Allotypes and Isozymes of Rabbit α_1-Aryl Esterase. Allelic Products with Different Enzymatic Activities for the Same Substrates*

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ABSTRACT: Rabbits were immunized with α_1-aryl esterase isolated from the serum of other rabbits by ion-exchange chromatography, gel filtration, and preparative disc electrophoresis. This elicited precipitating isoantibodies, anti-s1 and anti-s2, which distinguished two allotypes, Ess-1 and Ess-2, of rabbit α_1-aryl esterase. Progeny studies from the six possible matings revealed that Ess-1 and Ess-2 allotypic specificities are controlled by allelic genes at an autosomal locus. Differences in enzymatic activity for the same substrate were observed for the allelic products Ess-1 and Ess-2, since the Ess-1 allotype exhibited high esterase activity with several aromatic esters, *e.g.*, α-naphthyl acetate, β-naphthyl acetate, α-naphthyl butyrate, and *p*-nitrophenyl acetate; and the Ess-2 allotype exhibited little or no activity toward these same substrates. Two isozymes of each allotype were found by disc electrophoresis: (1) the serum from an *Ess*^{1} *Ess*^{1} homozygous animal revealed two α_1-aryl esterase staining bands with slightly different mobilities and both bands reacted with goat anti-rabbit α_1-aryl esterase and with anti-s1. (2) The serum from an *Ess*^{2} *Ess*^{2} homozygous animal revealed two bands with mobilities similar to the bands of *Ess*^{1} *Ess*^{1} serum; these bands had little or no esterase activity yet reacted with the goat anti-rabbit α_1-aryl esterase and with the anti-s2. Upon reelectrophoresis, each of the isozymes migrated as a single band with no change in mobility. The relationship of this α_1-aryl esterase to other esterases is discussed.

Genetic variants of several rabbit serum proteins have recently been identified by their antigenic properties and are called allotypes. The antigenic differences are usually identified by antisera prepared in the same species *i.e.*, allo- or isoantibodies (Oudin, 1956).

In the rabbit IgG[1] immunoglobulin system, several allotypic specificities have been characterized and shown to be controlled by two series of alleles at two genetic loci which are not closely linked (Oudin, 1960; Dray *et al.*, 1963; Hamers *et al.*, 1966; Dubiski and Muller, 1967; Kelus and Gell, 1967). These allotypic specifities serve as genetic markers and reflect differences in the amino acid sequence (Small *et al.*, 1965, 1966; Reisfeld *et al.*, 1965). The allotypes have provided an important means for studying the structure and biosynthesis of the immunoglobulin molecule (Dray and Nisonoff, 1963; Lark *et al.*, 1965; Pernis *et al.*, 1965; Adler *et al.*, 1966). In addition, allotypes of the following rabbit serum proteins have been identified and characterized in this laboratory: α_2-macroglobulin (Knight and Dray, 1968), low-density lipoprotein (Albers and Dray, 1968), IgA immunoglobulin (Conway *et al.*, 1969), and haptoglobin (Chiao and Dray, 1969).

During the course of cross-immunization of crude fractions of rabbit serum proteins, several isoantibodies to α_1, α_2, and β proteins have been obtained. One of these antibodies reacted with an α_1 protein which exhibited esterase activity using β-naphthyl acetate as substrate and which was present in some (positively reacting) and absent in other (negatively reacting) rabbit sera.

The negatively reacting animals were presumed to possess an alternate form of the protein (allotype). One purpose of this study was to immunize positively reacting rabbits with protein from the serum of negatively reacting animals to produce a second isoantibody. In this manner such a second antiserum was produced, and the two antisera facilitated the isolation and characterization of the α_1-protein esterase as well as a study of the genetic control of its biosynthesis.

Materials and Methods

Isolation of α_1-Aryl Esterase. The α_1-aryl esterase preparations used for immunization were isolated from the sera of individual rabbits by a combination of ammonium sulfate precipitation, ion-exchange chromotography, gel filtration, and preparative disc electrophoresis. First, whole rabbit serum was precipitated with 55% saturated ammonium sulfate. The precipitate was dissolved in saline, dialyzed overnight against 0.02 M Tris buffer (pH 7.2), and placed on a DEAE-cellulose column in the same buffer. The proteins which were not adsorbed to the column were discarded. The proteins eluted with the next buffer, 0.02 M Tris–0.05 M NaCl (pH 7.2), were also discarded. Following this, the α_1-aryl esterase was eluted with 0.02 M Tris–0.15 M NaCl (pH 7.2). After concentration by ultrafiltration, this fraction was placed on a Sephadex G-200 column equilibrated with saline–borate buffer (0.18% NaCl–0.2 M borate, pH 8.0). The optical density of the eluate was determined at 280 mμ and three peaks were obtained. The proteins in the third peak, containing the α_1-aryl ester-

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[1] Nomenclature recommended by the World Health Organization Committee on Nomenclature for Human Immunoglobins, 1964.

ase, were concentrated and further purified by preparative disc electrophoresis. The electrophoresis was performed at 50 mA in the Büchler preparative disc apparatus at 4° in Tris-glycine buffer (pH 8.9) with 7.5% polyacrylamide gel. After 12 hr, the proteins were eluted and fractions were collected in 5-ml volumes. The fractions with esterase activity, as determined by their reaction with β-naphthyl acetate, were pooled and concentrated by ultrafiltration.

In order to prepare a monospecific heterologous antiserum, a preparation of α_1-aryl esterase free of contaminants was needed. Analytical disc electrophoresis in 7.5% polyacrylamide gel (Williams and Reisfeld, 1964) was used to remove trace contaminants from the esterase fractions obtained from the preparative disc electrophoresis. Approximately 75 μg of protein was placed on each of 12 polyacrylamide gel columns. After electrophoresis, the gels were stained for 2–3 min with the esterase stain and the α_1-aryl esterase bands were rapidly cut out and crushed with a mortar and pestle.

Isolation of Immunoglobulin. The IgG immunoglobulin was obtained from the serum of each rabbit by two successive precipitations with 18 and 14% (w/v) sodium sulfate essentially by the procedure of Kekwick (1940). Further purification was obtained by chromatography on DEAE-cellulose using a modification of the procedure described by Levy and Sober (1960). The protein obtained from the DEAE-cellulose column with 0.02 M phosphate buffer (pH 6.8) was concentrated by ultrafiltration at 4°.

Antisera. The rabbits used in these experiments were crosses of closed colonies of Flemish giants and New Zealand whites bred in our own laboratory. Rabbits were injected with crude preparations of α_1-aryl esterase isolated by ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration. The third peak from Sephadex G-200 was concentrated to about 10 mg/ml and mixed with an equal volume of Freund's complete adjuvant (15% Arlacel A, 85% Bayol F, and *Mycobacterium butyricum*, 0.4 mg/ml). The rabbits were injected intradermally in the back and intramuscularly in the thighs every second day for 1 week; each rabbit received a total of approximately 40 mg of protein. Three weeks after the first injection, approximately 5 mg of protein in Freund's incomplete adjuvant was given and the animals were bled 7 days later.

A goat was injected with α_1-aryl esterase obtained from analytical disc electrophoresis. The crushed samples of acrylamide were mixed with Freund's complete adjuvant and a single injection was given in each thigh. After 3 weeks, another intramuscular injection was given in Freund's incomplete adjuvant. One week later, the goat was bled from the jugular vein and the serum was stored at −20°.

Allotyping of Rabbits. Rabbits were bled from the marginal ear vein and the serum was separated and stored at −20°. Sera for the allotyping were generally obtained at 8–10 weeks of age. Double-diffusion experiments in agar gel were performed in 1.5% Noble agar with 0.2 M borate–0.85% NaCl buffer (pH 8.0). A micro-Ouchterlony plate was used for typing of the sera using appropriate reference sera for controls as described previously (Dray *et al.*, 1963). Following development of precipitin bands, the plates were soaked in saline for 3–4 days, then in water for 1 day, and finally dried.

Enzymatic Assays. Esterase activity was determined using the method described by Uriel (1964). β-Naphthyl acetate (20 mg) was dissolved in 1 ml of acetone. Phosphate buffer (pH 7.5, 0.1 M, 100 ml) and diazo blue (40 mg) were added, and the mixture was filtered and used immediately. The dried immunoelectrophoresis and Ouchterlony plates were placed in the filtered solution for 5–10 min and then washed with 2% acetic acid. The polyacrylamide gels were placed in the staining solution immediately after electrophoresis and after 5–10 min placed in 2% acetic acid.

To follow the purification of the esterase through the various preparative procedures, 2–3 drops of the eluate from the columns (Sephadex, DEAE-cellulose, and preparative disc) were placed in a spot plate and 2–3 drops of the esterase staining solution were added. Development of a purple color indicated that the eluate had esterase activity and a very dark purple color was recorded as a 4^+ staining reaction. In some experiments, α-naphthyl acetate, indoxyl acetate, or α-naphthyl butyrate was used in place of β-naphthyl acetate. The test with propionylthiocholine iodide was performed according to Uriel (1964).

Enzymatic Inhibition. Inhibition of esterase activity was determined following the electrophoresis of whole rabbit serum on polyacrylamide gels. Individual gels were incubated at room temperature in 10^{-4} M *p*-mercuribenzoate, 10^{-5} M eserine, or a 0.01% Cu^{2+} solution. All of the solutions were prepared in a 0.1 M phosphate buffer (pH 7.5). After 60-min incubation, the gels were stained for esterase activity using β-naphthyl acetate and the relative intensity of the esterase positive bands was noted. A control gel was stained for esterase activity with prior incubation in 0.1 M phosphate buffer (pH 7.5). In some experiments the inhibition of esterase activity of the proteins in the third peak from Sephadex G-200 was determined. The protein solution was incubated with an equal volume of the inhibitor solution. After 60 min, 2 drops of this mixture were added to 2 drops of the esterase stain and the relative intensity of the purple color was noted.

Analytical Methods. Immunoelectrophoresis was performed in 1% (w/v) Agarose in 0.05 M sodium barbital buffer (pH 8.6) (Grabar and Williams, 1955). Analytical disc electrophoresis was performed in 7.5% polyacrylamide gel (pH 9.4) as described by Williams and Reisfeld (1964). In some experiments, portions of the disc gel were cut after electrophoresis, placed at the origin on a second set of polyacrylamide gels, and reelectrophoresced.

Agar gel diffusion using polyacrylamide disc gels was performed by two methods. In some experiments, portions of the polyacrylamide disc were cut out with a razor and inserted into wells on an Ouchterlony plate prepared with 1.5% agar in 0.2 M borate–0.85% NaCl buffer (pH 8.0). Antiserum was placed in an adjacent well and diffusion was allowed for 24 hr. In other experiments, the entire polyacrylamide disc gel was inserted into a trough on an Ouchterlony plate. Antiserum was placed in an adjacent trough and diffusion was allowed for 48 hr.

Immunochemical Determinatiom of the Amounts of α_1-Aryl Esterase in Serum. The relative amount of α_1-aryl esterase in different rabbits was determined by radial diffusion experiments as described by Mancini *et al.* (1963). A 1:20 dilution of the goat anti-α_1-aryl esterase, incorporated into 1.5% Noble agar in 0.2 M borate–0.85% NaCl buffer (pH 8.0), was poured into a 100 × 15 mm disposable Petri dish and allowed to harden. Several wells, 1 cm apart and 1 mm in diameter,

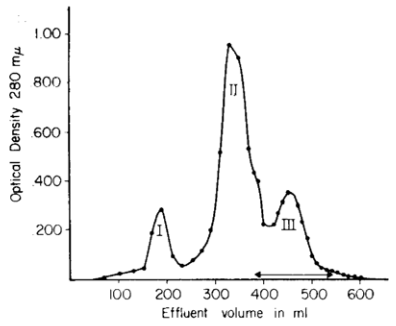

FIGURE 1: Gel filtration on Sephadex G-200 using saline–borate buffer (pH 8.0) of protein, eluted from DEAE-cellulose with 0.02 M Tris–0.15 M NaCl (pH 7.2). The acetate which exhibited esterase activity toward β-naphthyl acetate is indicated by the arrows.

were cut into the agar. Equal volumes of serum from several rabbits were carefully pipetted into individual wells. After 24 and 48 hr, the diameter of the precipitin rings formed was measured.

Results

Characterization of Purified α_1-Aryl Esterase. Figure 1 shows the elution pattern from Sephadex G-200 of the proteins eluted from DEAE-cellulose with 0.02 M Tris–0.15 M NaCl (pH 7.2). Three peaks were obtained from Sephadex G-200, and following concentration of the peaks by ultrafiltration, neither peak I nor peak II reacted with the antiallotype antisera. In addition, neither peak exhibited esterase activity with β-naphthyl acetate as substrate. If, however, the high- and low-density lipoproteins are not first removed by salting-out or by DEAE-cellulose, peak I and peak II from Sephadex G-200 will exhibit esterase activity due to the presence of the lipoproteins. The proteins in the third peak (Figure 1) had esterase activity and also reacted with the anti-allotype antisera. Preparative disc electrophoresis of the proteins in this peak from Sephadex G-200 resulted in the α_1-aryl esterase being eluted immediately following the elution of albumin. Since the optical density of the eluate at 280 mμ was only approximately 0.2 in 1-cm cells, elution of the esterase was monitored by reaction of eluate samples with β-naphthyl acetate. Fractions with esterase activity were pooled and concentrated. Immunoelectrophoresis of these preparations revealed that highly purified α_1-aryl esterase could be obtained. Figure 2 shows the immunoelectrophoresis of a purified preparation of α_1-aryl esterase. The reaction of the α_1-aryl esterase with a multispecific sheep anti-whole rabbit serum (Figure 2) revealed only a single precipitin arc, resulting from the reaction of a protein with α_1 electrophoretic mobility.

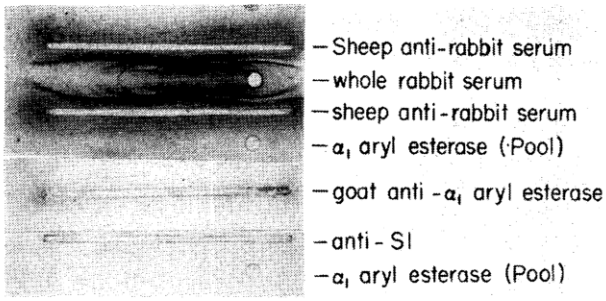

FIGURE 2: Immunoelectrophoresis in 1% Agarose showing the precipitin reaction between electrophoresced α_1-aryl esterase purified from a pool of rabbit sera and (1) sheep anti-whole rabbit serum, (2) goat anti α_1-aryl esterase, and (3) the two anti-allotype antisera. The reaction of electrophoresced rabbit serum is shown as a reference. The anode is at the left.

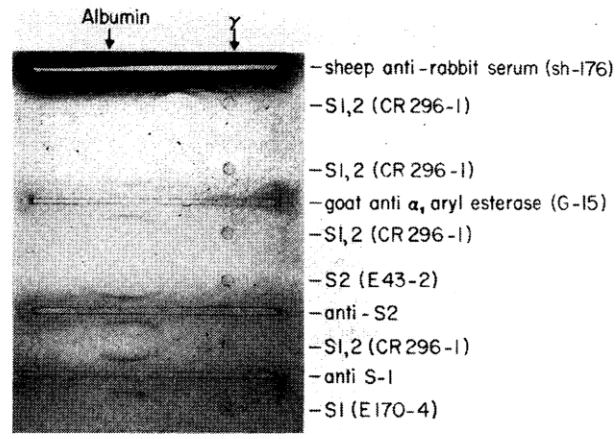

FIGURE 3: Immunoelectrophoresis in 1% Agarose showing the precipitin reaction between electrophoresced rabbit serum and (1) sheep anti-whole rabbit serum, (2) goat anti-α_1-aryl esterase, and (3) anti-allotype antisera. The α_1-aryl esterase phenotype of each rabbit is given in front of the parentheses. The anode is at the left.

Characterization of Goat Antiserum. Antiserum obtained from the goat 3 weeks after the first injection with a highly purified α_1-aryl esterase preparation gave on immunoelectrophoresis a single precipitin arc to a protein in the α_1 region (Figure 3). That this antiserum was directed against α_1-aryl esterase was confirmed by its reaction with the purified α_1-aryl esterase which on immunoelectrophoresis against sheep anti-whole rabbit serum resulted in a single precipitin arc in the α_1 region. The precipitin arc formed by the goat antiserum with the α_1-aryl esterase did not exhibit esterase activity with β-naphthyl acetate even though the purified esterase did react with β-naphthyl acetate. Later bleedings of the goat revealed two precipitin arcs with whole rabbit serum on immonoelectrophoresis. The major precipitin arc resulted from antibody to α_1-aryl esterase. The minor precipitin arc resulted from antibody to a protein with an electrophoretic mobility slightly more anodic than the α_1-aryl esterase.

Identification of α_1-Aryl Esterase Allotypes. By cross-immunization of rabbits with crude α_1-aryl esterase preparations, two antisera (2D42-3 and E170-3) were obtained which were specific for different serum allotypes, designated Ess-1 and Ess-2, and abbreviated to s1 and s2. All normal rabbit sera examined reacted with one antiserum, the other antiserum, or both. The presence of an allotypic specificity for each serum was determined in double-diffusion experiments by the coalescence of the precipitin band of the unknown serum with the precipitin band of the reference serum. Figure 3 is a photograph of an immunoelectrophoresis experiment showing the reaction between the anti-allotype antisera, anti-s1 and anti-s2, with sera from rabbits having one or both allotypes. Comparison of the precipitin arcs formed by

TABLE I: Progeny Test for Allelism of *Ess*-1 and *Ess*-2.

Genotype of Parents	No. of Litters	No. of Progeny		Genotype of Offspring s^1	Genotype of Offspring s^1s^2	Genotype of Offspring s^2	Probability
$s^2s^2 \times s^2s^2$	13	55	Exptl	0	0	55	
			Theor	0	0	55	
$s^2s^2 \times s^1s^1$	13	56	Exptl	0	56	0	
			Theor	0	56	0	
$s^1s^1 \times s^1s^1$	2	9	Exptl	9	0	0	
			Theor	9	0	0	
$s^1s^2 \times s^2s^2$	24	111	Exptl	0	57	54	0.90–0.80
			Theor	0	55.5	55.5	
$s^1s^2 \times s^1s^2$	16	68	Exptl	16	32	20	0.80–0.70
			Theor	17	34	17	
$s^1s^2 \times s^1s^1$	8	37	Exptl	18	19	0	0.95–0.90
			Theor	18.5	18.5	0	
Total	76	336					

sheep anti-rabbit serum with the precipitin arcs formed by the anti-allotype antisera shows that the allotypes migrate electrophoretically in the α_1 region. The appearance and position of the two anti-allotype precipitin arcs are similar to each other and also to the precipitin arc of goat anti-α_1-aryl esterase (Figure 3). Also, purified α_1-aryl esterase which gives only a single precipitin band against sheep anti-whole rabbit serum, reacts with both anti-allotype antisera, and on immunoelectrophoresis, the precipitin arcs are similar to the α_1-aryl esterase band formed with goat anti-α_1-aryl esterase (Figure 2).

Test for Allelism. Sera from 336 progeny from 76 families of rabbits were tested with anti-s1 and anti-s2. Table I shows the genotypes of the 336 offspring from all six possible parent combinations involving the allotypes s1 and s2. The probability values were obtained from the chi-squared test using the hypothesis that the allotypes s1 and s2 are controlled by allelic genes. All progeny from matings in which both parents were heterozygous reacted with anti-s1, anti-s2, or both. No rabbit was found that lacks both allotypes. The probability values for all matings is greater than 0.70 and the experimental findings are therefore consistent with the hypothesis that the allotypic specificities, s1 and s2, are controlled by allelic genes.

Test for No Association between the Ess Locus and Loci Controlling Other Serum Protein Allotypic Specificities. Figure 4 shows pedigrees of the *Ess* locus of the α_1-aryl esterase and the *Lpq* locus of low-density lipoproteins. If the *Ess* locus and *Lpq* locus are closely linked, a double heterozygous parent, q^1q^2 and s^1s^2, should transmit either the genes q^2s^1 in half the gametes and in the other half, q^1s^2, or the other combination of half q^2s^2 and half q^1s^1 but not both combinations. The pedigrees shown in Figure 4 have progeny with genotypes $q^1q^1s^2s^2$ and $q^1q^1s^1s^2$ showing that the *Ess* locus of the α_1-aryl esterase is not closely linked to the *Lpq* locus of the low-density lipoproteins. Similar pedigrees with the *Mtz* locus of the α_2-macroglobulin, the *a* (heavy chain) and *b* (light chain) loci of immunoglobulins, the *h* locus of haptoglobin, and the *f* locus of IgA-immunoglobulin reveal that the *Ess* locus is not closely linked to any of these loci. Finally, all 23 male progeny from reciprocal crosses of s^1s^1 and s^2s^2 rabbits had both allotypes indicating that the *Ess* locus is not sex linked.

Identification of Allotypes on Separate Molecules. Figure 5A shows the precipitin bands formed by the reaction of an s1,2 heterozygous serum with purified anti-allotype immunoglobulins, anti-s1 and anti-s2. The two precipitin bands crossed and no partial reaction of identity was observed. This indicates that the allotypic specificities s1 and s2 occur on separate α_1-aryl esterase molecules in an s^1s^2 heterozygous rabbit.

Esterase Activity of α_1-Aryl Esterase Allotypes. The α_1-

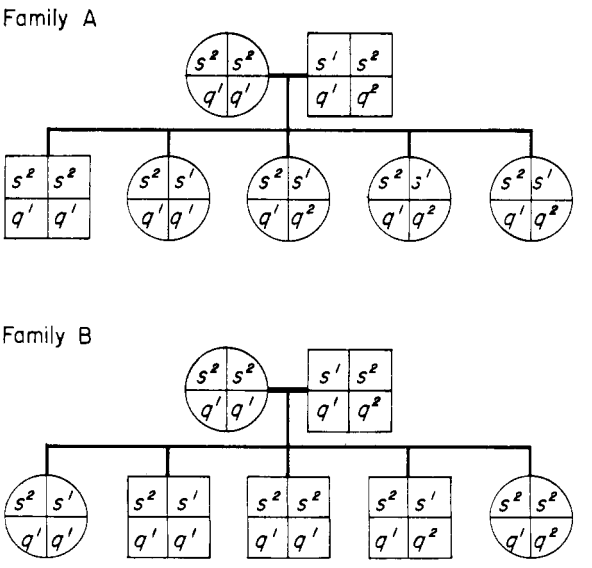

FIGURE 4: Pedigree of two rabbit families showing nonlinkage of the *Lpq* locus of low-density lipoproteins and the *Ess* locus. The low-density lipoprotein allotype is designated as ql or q2 and the α_1-aryl esterase alloytpe is designated as s1 or s2.

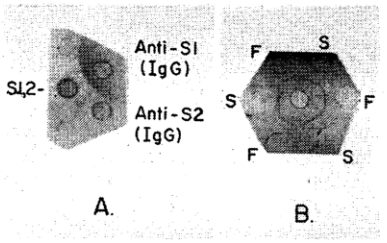

FIGURE 5: Double-diffusion experiments. (A) Showing crossing of the precipitin bands formed by the reaction of serum from an s1,2 heterozygote with anti-s1 and anti-s2 immunoglobulins. (B) Showing coalescence of the precipitin lines formed by the reaction of the fast (F) and slow (S) α_1-aryl esterase isozymes from an s^2s^2 rabbit, with goat anti-α_1-aryl esterase. The α_1-aryl esterase isozyme bands (fast and slow) were cut from polyacrylamide gels and placed in the outer wells. The center well contained goat anti-α_1-aryl esterase.

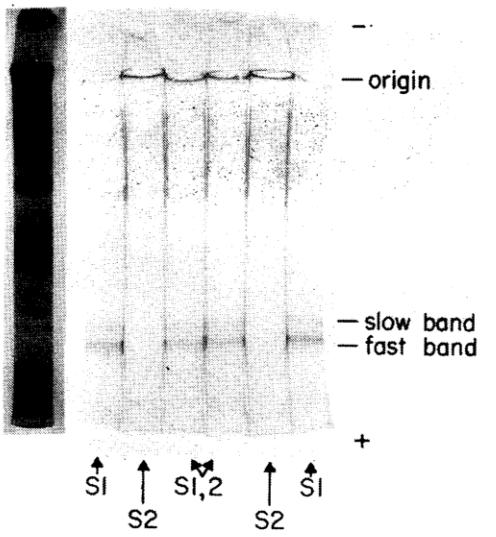

FIGURE 6: Analytical disc electrophoresis of whole serum on polyacrylamide gel. The disc gel on the left was stained for protein with 0.05% coomassie blue in 6% trichloroacetic acid; the remainder were stained with β-naphthyl acetate and diazo blue. The phenotype of the sera electrophoresced is indicated at the bottom of each disc gel.

aryl esterase allotypes were examined for esterase activity toward a variety of substrates using crude preparations of α_1-aryl esterase from rabbits of different genotypes (Table II). The allotypes were also examined for esterase activity on polyacrylamide gels following electrophoresis of whole serum from rabbits of different genotypes and in the precipitin bands in immunoelectrophoresis and agar gel diffusion experiments formed by the reaction of anti-s1 and anti-s2 with the s1 or s2 allotype, respectively. The results obtained were essentially the same as that shown in Table II.

Neither s1 nor s2 allotypes hydrolyzed propionyl thiocholine ester or methyl butyrate. The reaction with methyl butyrate was determined with crude esterase fractions only. With the addition of diazo blue, the s1 allotype with anti-s1 stained intensely with α-naphthyl acetate, β-naphthyl acetate, α-naphthyl butyrate, and indoxyl acetate, and weakly with *p*-nitrophenyl acetate. The s2 allotype stained only weakly with β-naphthyl acetate, α-naphthyl butyrate, and indoxyl acetate; no reaction with α-naphthyl acetate and a trace reaction with *p*-nitrophenyl acetate was observed. Precipitin bands formed with anti-s1 and sera from s1,2 heterozygotes stained with an intermediate intensity with α-naphthyl acetate, β-naphthyl acetate, α-naphthyl butyrate, and indoxyl acetate, *i.e.*, they stained less intensely than the s1 homozygotes with anti-s1 but more intensely than the s2 homozygotes with anti-s2.

The presence of antibody in the precipitin bands in double-diffusion experiments did not appear to influence the esterase activity since the enzymatic activity toward the various substrates was the same for the precipitin bands (presence of antibody) as for the crude preparations of α_1-aryl esterase (absence of antibody).

The relative amount of inhibition of esterase activity by 10^{-4} M *p*-mercuribenzoate, 10^{-5} M eserine, and 0.01% Cu^{2+} is also indicated in Table II. Both eserine and Cu^{2+} appeared to completely inhibit the esterase activity since the subsequent reaction with β-naphthyl acetate and diazo blue resulted in no visible color formation. *P*-mercuribenzoate partially inhibited the esterase activity as shown by the 1+ reaction compared with the 4+ reaction of esterase which was not incubated with *p*-mercuribenzoate. The results of the inhibition experiments were the same for the (1) esterase on polyacrylamide gels following electrophoresis of whole serum and (2) esterase from the partially purified third peak of Sephadex G-200 in which equal volumes of the esterase and inhibitor were mixed and incubated.

Isozymic Nature of α_1-Aryl Esterase Allotypes. Fifteen microliters of serum from rabbits with the genotypes s^1s^1, s^1s^2, and s^2s^2 was electrophoresced on polyacrylamide disc gel and subsequently stained for esterase activity with β-naphthyl acetate. As shown in Figure 6, sera from s^1s^1 and s^1s^2 rabbits revealed two bands with esterase activity in the α_1 region. The two bands are indicated as the fast and slow bands. With sera from s^2s^2 homozygotes, however, no esterase activity was observed in the α_1 region. Similar results

TABLE II: Reaction of α_1-Aryl Esterase Allotypes with Various Substrates and the Effect of Inhibitors.

Substrates	Inhibitor	Reaction	
		Ess-1	Ess-2
α-Naphthyl acetate		4+	0
β-Naphthyl acetate		3+	1+
α-Naphthyl butyrate		3+	1+
Indoxyl acetate		3+	1+
p-Nitrophenyl acetate		1+	0–1+
Propionylthiocholine iodide		0	0
Methyl butyrate[a]		0	*b*
β-Naphthyl acetate	*p*-Mercuribenzoate	1+	*b*
β-Naphthyl acetate	Eserine	0–1+	*b*
β-Naphthyl acetate	Cu^{2+}	0	*b*

[a] Assay kindly performed by Mr. Daniel Albertson according to the procedure described by Schwartz and Myers (1958). [b] Not done.

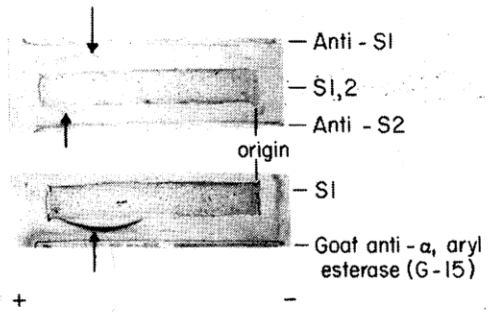

FIGURE 7: Agar gel diffusion showing precipitin reaction between whole rabbit serum electrophoresced on polyacrylamide gel and (1) goat anti-α_1-aryl esterase and (2) antiallotype antisera, anti-s1 and anti-s2.

were obtained with the partially purified α_1-aryl esterase preparations, isolated from sera of rabbits of genotypes s^1s^1, s^1s^2, and s^2s^2. In addition to the two α_1-esterase bands observed by the reactions of β-naphthyl acetate on electrophoresced s^1s^1 serum, a diffuse area in the α_2 to β region with esterase activity was seen. Figure 7 shows the results of the double-diffusion experiments done by inserting the entire disc gel, after electrophoresis of whole serum, into a trough cut in an agar gel plate, and allowing antiserum to diffuse from an adjacent trough. The goat anti-α_1-aryl esterase and both antiallotype antisera revealed precipitin arcs in the α_1 region, where the two esterase staining bands were previously observed. No precipitin bands were observed in the α_2 to β region. Figures 5B and 8 are photographs of a double-diffusion experiment performed in an effort to determine if either or both of the two esterase staining bands in the α_1 region is α_1-aryl esterase. The figures show that both bands with esterase activity cut from the disc and placed in an Ouchterlony plate, reacted with the anti-allotype antisera and with the specific goat anti-α_1-aryl esterase. The two esterase isozymes are immunologically very similar since their precipitin lines fuse, both with the goat anti-α_1 aryl esterase (Figure 5B) and with the anti-allotype antisera (Figure 8). The precipitin bands formed by the reaction of the isozymes from an s1 or s1,2 sera with anti-s1 exhibited esterase activity while the precipitin band formed by the reaction of the isozymes of s2 or s1,2 sera with anti-s2 exhibited little or no esterase activity. When the fast and slow bands were cut from the disc and reelectrophoresed separately, each of the isozymes migrated as a single band with no change in mobility (Figure 9).

TABLE III: Radial Precipitin Analysis of α_1-Aryl Esterase Allotypes.

Genotype	No. of Animals	Diameter (mm) Range (mm)	Diameter (mm) Av (mm)
s^1s^1	10	5.6–7.0	6.3
s^1s^2	10	6.0–7.0	6.5
s^2s^2	10	5.5–7.2	6.4

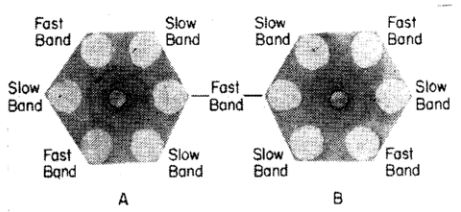

FIGURE 8: Double-diffusion experiment showing coalescence of the precipitin lines formed by the reaction of the fast and slow α_1-aryl esterase isozymes with anti-allotype antisera. The isozyme bands were cut from polyacrylamide disc gels following electrophoresis and placed in the outer wells. Anti-s1 was placed in the center well of pattern A; anti-s2 was placed in the center well of pattern B.

Quantitation of α_1-Aryl Esterase. The goat antiserum used for the immunochemical determination of relative amounts of the α_1-aryl esterase in rabbit serum gave two precipitin bands on immunoelectrophoresis with whole rabbit serum. One of the antibodies was directed against α_1-aryl esterase and the other against a contaminant protein in the α_0 region. A 1:20 dilution of the goat antiserum showed a single strong precipitin ring formed by whole rabbit serum in radial diffusion experiments. At this dilution, the second antibody showed no precipitin ring with whole rabbit serum. To determine if the one visible precipitin ring was formed by the reaction of the goat anti-α_1-aryl esterase with α_1-aryl esterase, the goat anti-esterase antibody was absorbed with purified α_1-aryl esterase. This purified preparation of α_1-aryl esterase used for absorption reacted with the anti-allotype antisera, had esterase activity, and reacted with the sheep anti-whole rabbit serum on immunoelectrophoresis to reveal only a single band. Radial diffusion experiments with this absorbed goat antiserum at 1:20 dilution revealed no precipitin ring with whole rabbit serum thus indicating that the precipitin ring observed with the 1:20 dilution of goat anti-α_1-aryl esterase (unabsorbed) and whole rabbit serum is due to the reaction of α_1-aryl esterase and anti-α_1-aryl esterase. Table III shows the results of radial diffusion experiments with sera from rabbits of genotypes s^1s^1, s^1s^2,-and s^2s^2 using

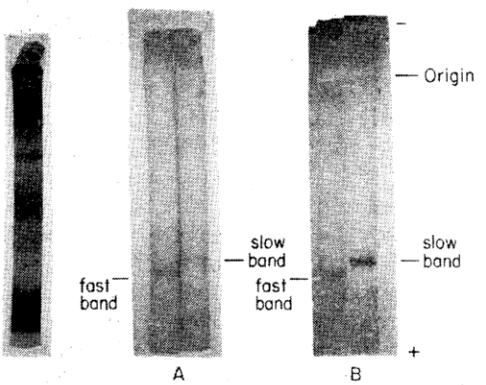

FIGURE 9: Reelectrophoresis on polyacrylamide gel of the fast and slow α_1-aryl esterase isozyme bands showing migration as a single band with an electrophoretic mobility similar to its original mobility. The disc gel on the left shows disc electrophoresis of whole serum stained for protein with 0.05% coomassie blue in 6% trichloroacetic acid. Disc gels in A were stained with β-napthyl acetate and diazo blue; disc gels in B were stained for protein with coomassie blue.

the unabsorbed goat anti-α_1-aryl esterase at 1:20 dilution. The diameter of the precipitin rings varied from 5.6 to 7.2 mm, and there appeared to be no significant difference between animals of different genotypes.

Discussion

Isoantibodies were obtained by cross-immunization of rabbits with α_1-aryl esterase preparations isolated from serum of other rabbits. These isoantibodies, anti-s1 and anti-s2, identified two allotypes of rabbit α_1-aryl esterase, designated s1 and s2. Progeny studies indicated that the two allotypes are controlled by allelic genes at an autosomal locus. The limited genetic data cannot eliminate the possibility, however, that the genes are closely linked rather than allelic as occurs in the heavy-chain chromosome region of immunoglobulins (Dray *et al.*, 1965; Natvig *et al.*, 1967). All 488 animals tested reacted with one or both antisera and no evidence was observed for the presence of a third allele in our colony.

Each of the isoantibodies used to type the rabbit sera revealed only one precipitin band on Ouchterlony analysis and immunoelectrophoresis. To minimize errors of typing due to nonspecific reactions or cross-reactions, coalescence of the precipitin band of the unknown sera with that formed by a reference serum was the criterion used to determine the presence of the allotypic specificity.

The allotypes described were identified as α_1-aryl esterase by isolating a protein fraction from pooled rabbit serum using salt precipitation, gel filtration, DEAE-cellulose, and preparative disc electrophoresis. This purified fraction was analyzed for its antigenic, immunoelectrophoretic, and enzymatic properties. On immunoelectrophoresis, this purified fraction revealed single similarly appearing precipitin arcs in the α_1 region with (1) sheep anti-whole rabbit serum, (2) monospecific goat anti-α_1-aryl esterase, and (3) each anti-allotype antiserum. The precipitin arcs formed by the anti-allotype antisera with whole serum or with purified esterase fractions had esterase activity using β-naphthyl acetate as substrate.

Uriel (1964) identified the following four precipitin arcs on immunoelectrophoresis of human serum which showed esterase activity using β-naphthyl acetate as substrate: (1) fast (high density) lipoproteins, (2) slow (low density) lipoproteins, (3) pseudocholinesterase, and (4) a protein found in the albumin region. In addition, an esterase with electrophoretic mobility slightly faster than albumin has been described and called ρ-esterase (Lichter and Dray, 1964). Clearly, the allotypes are not lipoproteins since: (1) they do not stain with the specific lipid stain, sudan black B; (2) the low-density lipoproteins migrate electrophoretically in the α_2 to β region; and (3) the lipoproteins are eluted from Sephadex G-200 in the first and second of the three peaks resulting from the separation of whole serum, and α_1-aryl esterase is eluted in the third peak. The allotypes are not cholinesterase since they do not hydrolyze propionylthiocholine iodide, a substrate hydrolyzed by cholinesterases (Uriel, 1963). These rabbit allotypes may possibly be the esterase described by Uriel as migrating with the albumin in immunoelectrophoresis or they may be the ρ-esterase. However, it had been reported that electrophoretic distribution of esterases vary widely in different species and are species specific (Holmes and Masters, 1968). It is therefore possible that the esterase described here would have a different electrophoretic mobility from the comparable esterases than in human serum. Furthermore, there are undoubtedly more esterases than observed by the technique of immunoelectrophoresis since (1) the enzymatic site may be blocked by combination with antibody and thus preventing its reaction with the substrate, and (2) the antiserum might not have antibody to all esterases present in normal serum and thus no precipitin bands would be formed.

Augustinsson (1961) divided vertebrate plasma esterases into the following three classes by means of electrophoretic mobility at pH 8.4 and substrate and inhibitor specificities: aryl esterases, aliesterases, and cholinesterases. He reported that aryl esterases hydrolyze aromatic esters but not aliphatic nor choline esters, and migrate electrophoretically at pH 8.4 in the albumin region, and are inhibited by *p*-mercuribenzoate and Cu^{2+}. The allotypes described here appear to be α_1-aryl esterase since they hydrolyze all aromatic esters examined (β-naphthyl acetate, indoxyl acetate, α-naphthyl butyrate, and *p*-nitrophenyl acetate). They migrate electrophoretically in the α_1 region, and are inhibited by *p*-mercuribenzoate and Cu^{2+} (Table II). The allotypes appear not to be aliesterases since they do not hydrolyze methyl butyrate and also since aliesterases are not inhibited by *p*-mercuribenzoate. The enzymatic activity of the allotypes is inhibited by eserine and the only esterase described by Augustinsson inhibited by eserine is cholinesterase. However, as already stated, the allotypes are not cholinesterase since they do not hydrolyze choline ester and they have a faster electrophoretic mobility. Therefore, due to this difference in inhibitors, the allotypes do not appear with be identical with any of the esterases described by Augustinsson.

The isozymic nature of the allotypes was recognized by the polyacrylamide gel electrophoresis of sera from rabbits of various genotypes. Disc electrophoresis of whole rabbit serum from an s^1s^1 rabbit revealed two bands with esterase activity having an electrophoretic mobility slightly less than albumin. Each isozyme band reacted with the goat anti-α_1-aryl esterase and the anti-s1 anti-allotype antiserum. Both isozymes possessed the same isotypic antigenic determinants (using goat anti-α_1-aryl esterase) and the same allotypic antigenic determinants (using rabbit anti-s1) as observed by the coalescence of the two precipitin lines. Disc electrophoresis of serum from an s^2s^2 rabbit revealed no esterase staining bands in the same region as observed for the s^1s^1 rabbit. On staining with the protein stain, Amido Black, two bands migrating close to the albumin band were seen, each of which reacted with the anti-s2 antiserum and goat anti-α_1-aryl esterase; each antiserum formed precipitin lines of identity with the two isozyme bands showing again that both isozymes have the same isotypic and allotypic determinants. Therefore, in addition to allotypic variations, the α_1-aryl-esterase occurs in at least two electrophoretic polymorphic forms which are thus far indistinguishable immunologically. Whereas a rabbit may have different allotypes of α_1-aryl esterase, *i.e.*, either s1, s2, or s1 and s2, all rabbits exhibit the two electrophoretically different isozymes, *i.e.*, fast and slow, of α_1-aryl esterase.

The possibility that the isozymes were simply due to aggregation products of the same protein was investigated. The two α_1-aryl esterase bands observed in disc electrophoresis were cut from the disc and reelectrophoresced individually.

Each band migrated as a single band with an electrophoretic mobility identical with its original mobility. This suggests that the two bands observed in electrophoresis of whole serum are not the result of simple aggregation but in fact represent different molecular species, *i.e.*, isozymes. However, we cannot at this time rule out the possibility that the isozymes result from deamidination and differ only in the number of amide groups on the molecule.

The esterase activities of the allotypes were found to be markedly different. The s1 allotype exhibited strong esterase activity toward all aryl esters tested and the s2 allotype reacted only weakly with these same esters. Precipitin bands formed by sera from s^1s^2 heterozygotes reacted with intermediate intensity. The decrease in enzymatic activity of the s2 allotype observed in precipitin bands is not due to the blocking of the enzymatic site of the s2 allotype by the antibody since similar staining reactions were obtained with sera electrophoresced on polyacrylamide gel without the presence of the antibody. In contrast, the reaction of goat anti-α_1-aryl esterase with the allotypes does block the enzymatic activity of both s1 and s2. Quantitative analysis of the allotypes with the goat anti-α_1-aryl esterase revealed that the relative amount of α_1-aryl esterase is similar for animals of the three known phenotypes, s1, s1,2, and s2 indicating that the decreased enzymatic activity of s2 is also not due simply to a lesser amount of enzyme present. A quantitative enzymatic assay of each of the allotypes needs to be done to determine the actual difference in enzymatic activities.

There appears to be a relationship between the allotypic specificities and the enzymatic site of the α_1-aryl esterase molecule. It may be that a part (some amino acid residues) of the allotypic determinant contributes to the enzymatic site, and that the s2 determinant interferes with the enzymatic activity while the s1 determinant enhances it.

We are not aware of any other example in mammals where both allelic enzyme products have different quantitative enzymatic activities. However, since we have not yet identified the natural biological substrate for α_1-aryl esterase, the possibility exists that such quantitative differences might not hold *in vivo*. Also, since the biological function of α_1-aryl esterase is not yet known, we cannot be certain that it functions as an esterase *in vivo*. If, however, it does indeed function as an esterase, it would be important to determine its biological substrate. Experimental liver damage in mice and cirrhosis in man have been shown to be accompanied by a marked decrease in liver and serum esterase activity (Takahashi *et al.*, 1967), using phenyl acetate as substrate. The phenyl acetate esterase, was described as an aryl esterase which migrated in the albumin region on starch block electrophoresis and may correspond to the α_1-aryl esterase described in our study.

Preliminary studies indicate that the s1 and s2 specificities are on different molecules in an s^1s^2, heterozygous animal. When the isolated immunoglobulins from anti-s1 and anti-s2 antisera were reacted with serum from a heterozygous animal, in a double-diffusion experiment, the resulting precipitin bands did not coalesce, *i.e.*, they crossed, thus suggesting that the molecules do not have both s1 and s2 allotypic specificities. Also, consistent with this is the observation that the precipitin line formed by the reaction of anti-s1 and serum from an s1,2 heterozygote exhibited strong esterase activity, but the precipitin band formed with the same s1,2 serum and anti-s2, had very little esterase activity. Quantitative evidence to determine if any molecules in a heterozygous animal have both specificities is being obtained by precipitation of radioactively labeled purified α_1-aryl esterase with the anti-allotype antisera.

It is difficult to determine how the allotypes of rabbit α_1-aryl esterase relate to the genetic studies of serum aryl esterases previously reported.

By starch gel electrophoresis of serum, Petras (1963) described three phenotypes of a mouse aryl esterase with fast mobility and Augustinsson and Henricson (1966) described two phenotypes of a rat aryl esterase also with a fast mobility. In both studies only one gene product was identified. No immunological nor enzymatic studies were undertaken to identify an allelic product in phenotypes which lacked esterase activity. Also, no isozymes were observed and in neither study was the esterase isolated.

No strutural studies have yet been reported on α_1-aryl esterase. However, since α_1-aryl esterase is eluted in the third peak from gel filtration of whole serum on Sephadex G-200, the molecular weight is probably less than 100,000. The difficulty in obtaining large amounts of purified α_1-aryl esterase has hindered extensive structural studies; however, the availability of specific antisera for α_1-aryl esterase will be useful for the purification of larger quantities of this protein. Also, the anti-allotype antisera will be useful for studying the structure and biosynthesis of α_1-aryl esterase in a manner similar to that done with the immunoglobulins (Dray and Nisonoff, 1965; Pernis *et al.*, 1965; Adler *et al.*, 1966).

References

Adler, F. L., Fishman, M., and Dray, S. (1966), *J. Immunol. 97*, 554.

Albers, J. J., and Dray, S. (1968), *Biochem. Genet. 2*, 25.

Augustinsson, K. B. (1961), *Ann. N. Y. Acad. Sci. 94*, 844.

Augustinsson, K. B., and Henricson, B. (1966), *Biochem. Biophys. Acta 124*, 323.

Chiao, J. W., and Dray, S. (1969), *Biochem. Genet. 3*, 1.

Conway, T., Dray, S., and Lichter, E. (1969), *J. Immunol. 102*, 544.

Dray, S., and Nisonoff, A. (1963), *Proc. Soc. Exptl. Biol. Med. 113*, 20.

Dray, S., and Nisonoff, A. (1965), *in* Molecular and Cellular Basis of Antibody Formation, Sterzl, J., Ed., New York, N. Y., Academic.

Dray, S., Potter, M., and Lieberman, R. (1965), *J. Immunol. 95*, 823.

Dray, S., Young, G. O., and Gerald, L. (1963), *J. Immunol. 91*, 403.

Dubiski, S., and Muller, P. J. (1967), *Nature 214*, 696.

Grabar, P., and Williams, C, A. (1955), *Biochim. Biophys. Acta 17*, 67.

Hamers, R., Hamers-Casterman, C., and Lagnaux, S. (1966), *Immunology 10*, 399.

Holmes, R. S., and Masters, C. J. (1968), *Biochim. Biophys. Acta 151*, 147.

Kekwick, R. A. (1940), *Biochem. J. 34*, 1248.

Kelus, A. S., and Gell, P. G. H. (1967), *Progr. Allergy 11*, 141.

Knight, K. L., and Dray, S. (1968), *Biochemistry 7*, 1165.

Lark, C. A., Eisen, H. N., and Dray, S. (1965), *J. Immunol. 95*, 404.

Levy, H. B. and Sober, H. A. (1960), *Proc. Soc. Exptl. Biol. 103*, 250.
Lichter, E. A., and Dray, S. (1964), *J. Immunol. 92*, 91.
Mancini, G,, Vaerman, J. P., Carbonara, A. D., and Heremmans, J. F. (1963), *Proc. XI Colloq. Protein Biol. Fluids 11*, 370.
Natvig, J. B., Kunkel, H. G., and Litwin, S. D. (1967), *Cold Spring Harbor Symp. Quant. Biol. 32*, 173.
Oudin, J. (1956), *Compt. Rend. 242*, 2606.
Oudin, J. (1960), *J. Exptl. Med. 112*, 107.
Pernis, B., Chiappino, M. B., Kelus, A. S., and Gell, P. (1965), *J. Exptl. Med. 122*, 853.
Petras, M. L. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 112.
Reisfeld, R. A., Dray, S., and Nisonoff, A. (1965), *Immunochemistry 2*, 155.
Schwartz, M., and Myers, T. C. (1958), *Anal. Chem. 30*, 1150.
Small, P. A., Reisfeld, R. A., and Dray, S. (1965), *J. Mol. Biol. 11*, 713.
Small, P. A., Reisfeld, R. A., and Dray, S. (1966), *J. Mol. Biol. 16*, 328.
Takahashi, Y., Aoyama, I., Ito, F., and Yamamura, Y. (1967), *Clin. Chim. Acta 18*, 21.
Uriel, J. (1963), *Ann. N. Y. Acad. Sci. 103*, 956.
Uriel, J. (1964), *in* Immunoelectrophoretic Analysis, Grabar, P., and Burton, P., Ed., Elsevier, Amsterdam, p 50.
Williams, D. E., and Reisfeld, R. A. (1964), *Ann. N. Y. Acad. Sci. 121*, 373.

Dehydrogenase-Reduced Coenzyme Difference Spectra, Their Resolution and Relationship to the Stereospecificity of Hydrogen Transfer*

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ABSTRACT: The binding of reduced diphosphopyridine nucleotide to any specific dehydrogenase causes changes in the ultraviolet absorption spectrum of the reduced coenzyme. The 340-mμ regions of the resulting difference spectra can all be resolved into two simple operations on a reduced diphosphopyridine nucleotide spectrum; a shift of the band itself to a higher or lower wavelength without any change in shape and a uniform hyper- or hypochromicity of that peak. We find that A stereospecific dehydrogenases produce blue shifts while B stereospecific dehydrogenases produce red shifts (with the possible exception of mitochondrial malate dehydrogenase). The size of most of the shifts observed here is sufficiently large that the concentration of reduced diphosphopyridine nucleotide involved can be calculated. We have previously shown that changes in conformation of reduced diphosphopyridine nucleotide produce difference spectra resolvable into various combinations of the same components. Most of the shifts of the coenzyme spectrum in dehydrogenase complexes, however, are far too large to be accounted for by a simple opening or closing of the reduced diphosphopyridine nucleotide molecule.

In previous studies we have shown that the shift in the ultraviolet absorption spectrum of DPNH produced by the binding of the reduced coenzyme to either liver alcohol dehydrogenase, liver L-lactate dehydrogenase, or mitochondrial malate dehydrogenase described by Theorell and Bonnichsen (1951), Chance and Neilands (1952), and Pfleiderer and Hohnholz (1959) is not a unique property of those three enzymes, but occurs in the binary complexes of other dehydrogenases (Fisher and Cross, 1966). In a more recent paper (Cross and Fisher, 1969), we have shown that changes in the conformation of DPNH (or TPNH) in solution generate rather similar difference spectra, and that all such spectra can be resolved into combinations of simple shifts and hypochromicities of the 340-mμ band of DPNH. While the difference spectra resulting from DPNH conformational changes differ from those due to dehydrogenase complexing, and while no two dehydrogenase binary complex difference spectra are themselves identical, all of these difference spectra can be resolved into different combinations and algebraic senses of the same two simple operations that sufficed for the resolution of conformation difference spectra.

We present here a general survey of the difference spectra of dehydrogenase-reduced coenzyme binary complexes, the resolutions of the reduced nicotinamide absorption regions of those difference spectra into two simple and physically relevant operations, and consider the possible implications of these resolutions. The scope of this particular paper is limited to those things which are characteristic of dehydrogenase-reduced coenzyme difference spectra as a group—more detailed studies of

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