ligand interaction utilizes almost the full capacity of the lactosyl moiety for hydrogen bond formation in an antibody site and that the ΔH term is primarily due to multiple hydrogen bonding. It is to be noted that the H-bond contribution is the difference in energy between lactose hydrogen bonded to solvent molecules and its binding to donor and acceptor groups of the contact amino acids. The near constancy of the ΔH term is also seen in the binding characteristics of purified antibody specific for the *p*-azophenyl β-lactoside group and with equine anti-lactose IgM antibody (Kim and Karush, 1973).

A comparison of the 1472 fractions with 1480 reveals a difference in the ΔS_u values which may reflect structural features common to the former but absent in the latter. This rather tentative inference is supported by a parallel difference in the CD spectra of the bound hapten (Figure 4). An attractive but speculative explanation for these differences is the notion that the isoelectric fractions of 1472 represent monoclonal products controlled by variable region genes derived somatically from a common V_L germ-line gene and/or a common V_H germ-line gene. In the other rabbit, 1480, one or both of the germ-line genes would be different from that of 1472.

It has been noted that in the van't Hoff plots for the temperature range 4–37° there was no significant curvature. Thus the values of ΔH (Table II) obtained from the slopes of the curves appear to be constant in this range and it may be concluded that there is no significant change in heat capacity associated with the formation of the antibody–hapten complex.

The optical properties of the antibody–ligand complexes have led to the recognition of subtle structural differences among the isoelectric fractions which were not evident from the energetics of complex formation. The most revealing optical probe has been the evaluation of the difference spectrum between bound and free Lac dye. As shown in Figure 3, the difference spectrum is characterized in each case by a hyperchromicity for the bound hapten and the existence of two spectral bands. Nevertheless, the quantitative expression of these features is distinctive for each isoelectric fraction as is apparent from the variety of shapes of the difference spectra. Thus, if the isoelectric fractions represent monoclonal products, as has been inferred from chain recombination experiments (Ghose and Karush, 1974), then the difference spectrum, as well as the affinity, can be used for phenotypic characterization. From this point of view it is of particular interest to note that the difference spectra for the three isoelectric fractions of rabbit 1472 can be readily distinguished.

The difference spectra do not lend themselves readily to structural interpretation but it can be surmised that the common feature of hyperchromicity does not arise from a reduced dielectric constant in the region of the antibody combining site. This conclusion is based on the finding that in dioxane the Lac dye exhibits a hypochromicity and a shift of the absorption band to shorter wavelengths and in 2 M NaCl there is no spectral change. One likely explanation for the hyperchromicity is an increased rigidity of the chromophoric group of the Lac dye when it is bound to the antibody. The observed spectral effects are consistent with the notion that this interaction lends stability to the planar phenylazophenyl structure.

Another optical probe which has revealed structural differences among the antibody combining sites is the measurement of the induced circular dichroism of the Lac dye. In contrast to the weak negative CD band intrinsic to the ligand in solution, the interaction with antibody generates a positive CD band of various magnitudes (Figure 4). The CD spectra associated with different animals are readily distinguishable and demonstrate the utility of this technique for structural analysis. This utility has previously been emphasized on the basis of CD studies with nitrophenyl-binding immunoglobulins (Glaser and Singer, 1971). A variation of this approach has been utilized with homogeneous rabbit anti-oligosaccharide antibodies. In this case the change in the intrinsic CD spectrum of the antibody resulting from specific ligand interaction was evaluated and found to be distinctive for each of three homogeneous fractions (Holowka *et al.*, 1972).

The common feature of a positive CD band in all cases suggests that the chromophoric group of the Lac dye is subjected, in its bound form, to a generally similar but individually distinct asymmetric environment. This similarity could arise from the common specificity of the antibody sites which might require the same contact amino acids in a similar array in order to achieve this specificity. It is also necessary, however, to include the possibility that the acquired rigidity of the bound ligand would modify the vicinal effect of the optically asymmetric lactosyl group on the covalently linked chromophore. It is apparent that both factors might contribute to the generation of the positive CD bands and to a different extent in each case.

Another noteworthy feature of the CD results is the virtual identity of the spectra for the three isoelectric fractions of rabbit 1472. This finding suggests more limited structural differences among these antibody fractions than among the antibodies of different animals. The correlation of this observation with the energetics of binding has already been pointed out and a possible genetic explanation suggested (see above).

Although not as discriminating as the other optical probes the fluorescence spectra also revealed structural differences between one of the isoelectric fractions of rabbit 1472 and the antibody of rabbit 1480 (Figure 5). The observation that bound lactose can lead either to the enhancement or the quenching of antibody tryptophan fluorescence is in accord with findings in another immunoglobulin system involving anti-carbohydrate specificity (Jolley *et al.*, 1973). In this case mouse myeloma IgA proteins with binding activity against multiple $\beta(1\rightarrow6)$-linked D-galactopyranose units showed a variety of modifications of the protein fluorescence when complexed with a tetrasaccharide of galactose. Out of six such myeloma proteins two exhibited an enhancement with a maximum of 27%, one was quenched by 8%, and three were unaffected. It thus appears that fluorescence spectra are useful in recognizing differences involving tryptophan residues in or near the antibody combining site even when no energy transfer to a bound ligand is involved.

It will be noted (Table II) that the anti-Lac antibody, induced by the *p*-azophenyl β-lactoside group coupled to carrier protein, exhibits similar energetics to anti-lactose antibody and shows a similar difference spectrum with Lac dye (Figure 3). This comparison implies that the anti-Lac antibody carried very little specificity for the aglycoside. Such a conclusion is consistent with the fact that the anti-Lac antibody was obtained after only 4 weeks of immunization and bound Lac dye with an average association constant of 1×10^5 M^{-1} at 25°. On prolonged immunization in the horse, on the other hand, the value for IgG anti-Lac antibody reached 1×10^7 M^{-1} (Klinman and Karush, 1967).

Finally, there is the question regarding the site of interaction of the chromophoric group of the Lac dye when this ligand is bound by anti-lactose antibodies. This question relates to the interpretation of the observed optical effects in terms of a direct role of the contact amino acids of the combining site. Although the interaction of the β-linked aglycoside of the Lac dye, *p*-(*p*-dimethylaminophenylazo)benzene, with anti-lactose antibody is nonspecific, it probably takes place within the antibody site defined by the total antigenic determinant(s) provided by the diheteroglycan of the bacterial cell wall. This conclusion is based on the frequent observation that anti-carbohydrate antibodies are usually specific for ligands which are at least as large as a trisaccharide (Goodman, 1969).

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An Electrophoretic Method for the Quantitative Isolation of Human and Swine Plasma Lipoproteins†

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ABSTRACT: A procedure for the isolation and purification of human and swine plasma lipoproteins using the combination of ultracentrifugation and Geon–Pevikon block electrophoresis has been described. Normal human and swine lipoproteins isolated by this procedure were compared to lipoproteins isolated by the standard method of ultracentrifugation and were found to be essentially identical with respect to chemical composition, immunochemical reactivity, size by electron microscopy, and apoprotein content by polyacrylamide gel electrophoresis. This procedure allowed the isolation of plasma lipoproteins in a shorter period of time without subjecting the lipoproteins to repetitive ultracentrifugation and washing procedures. In addition, the plasma lipoproteins from cholesterol-fed swine, which could not be separated by ultracentrifugation alone, were purified by the Geon–Pevikon electrophoretic procedure. These swine developed a hyperlipoproteinemia characterized by the presence of two different lipoproteins which had overlapping densities in the low density fraction.

We have previously reported the use of Geon–Pevikon block electrophoresis for the isolation of canine plasma lipoproteins (Mahley *et al.*, 1973). The dog has two distinctly different lipoproteins with overlapping densities in the low density range which were easily purified by the block electrophoretic procedure. The relative ease, the rapidity of separation compared with repetitive ultracentrifugations, and the high degree of purity suggested that the Geon–Pevikon block electrophoretic procedure might be of value under certain circumstances for purification of the lipoproteins of other species. A method for the isolation of plasma lipoproteins from the human and swine is reported. In addition the method is applied to the separation and purification of lipoproteins of cholesterol-fed swine, where ultracentrifugation alone results in a mixture of lipoprotein types.

Materials and Methods

Human plasma was obtained from laboratory workers who had normal lipoprotein profiles as judged by lipid values and lipoprotein electrophoresis. Porcine plasma was obtained from miniature swine derived from the Hormel breed following an overnight fast. Control swine were on a low-fat commercial hog chow. A high-fat, high-cholesterol diet was prepared by blending 15% lard and 1% crystalline cholesterol by weight with commercial hog chow. The swine were fed this diet for 6 months to 1 year prior to lipoprotein analysis. Plasma lipid concentrations and type of atherosclerosis produced in similar animals have previously been reported from this laboratory (Flaherty *et al.*, 1972).

Ultracentrifugation. Plasma was subjected to ultracentrifugation using established procedures (Havel *et al.*, 1955). All centrifugations were performed in a Beckman 60 ti rotor at 59,000 rpm for variable periods of time. Fractions isolated at $d = 1.006$ were centrifuged for 12 hr; at $d = 1.02$, 1.04, 1.06, and 1.08 for 16–18 hr; and at $d = 1.21$ for 24 hr. The $d = 1.006$ fraction was washed in saline ($d = 1.006$) by ultracentrifugation. Following ultracentrifugation the fractions were dialyzed against 0.15 M NaCl–0.01% EDTA and concentrated using the Amicon Diaflo apparatus.

Immunochemical Analyses. Antisera were produced to purified fractions of lipoproteins as previously described (Bersot *et al.*, 1970). Immunoelectrophoresis was performed according to the method of Levy and Fredrickson (1965).

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