that a significant concentration of the doubly charged phosphonate ion remains at *p*H 6 and 7.

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Effects of the Amidination Reaction on Antibody Activity and on the Physical Properties of Some Proteins*

LEON WOFSY†‡ AND S. J. SINGER‡

From the Department of Chemistry,§ Yale University, New Haven, Connecticut

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Essentially all lysine residues of proteins can be amidinated under mild conditions without introducing gross changes in protein structure and physical properties. Partially and exhaustively amidinated bovine serum albumin and similarly modified rabbit antibody to benzenearsonic acid have been studied with respect to electrophoretic properties, sedimentation in the ultracentrifuge, optical rotation, and ultraviolet absorption. Six rabbit antibodies—three of them to antigenic or haptenic determinants bearing negatively charged groups—have been exhaustively amidinated to determine whether the particular antibody sites might involve complementary, positively charged ϵ-ammonium lysine residues. In each instance, antibody binding capacity was largely retained despite such modification. It has been concluded that lysine residues cannot be vital components of the reactive sites of the antibodies examined.

It has been suggested that in certain antigen-antibody systems a positively charged lysine ammonium group on the antibody molecule might be complementary to a negatively charged antigenic function (Heidelberger and Kabat, 1929; Chow and Goebel, 1935; Kleinschmidt and Boyer, 1952; Singer, 1955; Epstein and Singer, 1958). Previous investigations into this possibility have proceeded by chemically modifying the free amino groups of certain rabbit antibodies, and determining the effects of such modification on the antibody activity. Such studies have made use of either or both of two modification reactions: acetylation (Chow and Goebel, 1935; Marrack and Orlans, 1954; Singer 1955; Nisonoff and Pressman, 1958) and guanidination (Singer, 1955; Habeeb *et al.*, 1959). Each of these reactions, however, presents serious problems and limitations. In neither case can all lysine residues be reacted under conditions that do not cause nonspecific inactivation or denaturation of antibody. Extensive acetylation of amino groups results in a substantial increase in the net negative charge on a protein at neutral *p*H, since neutral functions replace positively charged groups. Intramolecular electrostatic repulsions may become sufficiently pronounced to produce gross conformational changes in the antibody molecules (Habeeb *et al.*, 1959). Such nonspecific conformational alterations in antibody molecules inevitably confuse the issue of whether any observed inactivation of antibody may conceivably be attributed to a specific chemical change within the binding sites. (Thus, it has been shown by Nisonoff and Pressman [1958] that antibody to a conjugated benzoate protein undergoes a severe loss in precipitating capacity after only 20% acetylation of amino groups—but the reactive sites have not lost their ability to bind a benzoate hapten up to the point of some 70% acetylation. If more extensive acetylation is produced, all antibody activity is eventually lost.)

Guanidination does not significantly change the charge on protein molecules at neutral *p*H, and does not appear to produce gross conformational changes in the molecules. However, in certain cases it is necessary to subject a protein to highly undesirable reaction conditions, a *p*H of 10.0–10.5 for 72 hours, in order to guanidinate a maximum of only about 70% of the amino groups (Hughes *et al.*, 1949).

By utilizing the amidination reaction, it has been possible in the present studies to modify chemically under mild conditions essentially all of the ϵ-NH_2-lysine residues of rabbit antibodies, while producing

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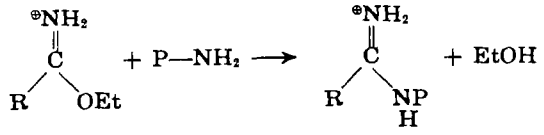
† Monsanto Chemistry Fellow, 1961.

‡ Present address: Department of Biology, University of California, San Diego, La Jolla, Calif.

§ Contribution No. 1704.

only minimal, if any, gross structural alterations in the molecules. This has permitted the determination of whether one or more lysine side-chains might be critically involved in the reactive sites of particular antibodies.

Our interest in the amidination reaction as the most useful tool for the present study, and as a valuable addition to available methods for the chemical modification of proteins, was evoked by the findings of Ludwig and Hunter (1959) on the reaction of substituted imidoesters with typical α- and ϵ-amino groups of glycylglycine and ϵ-amino-caproic acid to form amidines:

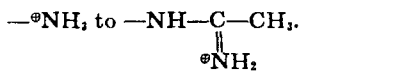


(R = alkyl or aryl, P = peptide)

They reported that the reaction proceeds "rapidly in aqueous solution near neutral *p*H even at +1°C," and that, under these conditions, the imidoester reagent does not react "with model compounds containing sulfhydryl groups, phenolic groups, imidazole groups or peptide bonds." Further, they noted that the net charge of the peptide is unchanged through most of the *p*H range, the amidine function having a *pK* near 12.

Hunter and Ludwig (1962) and Hand and Jencks (1962) have gone on to fundamental investigations of the kinetics and mechanism of the reaction of various imidoesters with peptides and proteins. Our utilization and study of the amidination reaction has been directed primarily at achieving exhaustive modification of the amino groups of proteins, and at examining the effects of such modification on protein structure and biological activity.

The amidinating reagent we have used in all cases is ethyl acetimidate hydrochloride. When this reagent reacts with an ammonium group, the latter is converted to an acetamidinium group,



This choice was determined by the desire for a relatively small and highly reactive amidinating reagent. The object was to facilitate extensive reaction, while not introducing on the protein molecule excessively bulky and hydrophobic residues with concomitant undesirable side-effects.

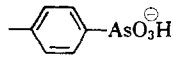
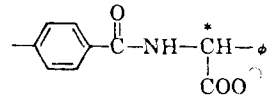
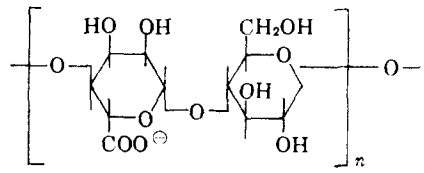
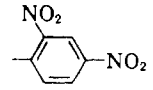
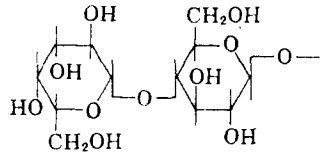
At the same time, it should be noted that the reagent used brings about an increase in size of the ϵ-*N* substituent of lysine which is the same as, or larger than, the comparable change resulting from acetylation or guanidination. This fact is relevant to the fundamental premise of this and similar investigations. It is assumed that the chemical modification of a putative critical amino function within an antibody reactive site will, by virtue of changes in the complementarity of the site to the antigenic determinant, substantially reduce antigen-binding capacity of the antibody. Based on this assumption (see Discussion), the present study took the following course:

(1) Conditions were defined which, while reasonably mild, permitted the exhaustive amidination of the lysine residues of antibodies and other proteins. This result was verified by developing suitable methods of amino acid analysis.

(2) In order to evaluate the significance of nonspecific changes caused by the extensive chemical modification, the physical properties of amidinated proteins were investigated with respect to sedimentation in the ultracentrifuge, electrophoresis, optical rotation, and ultraviolet absorption.

(3) The immunochemical activities of the partially and exhaustively amidinated antibodies (and one amidinated antigen) were assayed. The antibodies studied included two directed against negatively charged haptens, one against a negatively charged biuronic acid polysaccharide, one against a native protein antigen, and two against neutral, polar haptens. (In Table I, these antibodies are identified, and the abbreviated designations used in this paper to refer to particular antigens and haptens are indicated.)

TABLE I

Antibody	Antigen or Determinant
Anti-BSA	BSA = bovine serum albumin
Anti-benzene-arsonate	
Anti-D-benzoyl-aminophenyl acetate	
Anti-SIII	 SIII = biuronic acid repeating unit of Type III pneumococcal polysaccharide
Anti-DNP	 DNP = -2,4-dinitrophenyl
Anti-β-lactoside	

EXPERIMENTAL

Materials.—Crystalline BSA was obtained from Pentex Incorporated, Lot No. Bx4.

Anti-BSA antibody was prepared in rabbits by standard procedures, by use of the Freund adjuvant technique (Freund and Bonanto, 1944). It was studied as part of the γ-globulin fraction of pooled, high-titer anti-BSA antisera which had been precipitated with 40% saturated $(NH_4)_2SO_4$ and stored at 5° until use. A small amount of pure anti-BSA antibody, prepared by the thiolation method of Singer *et al.* (1960), was also utilized.

Pure anti-benzenearsonate antibody was prepared from pooled, high-titer antisera from rabbits immunized against the conjugated antigen, bovine γ-globulin-azobenzenearsonate. The injecting antigen was a highly coupled diazotization product (Pepe and Singer, 1959) of bovine γ-globulin (Armour and Co.) and arsanilic acid; use was made of the Freund adjuvant technique. The pure anti-hapten antibody was isolated by Epstein's method B (Epstein *et al.*, 1956), as adapted by Pepe and Singer (1959). This involves precipitation of the

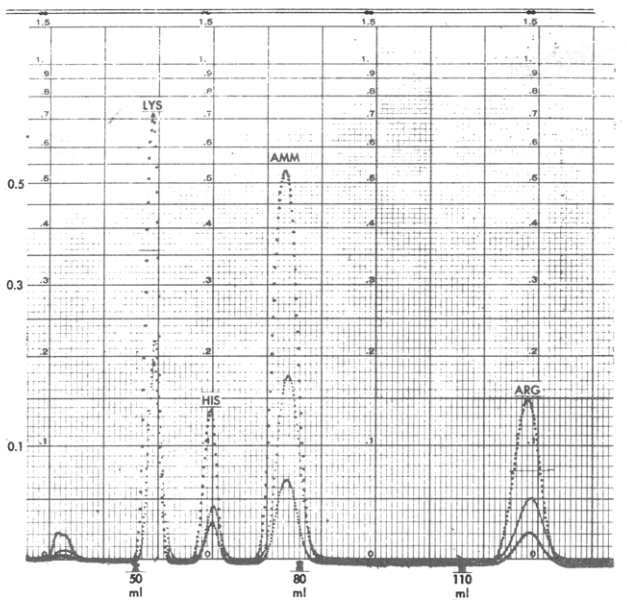
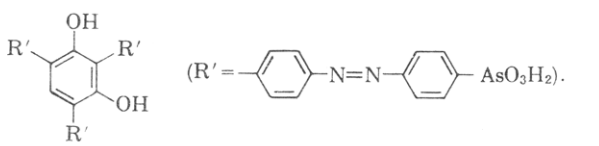


FIG. 1.—Chromatographic analysis of the basic amino acids in a hydrolysate of anti-BSA (γ-globulin fraction) on a 15 cm column. In this, and in the other analyses represented in Figures 2, 3, 4, and 5, the buffer used was 0.35 N Na citrate, *p*H 5.28, at a flow rate of 30 ml per hour, and at 50°. (Known peaks are labeled with the usual abbreviations: lys = lysine, his = histidine, amm = ammonia, arg = arginine.)

anti-benzenearsonate antibody from a solution of whole antisera in 0.17 M borate, 0.12 M NaCl buffer, *p*H = 8.0, with a trivalent hapten, R′$_3$-resorcinol:



The R′$_3$-resorcinol was kindly supplied by Drs. D. Pressman and D. H. Campbell.

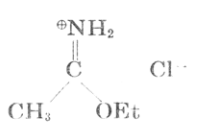
The hapten used in equilibrium dialysis studies with antibenzenearsonate antibody was *p*-(*p*′-dimethylaminophenylazo)benzenearsonate, sodium salt, purchased from the Mann Research Laboratories.

Rabbit anti-SIII antibody, as well as a Squibb Co. preparation of the SIII polysaccharide (Markowitz and Heidelberger, 1954), was generously contributed for this work by Dr. M. Heidelberger.

Pure rabbit anti-D-benzoylaminophenyl acetate antibody and the homologous azohapten, D-phenyl(*p*-(*p*′-dimethylaminobenzeneazo) - benzoyl - amino) - acetate, were obtained through the generosity of Dr. F. Karush. Dr. Karush also provided a preparation of pure rabbit anti-β-lactoside antibody and the hapten, *p*-(*p*′-dimethylaminobenzeneazo)-phenyl β-lactoside. The methods used for preparation and purification of both of these antibody-hapten pairs have been described in detail (Karush, 1956 and 1957).

Pure rabbit anti-DNP antibody was the generous gift of Dr. H. N. Eisen. Its preparation has been described by Farah *et al.* (1960).

The amidinating reagent used was ethyl acetimidate hydrochloride,



It was prepared by the method of Pinner (1883), as adapted by McElvain and Nelson (1942), by passing HCl through an ether solution of acetonitrile and ethanol.

Glycyl-lysine sulfate and ϵ-*N*-DNP lysine were obtained from the Mann Research Laboratories.

Amidination Procedures

1. Partial Amidination. To achieve 62–65% amidination of the ϵ-amino groups of proteins, the following procedure was adopted. To 0.14 g ethyl acetimidate hydrochloride was added 0.1 ml 5 M NaOH, then 5 ml of an approximately 1% protein solution in borate buffer, *p*H 8.5, μ = 0.1. (The amount of reagent was calculated to be about 0.2 M in a total of 6 ml. The amount of 5 M NaOH mixed with the acid reagent prior to the addition of the protein solution was always somewhat less than required for neutralization; the preliminary mixing of the acid and base was performed in order to prevent subjecting the protein to any extremes of *p*H in initiating the reaction.) The *p*H was adjusted and maintained at 8.3–8.6 for 2 hours, the reaction solution being stirred and kept at 0°. After the 2 hours, dialysis against borate buffer was begun.

To attain 85% amidination, the same procedure was used with an increase in reagent concentration to 1 M.

2. Exhaustive Amidination. The procedure adopted to achieve 98–100% amidination required several successive additions of reagent, in the following stages:

(*a*) To 1.0 g reagent in 0.9 ml 5 M NaOH was added 14 ml of an approximately 1.0% solution of protein in borate buffer, *p*H 8.5, μ = 0.1. The mixture was kept at 0° for 30 minutes, during which time the *p*H increased from about 8.4 to about 9.0.

(*b*) The mixture was then brought to room temperature, adjusted to *p*H 9.5, and allowed to react for another hour.

(*c*) An additional 0.5 g reagent in 0.4 ml 5 M NaOH was added, and the *p*H was maintained between 9.2 and 9.5 for another hour.

(*d*) Step *c* was repeated.

(*e*) Finally, 1.0 g reagent in 0.9 ml 5 M NaOH was added. This was found to reduce the *p*H to about 8.9. After the mixture was kept at 4° overnight, the *p*H had risen to 9.9. It was then dialyzed against the borate buffer.

At no stage of the process did the concentration of the reagent exceed 1.2 M.

Amino Acid Analysis.—In the first several amidination experiments, the degree of reaction was determined by Van Slyke analyses (as described by Morrow and Sandstrom, 1935) of free amino groups remaining. Automatic amino acid analyses, however, proved to be more practical and accurate where only small quantities of a given protein (*e.g.*, purified antibody) were at hand.

Automatic amino acid analyses were performed with a Spinco Model 120 Amino Acid Analyzer, based on the method of Spackman *et al.* (1958). Adaptations of this method to the particular problems of analysis for ϵ-*N*-substituted lysine derivatives will be described in detail in a separate publication (Cherry, Wofsy, and Singer, to be published).

The problem in effecting a quantitative analysis of the degree of amidination is that ϵ-amidinated lysines are subject to attack during acid protein hydrolysis; thus, some of the amidinated lysines reappear in amino acid analysis as free lysine. Therefore, prior to hydrolysis, the amidinated proteins were treated with 2,4-dinitrofluorobenzene (DNFB) to block the still un-

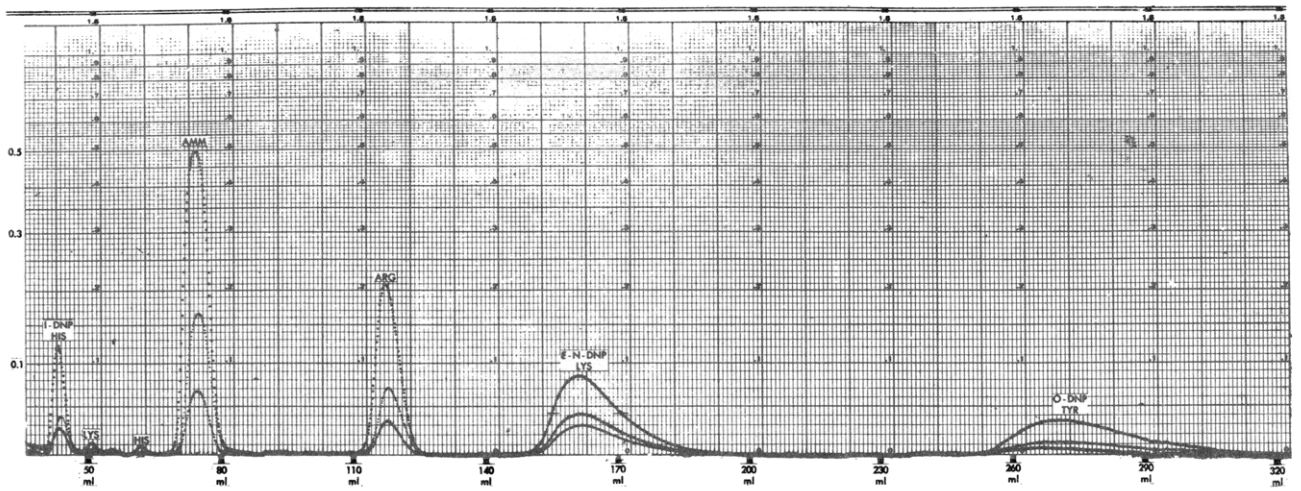


FIG. 2.—Chromatographic analyses on a 15 cm column of the basic amino acids in a hydrolysate of rabbit normal γ-globulin, which was reacted exhaustively with DNFB prior to hydrolysis. (i-DNP-his = imidazole-DNP-histidine; ε-N-DNP lys = ε-N-DNP lysine; O-DNP-tyr = O-DNP-tyrosine).

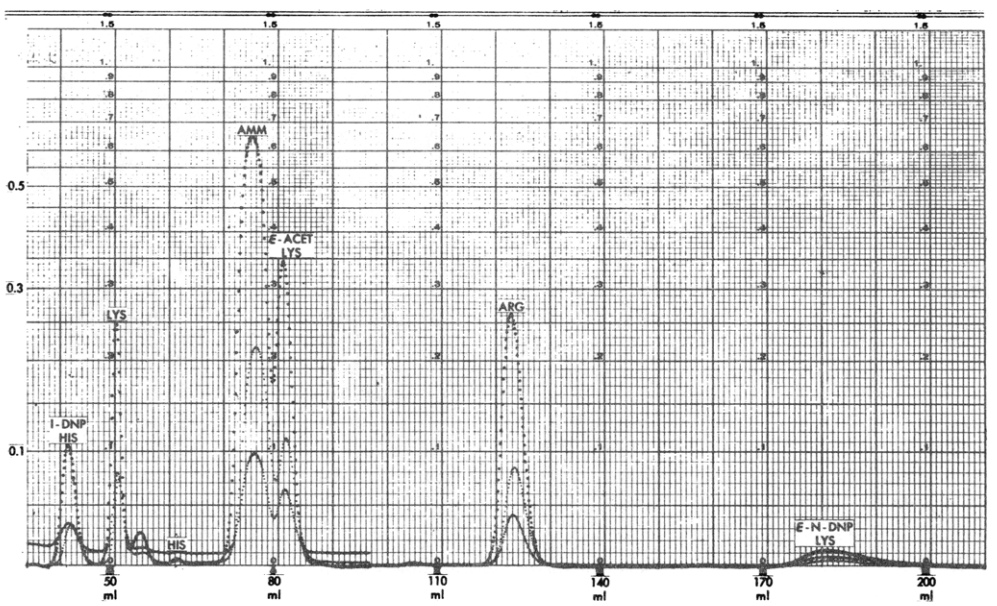


FIG. 3.—Chromatographic analysis on a 15 cm column of the basic amino acids in a hydrolysate of partially amidinated pure anti-benzenearsonate, which had been reacted with DNFB prior to hydrolysis. (ε-acet lys = ε-acetamidine lysine).

reacted amino groups.[1] When hydrolysates of DNFB-treated proteins were examined, an ε-*N*-DNP lysine peak appeared on the short column chromatogram about $1^1/_2$ to 2 hours (45–60 ml) after arginine. Measurement of the area under this peak, calibrated with an authentic sample of ε-*N*-DNP lysine, gave the per cent of the lysine residues which had remained unmodified by the amidinating reagent. DNFB-treated proteins were hydrolyzed for amino acid analysis in refluxing 4 N HCl for 24 hours. Under these conditions, ε-*N*-DNP lysine is stable (Porter and Sanger, 1948), and reproducible, essentially complete release of lysine residues is obtained.

In Figures 1, 2, 3, and 4 various short column chromatograms are reproduced. Figure 1 is the analysis of a sample of a rabbit γ-globulin fraction containing anti-BSA antibody. Figure 2 shows the result obtained with a nonamidinated rabbit normal γ-globulin sample which had been reacted exhaustively with DNFB prior to hydrolysis. In this chromatogram, three new peaks are apparent, corresponding to imidazole-DNP-histidine, ε-*N*-DNP-lysine, and *O*-DNP-tyrosine; there is about one residue of free lysine and less than one residue of free histidine per mole protein. From long column chromatograms, not reproduced here, it appears that the amount of free tyrosine remaining in the DNP-protein hydrolysates was also no more than 0–2 residues per mole.

In Figure 3 is shown the hydrolysate pattern of a partially amidinated antibody (pure anti-benzenearsonate), which had been treated with DNFB prior to

[1] The procedure used in the DNFB treatment was based on similar methods described by Sanger (1945) and by Porter and Sanger (1948): Several mg of protein in 1 ml solution were denatured by addition of 2 ml ethanol. After an hour or more, 0.2 g $NaHCO_3$ and 0.1 ml DNFB were added. The mixture was put on a shaker for 2 hours at room temperature. The insoluble protein product was centrifuged down, and two water, two alcohol, and two ether washes were performed successively.

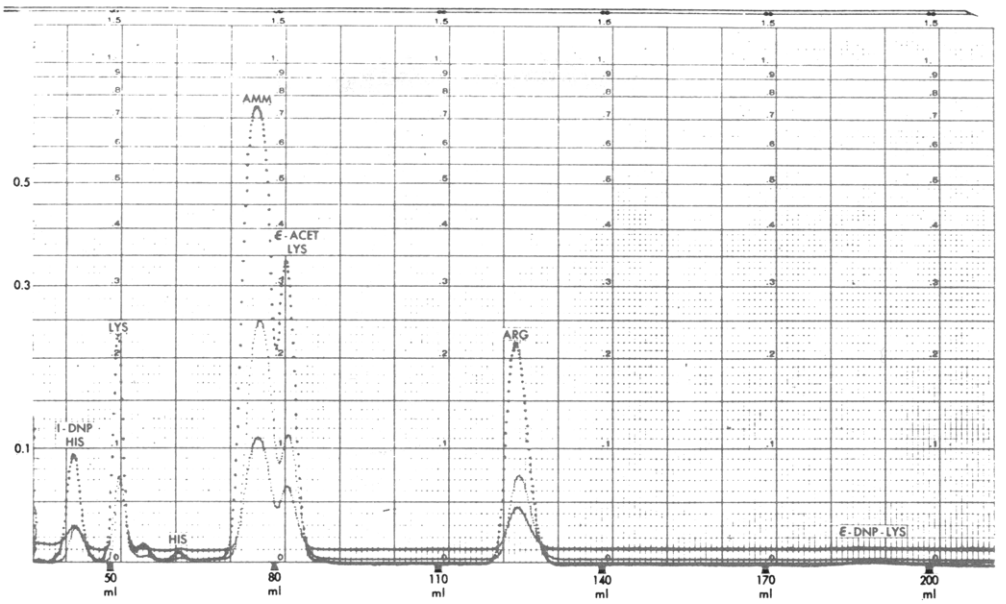


FIG. 4.—Chromatographic analysis on a 15 cm column of the basic amino acids in a hydrolysate of exhaustively amidinated pure anti-benzenearsonate, which had been reacted with DNFB prior to hydrolysis.

hydrolysis. Despite the DNFB treatment, free lysine is now present, having been released by the hydrolysis of some ε-acetamidine-lysine residues. Some unhydrolyzed ε-acetamidine-lysine is also present, seen as a slow moving shoulder on the "ammonia" peak. This peak was identified as follows: glycyl-lysine sulfate was reacted at room temperature in slightly alkaline solution with a large excess of ethyl acetimidate hydrochloride (1:20 molar ratio, 0.2 M in reagent). The products of this reaction were not isolated. After standing overnight, the reaction solution was added to an equal volume of 2 N HCl and refluxed for 11 hours. Amino acid analysis showed a peak for free glycine, one for lysine, one for ammonia, and one that corresponded exactly with the slower component in the split "ammonia" peak in Figure 3.

Figure 4 reproduces the assay of a hydrolysate of fully amidinated anti-benzenearsonate antibody which was reacted with DNFB after amidination. Here the ε-*N*-DNP lysine peak is absent, and this is taken as evidence for the fact that exhaustive amidination of ε-NH_2 groups was achieved. The fact that no ε-*N*-DNP lysine was present also proves that the amidine functions are not removed during the DNFB reaction, for any lysine released would have reacted with DNFB. There was no detectable increase of free histidine or free tyrosine in hydrolysates of DNFB-treated samples of amidinated proteins, as should have been observed had any appreciable amidination of these residues been produced. This further indicates that amidination takes place specifically at the NH_2 groups of proteins.

A more direct analytical procedure for the extent of amidination was also formulated on the basis of modifying the chromatographic technique so as to permit adequate resolution of ε-acetamidine lysine (Fig. 5). In this procedure, it was still necessary to react the free amino groups of an amidinated protein with DNFB, but after hydrolysis, the degree of amidination could be determined directly from the sum of free lysine plus ε-acetamidine lysine.

Techniques Used to Study the Properties of Amidinated Proteins.—Detailed chemical and physical studies were made of preparations of amidinated BSA and amidinated anti-benzenearsonate antibody. Two modified BSA samples—one with the ε-NH_2 groups 98%, and the other 86%, amidinated—were examined along with an unmodified control. These samples, including the control, were kept in solution in borate buffer, *p*H 8.5, $\mu = 0.1$, and were stored frozen for one month prior to the study of their physical properties. Pure anti-benzenearsonate antibody samples were amidinated—one to the extent of 99% and another 89%—and studied alongside the unmodified antibody. After amidination, the antibody samples used in these studies were preserved with merthiolate (1 part per 10,000) and stored in borate buffer, *p*H 8.5, $\mu = 0.1$, at 5°.

Most of the physical studies were made at *p*H 8.5–8.6, especially where protein solutions of 1% or higher were required, in order to avoid any complications from low solubilities that might arise with the heavily modified samples at lower *p*H.

1. Electrophoresis. Electrophoresis studies on amidinated proteins were conducted with a Spinco Model H Electrophoresis-Diffusion instrument, with solutions dialyzed to equilibrium against barbital buffer, *p*H 8.58, $\mu = 0.1$, at a field strength of 5.71 volts/cm.

Electrophoresis of soluble complexes (in antigen excess) of fully amidinated anti-BSA antibody and fully amidinated BSA was performed in a Perkin-Elmer Model 38 Tiselius apparatus.

2. Ultracentrifugation. Sedimentation constants were determined with a Spinco Model E Ultracentrifuge at speeds of 59,780 rpm near room temperature, in borate buffer, *p*H 8.5, $\mu = 0.1$.

3. Ultraviolet Absorption and the Determination of Protein Concentration. Optical densities of protein solutions were determined with a Beckman Model DU spectrophotometer, at 279 mμ for BSA and amidinated BSA, and at 280 mμ for anti-benzenearsonate and amidinated anti-benzenearsonate antibody. Extinction coefficients for 1% solutions ($\epsilon\ ^{1\%}_{1cm}$) in borate buffer, *p*H 8.5, $\mu = 0.1$, were determined on the basis of quantitative analyses for nitrogen by standard Nessler procedures, supplemented in some cases by Kjeldahl analyses. The per cent nitrogen of a particular amidinated protein sample was calculated, taking into account the increase in molecular weight and nitrogen content introduced by the amidine groups. For unmodified

BSA the molecular weight, 65,000, and per cent nitrogen, 16.07%, were taken as reported by Hughes (1954); for control anti-benzenearsonate antibody, the molecular weight was taken as 160,000 (Isliker, 1957), and the per cent nitrogen as 16.00% (Smith *et al.*, 1955).

4. Optical Rotation. All optical rotation determinations were made at the Na_D line on a Schmidt and Haensch polarimeter, with a Rudolph photoelectric attachment, in 2 decimeter cells with a 3.5 mm bore. Solutions were prepared at 1% concentrations in borate buffer, *p*H 8.5, $\mu = 0.1$. Specific rotations were calculated assuming the rotation to be a linear function of protein concentration.

Methods for Assaying Antibody Activity

1. Equilibrium Dialysis. All equilibrium dialysis experiments were conducted with specially designed cells and by procedures modeled after those described by Karush (1956). Results were evaluated from the relationship:

$$K_i = \frac{r}{(n - r)c} \qquad (1)$$

where K_i = intrinsic association constant; r = moles hapten bound per mole antibody; n = number of binding sites per antibody molecule; and c = equilibrium concentration of free hapten.

Assays were run at least in duplicate, allowing 24 hours for equilibrium to be attained. All buffers were of ionic strength equal to or greater than 0.1, and protein concentrations ranged from 1×10^{-5} to 2×10^{-5} M, so that no correction had to be made for the Gibbs-Donnan effect. Corrections for the binding of hapten by the dialysis membrane were determined on the basis of blanks.

In studying the anti-benzenearsonate system, where a reasonably adequate supply of pure antibody was available, an equilibrium binding curve at 25° was determined for unmodified anti-benzenearsonate antibody on the basis of equilibrium dialysis at total hapten concentrations ranging from 1×10^{-5} M to 8×10^{-5} M. This served as the basis for the choice of suitable concentrations of antibody and hapten at which to compare the activities of several different amidinated samples and the unmodified control. Where the amount of antibody was limited, only two modified samples—one partially and one exhaustively amidinated—were prepared and compared with the appropriate control at the same total hapten concentration. In the latter instances, concentrations of antibody and hapten were selected for equilibrium dialysis experiments by use of the binding curves previously reported in the literature for the particular antibody-hapten pair. In general, initial concentrations of antibody and hapten were selected so that, for the unmodified control, $r \geqq 1$ and the difference between initial and final concentrations of free hapten could readily be determined spectrophotometrically.

Optical densities of hapten solutions were determined with a Beckman Model B spectrophotometer. The r/c values were used as the scale for comparing activities in a set of antibody samples, with the r/c value of the unmodified antibody taken as 100. This scale, while somewhat arbitrary in the absence of complete equilibrium binding curves, is sensitive to very small alterations of antibody-hapten binding affinity (see Discussion, under Amidination and Immune Activity).

2. Precipitin Assays. Precipitin curves for anti-benzenearsonate antibody with R′$_3$-resorcinol were determined in 0.1 M borate–0.12 M NaCl buffer, *p*H 8.0. BSA-anti-BSA precipitin curves were obtained

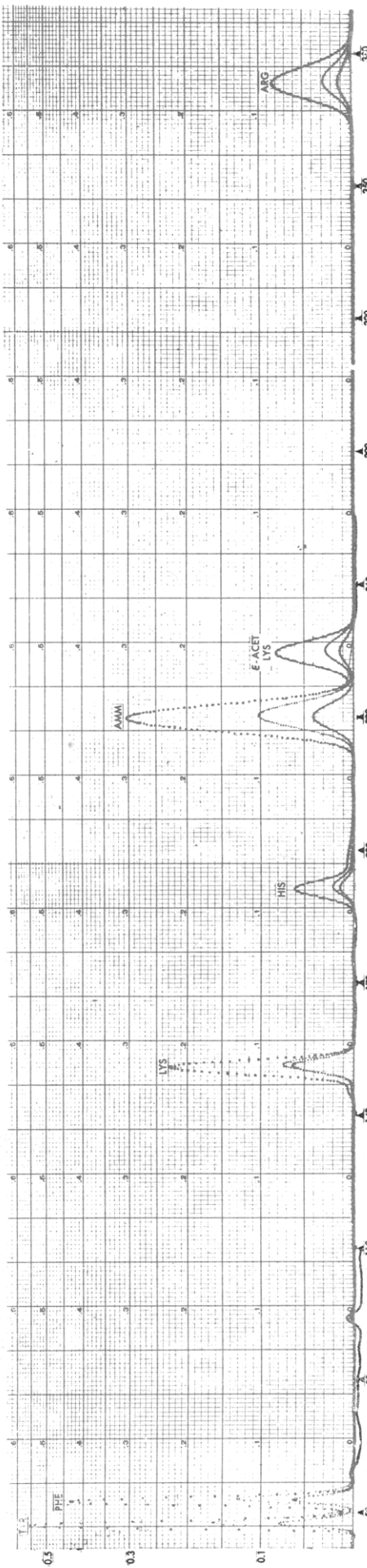


FIG. 5.—Chromatographic analysis on a 50 cm column of the basic amino acids in a hydrolysate of partially amidinated anti-BSA (γ-globulin fraction). The protein was *not* previously treated with DNFB, since the object of this particular assay was simply the resolution of a separate peak for ε-acetamidine lysine. (tyr = tyrosine; phe = phenylalanine.)

TABLE II
PHYSICAL PROPERTIES OF AMIDINATED PROTEINS

Protein	% Amidination	Electrophoretic[a] Mobility × 10^5 cm^2/volt sec.	$S_{20}^W \times 10^{13}$ [b]	$[\alpha]_D^{25}$ [b]	$\epsilon_{1\,cm}^{1\%}$ [b,c] Found	$\epsilon_{1\,cm}^{1\%}$ [b,c] Expected
BSA	0	−6.5	3.7	−64.5°	7.15	—
BSA	86	−6.5 (75%) −5.8 (25%)	—	−59.0°	6.88	7.05
BSA	98	−6.8[d]	3.6	−66.3°	7.80	7.02
Anti-benzenearsonate	0	−1.5	6.9	−56.5°	15.1	—
Anti-benzenearsonate	89	—	—	−52.8°	14.5	14.9
Anti-benzenearsonate	99	−1.7	6.8	−57.1°	15.9	14.8

[a] Ascending mobilities, determined in barbital buffer, *p*H = 8.58, μ = 0.1. [b] Values listed in the last four columns were obtained with solutions in borate buffer, *p*H 8.5, μ = 0.1. [c] Extinction coefficients, $\epsilon_{1\,cm}^{1\%}$, for BSA solutions are at 279 mμ; those for anti-benzenearsonate antibody solutions are at 280 mμ. The expected values, in the last column, are the native values corrected for molecular weight changes produced by amidination. [d] Asymmetric spread boundary, mobility measured at maximum of curve.

in borate buffer, *p*H 9.5, μ = 0.1. Precipitates were incubated at 37° for 20 minutes, then maintained at 5° for 18–20 hours. The weight of nitrogen in precipitates was determined by Nessler analyses.

In the anti-SIII system, complete precipitin curves could not be run. The tests were performed at two points, duplicating the conditions reported by Markowitz and Heidelberger (1954) for similar anti-SIII antibody preparations, and using their published precipitin curves as a guide.

RESULTS

Some Physical Properties of Amidinated Proteins.—The results obtained from physical studies of amidinated proteins show only relatively small changes in any of the properties investigated. In Table II are summarized the results of the studies conducted with BSA, 86% amidinated BSA, and 98% amidinated BSA; and with anti-benzenearsonate, 89% amidinated anti-benzenearsonate, and 99% amidinated anti-benzenearsonate.

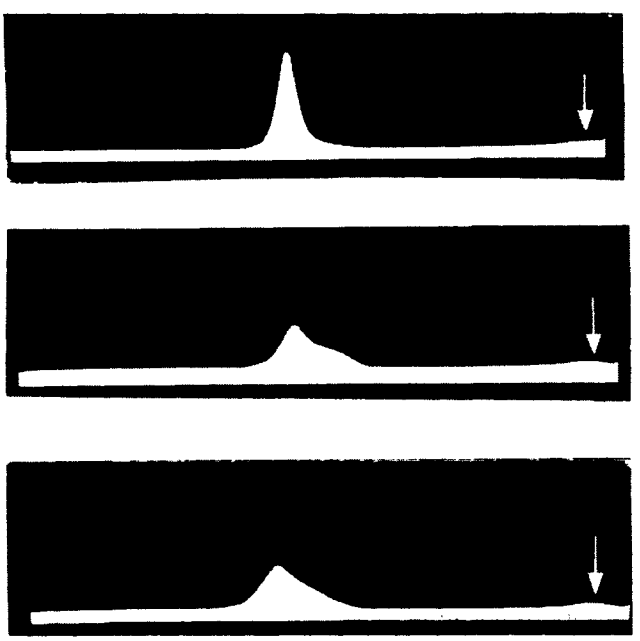


FIG. 6.—Ascending electrophoresis patterns, 0.25% protein concentration, in barbital buffer, *p*H 8.6, μ = 0.1, after 10,800 seconds at a field strength of 5.71 volts/cm, of (*a*) unmodified BSA, (*b*) 86% amidinated BSA, and (*c*) 98% amidinated BSA. The arrows indicate the starting positions. Experiments were conducted with a Spinco Model H Electrophoresis-Diffusion Instrument.

Schlieren patterns for comparable electrophoresis runs with unmodified and amidinated BSA are reproduced in Figure 6. Some asymmetry can be noted in the patterns for the amidinated BSA solutions, whereas those for the native protein are symmetrical. In the case of the extensively, but incompletely, amidinated BSA (Fig. 6b), 75% of the protein migrated with the same negative mobility as unmodified BSA, and about 25% comprised a slower moving shoulder (Table II). The totally modified BSA showed greater electrophoretic heterogeneity (Fig. 6c); the mean negative mobility, measured at the peak maximum, was slightly enhanced over that of native BSA (Table II).

Schlieren patterns from ultracentrifuge runs with fully amidinated and unmodified proteins are shown in Figures 7 and 8. The small amount of faster-moving material revealed in the anti-benzenearsonate patterns (Fig. 7) can probably be attributed to aggregates formed between antibody and residual R′$_3$-resorcinol, the trivalent hapten used in the purification process. A component which is probably BSA dimer is evident in the BSA patterns (Fig. 8a,b), more in the case of amidinated BSA than of native BSA.

Figure 8c shows the pattern from another ultracentrifuge run with fully modified BSA about 1 hour after the solution was treated in the following manner: 0.05 ml of a 0.1 M cysteine solution, adjusted to *p*H 8.5, was added to 1 ml of the 1% solution of amidinated BSA, making it about 5×10^{-3} M in cysteine. The peak attributed to dimer is practically eliminated by this treatment.

The Effects of Amidination on Immune Activity.—Data obtained by means of equilibrium dialysis assays show that the binding capacities of anti-benzenearsonate, anti-D-benzoylaminophenyl acetate, and anti-β-lactoside antibodies are hardly, if at all, diminished by amidination. Table III records the results of duplicate assays, at antibody concentrations of about 1×10^{-5} M to 2×10^{-5} M, with several antibody preparations modified to varying degrees.

The other anti-hapten system, anti-DNP antibody and ϵ-*N*-DNP lysine, could not be investigated by the same method of equilibrium dialysis. Anti-DNP antibody is characterized by unusually high binding affinities for its antigens and homologous haptens, especially for ϵ-*N*-DNP lysine; the intrinsic dissociation constant for the latter is of the order of 10^{-8} (Velick *et al.*, 1960). At antibody and hapten concentrations of 10^{-5} M, generally required for equilibrium dialysis assays, ϵ-*N*-DNP lysine is therefore bound stoichiometrically by anti-DNP antibody. To ascertain any gross effect of amidination on antibody binding capacity, fully amidinated anti-DNP was titrated with

TABLE III
EFFECT OF AMIDINATION ON ANTIBODY BINDING CAPACITY

Antibody	% Amidinated	r	$r \times 10^{-4}$ / c	Activity Scale
Anti-benzenearsonate[a]	0	1.28	6.0	100
	65	1.25	5.6	93
	73	1.21	5.2	87
	88	1.08	4.6	77
	100	1.03	4.4	73
Anti-D-benzoyl-aminophenyl acetate[b]	0	1.33	9.8	100
	75	1.28	9.5	97
	100	1.27	9.5	97
Anti-β-lactoside	0	0.76	4.2[c]	100
	75	0.73	4.0	95
	100	0.68	3.9	93

[a] Buffer: 0.12 M NaCl, 0.17 M borate, pH 8.0. [b] Buffer: 0.15 M NaCl, 0.02 M phosphate, pH 7.4. [c] The r/c values obtained with modified and unmodified anti-β-lactoside antibody were uniformly lower, by about 40%, than those indicated by the binding curve reported by Karush (1957) for a similar anti-β-lactoside antibody preparation. Thus, there may have been some deterioration of this preparation, but the relative activities of amidinated and control antibody were the same, within experimental error.

ε-*N*-DNP lysine. The value of r (moles hapten bound per mole antibody) was found to be the same, $r = 1.7 \pm 0.1$, as that for unmodified anti-DNP with the same hapten. The titrations were performed at 2×10^{-5} M antibody concentrations in 0.15 M NaCl, 0.02 M phosphate buffer, pH 7.4, utilizing the cells designed for equilibrium dialysis studies.

Precipitin results were obtained as a measure of the effects of amidination on anti-benzenearsonate, anti-SIII, and anti-BSA antibodies, and on BSA antigen. Up to 90% amidination of anti-benzenearsonate antibody causes no substantial loss of precipitating capacity with the trivalent R'_3-resorcinol hapten (Fig. 9). A comparison of the mg of nitrogen precipitated at the maxima of the curves shown in Figure 9 demonstrates that even exhaustively amidinated anti-benzenearsonate antibody retains over 60% of its precipitating capacity.

Precipitin results for partially and fully amidinated anti-SIII, as well as for the native antibody, with two different concentrations of the SIII polysaccharide, are recorded in Table IV.

The partially amidinated anti-SIII antibody lost essentially none of its precipitating capacity. The results with fully modified anti-SIII antibody are somewhat obscure. Even after extensive centrifugation of the tubes in which the precipitin reaction with the exhaustively amidinated antibody was performed, the supernatant remained very turbid. This was not so with the control or with the less drastically modified

TABLE IV
EFFECT OF AMIDINATION ON ANTI-SIII ANTIBODY PRECIPITATING CAPACITY

Anti-SIII % Amidinated	μg SIII	mg N ppt.[a]
0	45	0.47
0	90	0.56
75	45	0.46
75	90	0.54
97	45	>0.13
97	90	>0.20

[a] The precipitin tests were conducted with 1 ml aliquots of 2% antisera solutions in phosphate buffer, pH 6.7, $\mu = 0.1$.

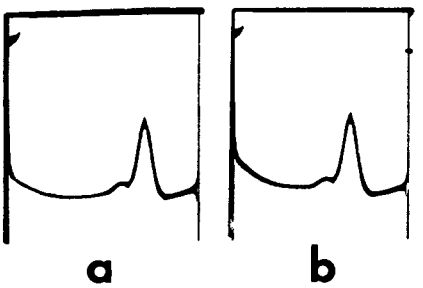


FIG. 7.—Ultracentrifuge diagrams of (*a*) unmodified anti-benzenearsonate and (*b*) exhaustively amidinated anti-benzenearsonate after 2880 seconds at 59,780 rpm. Both solutions were 0.7% in borate buffer, pH 8.5, $\mu = 0.1$. Sedimentation is to the left.

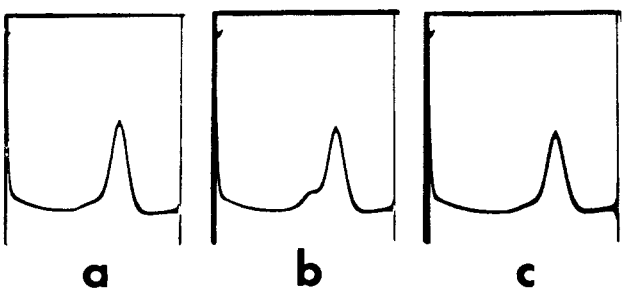


FIG. 8.—Ultracentrifuge diagrams, after 5760 seconds at 59,780 rpm, of 1% BSA solutions in borate buffer, pH 8.5, $\mu = 0.1$: (*a*) unmodified BSA, (*b*) exhaustively amidinated BSA, (*c*) exhaustively amidinated BSA in 5×10^{-3} M cysteine. Sedimentation is to the left.

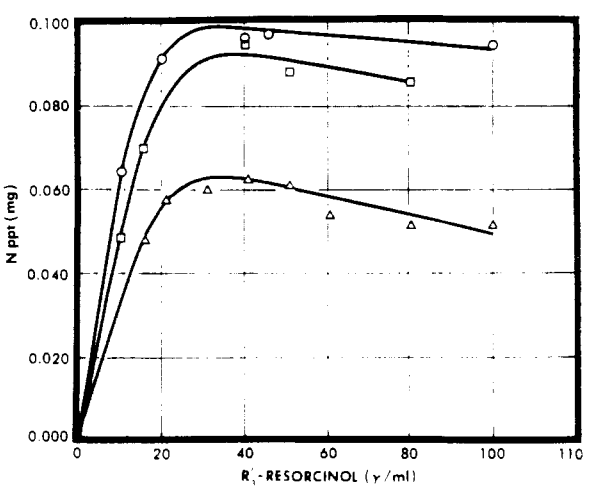


FIG. 9.—Precipitin curves for unmodified (○), 89% amidinated (□), and exhaustively amidinated (△) anti-benzenearsonate with R'_3-resorcinol in borate-NaCl buffer, pH 8.0, $\mu = 0.1$. One ml hapten solutions were mixed with 1 ml aliquots of 0.1% antibody.

sample. Presumably antigen-antibody complexes had formed, but were not capable of being centrifuged at the low centrifugal fields employed. This point was not further investigated, however.

The antigenic activity of BSA, as indicated by precipitin determinations, is not affected significantly even by greater than 90% amidination (Table V).

Although 65% amidinated anti-BSA antibody retained precipitating capacity almost entirely (Fig. 10), exhaustive amidination destroyed the ability to precipitate with unmodified BSA.

Although these results imply that fully amidinated

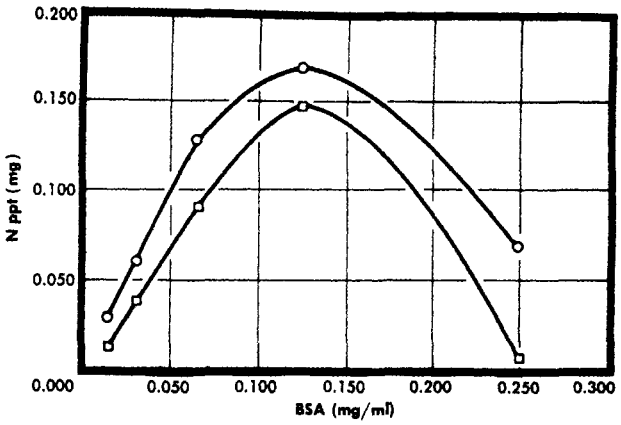


FIG. 10.—Precipitin curves for unmodified anti-BSA (○) and 65% amidinated anti-BSA (□) with BSA in borate buffer, *p*H 9.5, μ = 0.1. One ml BSA solutions were added to 1 ml aliquots of γ-globulin solution containing anti-BSA antibody, 4 mg protein/ml.

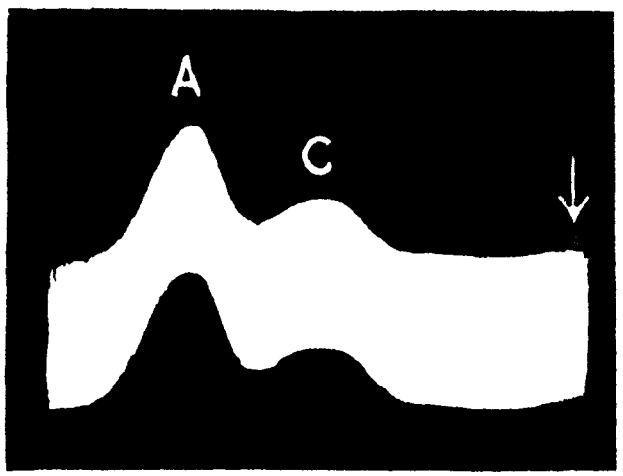


FIG. 11.—Ascending electrophoresis pattern of a mixture of exhaustively amidinated BSA and exhaustively amidinated anti-BSA, the former present in 3:1 excess, after 15,500 seconds, at a field strength of 3.03 volts/cm, in borate buffer, *p*H 9.5, μ = 0.1. The A peak represents free antigen, the C peak represents antigen-antibody complexes. A Perkin-Elmer Model 38 Tiselius apparatus was used. The arrow indicates the starting position.

anti-BSA antibody has lost its combining capacity for antigen, electrophoresis of a mixed solution of fully amidinated pure anti-BSA antibody and fully amidinated BSA in borate buffer, *p*H 9.5, μ = 0.1, revealed the presence of two components: one, with a mobility of -6.4×10^{-5} cm²/volt sec., is attributable to the uncombined antigen,[2] present in excess; the other, with a mobility of -4.4×10^{-5} cm²/volt sec., is due to antigen-antibody complexes (Fig. 11). This indicates that fully amidinated anti-BSA antibody is still capable of binding antigen in antigen excess, although probably too weakly to give a precipitate at equivalent proportions.

DISCUSSION

The Amidination of Proteins.—It has been demonstrated that the free amino groups of two proteins, BSA and rabbit γ-globulin, can be amidinated exhaustively under reasonably mild conditions. The amino acid analyses clearly establish that essentially all of the ϵ-NH_2-lysines can be converted to ϵ-acetamidine lysine residues. The reaction may be carried out without subjecting a protein to *p*H values higher than 9.5–9.9, in a reaction time of less than one day, with no more than 3 hours of this period at room temperature.

[2] In borate buffer, *p*H 9.5, μ = 0.1, the mobility of native BSA is -6.4×10^{-5} cm²/volt sec.; that of native rabbit γ-globulin is about -1.7×10^{-5} cm²/volt sec. (S. J. Singer, unpublished data).

TABLE V

EFFECT OF AMIDINATION ON ANTIGENIC ACTIVITY OF BSA

BSA % Amidinated	mg N Ppt.[a] at max.	% Activity
0	0.110	100
66	0.110	100
85	0.101	92
90	0.100	91
94	0.094	85

[a] The precipitin tests were conducted with 1 ml aliquots of a solution of a γ-globulin fraction containing anti-BSA antibody, 4 mg protein/ml, in borate buffer, *p*H 9.5 μ = 0.1.

With respect to the specificity of the reaction, the amino acid analyses offer additional substantiation of the finding reported by Hunter and Ludwig (1962) that imidazole and phenolic groups are not affected under somewhat similar amidinating conditions. When hydrolysates of fully amidinated proteins are analyzed and chromatographed with the Amino Acid Analyzer, the tyrosine and histidine peaks are not reduced relative to unmodified control hydrolysates; only the relative amount of lysine is decreased, and only one new component appears on the chromatogram (Fig. 5). It might be considered possible that tyrosine or histidine residues react with the reagent to form unstable derivatives and then, upon protein hydrolysis, are released as the unmodified amino acids. However, when the fully amidinated proteins were first reacted with DNFB, and then hydrolyzed, only trace amounts, if any, of free tyrosine and histidine were detected. Since ϵ-acetamidine lysine was partially hydrolyzed in 4 N HCl, yielding appreciable amounts of free lysine, one would certainly not expect heteroatom analogs of amidines to survive the protein hydrolysis. The evidence is consistent with the conclusion that a high degree of specificity characterizes the amidination reaction.

The highly amidinated BSA and anti-benzenearsonate antibody preparations do not appear to have undergone appreciable denaturation or gross changes in the properties studied. The results obtained are in considerable measure similar to those reported by Hughes *et al.* (1949) in the somewhat analogous studies on guanidinated human serum albumin. Amidinated protein derivatives, like their guanidinated counterparts, show no major changes in electrophoretic mobilities, in sedimentation constants, in optical rotation, or in ultraviolet absorption (Table II). Thus, the net charge and molecular structure of the proteins are essentially preserved. In this connection, it should be emphasized that the guanidinated derivatives studied by Hughes *et al.* were no more than 70% modified, whereas all of our physical studies dealt with 85–90% and 98–100% modified proteins.

Although amidinated proteins, under relatively gross examination, retain most of their original properties, more subtle study indicates that small but definite structural changes are produced upon exhaustive amidination. It may be instructive to compare qualitatively the consequences of 85–90% amidination with the physical alterations noted after modification is

exhaustive. In the former case, there is no distinct indication of changes in structure or properties that suggests any significant unfolding or denaturation.

For example, after 85–90% amidination, the slightly reduced values of specific extinction coefficients observed for ultraviolet absorption (Table II) are close to the values predictable from the calculated increase in molecular weight of the proteins. However, with the completely modified proteins, there are definite increases by about 7–9% over the expected extinctions. This increased aromatic absorption suggests that some conformational changes are induced in the exhaustively amidinated proteins. Results of optical rotation studies for the 86% and 89% amidinated BSA and anti-benzenearsonate preparations, respectively, both show a small but distinct decrease in levorotation when compared with unmodified controls (Table II). The direction of this change is the same as that observed by Hughes *et al.* (1949) with guanidinated human serum albumin, and is *opposite* to the direction of change in optical rotatory properties generally associated with protein denaturation phenomena. At least a part of this small change in the value of $[\alpha]_D^{25}$ may also reflect the alteration in molecular weight. With the exhaustively amidinated proteins, the observed values for $[\alpha]_D^{26}$ are perhaps even slightly more levorotatory than those of the unaltered proteins; clearly, however, the fully amidinated proteins exhibit significantly increased levorotations compared to their partially amidinated counterparts. The increases in levorotation observed for the transition from partially to exhaustively amidinated proteins are much less than those associated with the extensive denaturation of the respective native proteins (Urnes and Doty, 1961), suggesting that only slight denaturation was produced on complete reaction.

The electrophoresis patterns for both partially and fully amidinated BSA (Fig. 6) show that the modified proteins have become somewhat heterogeneous. (Such an effect is difficult to detect in the case of modified rabbit antibody, which is so heterogeneous to begin with in the unmodified state.) The 86% amidinated BSA appears to contain two components: about 75% of the protein shows a mobility identical with that of native BSA (-6.5×10^{-5} cm²/volt sec.); the other 25% shows a reduced negative mobility (-5.8×10^{-5} cm²/volt sec.). One possible explanation for this electrophoretic separation is that amidination of the single α-NH_2 group of BSA is incomplete (Hunter and Ludwig, 1962): at *p*H 8.6, amidination of the α-NH_2 group ($pK \sim 7.8$) would add a positively charged group ($pK \sim 12$) to a protein molecule, thus decreasing the magnitude of its negative mobility.[3]

Exhaustively amidinated BSA, however, shows a slightly enhanced average negative mobility, and a more extensive heterogeneity is revealed in the broadened boundary. Presumably the α-amino groups should have been even more extensively modified, and, other things being equal, a more pronounced slow-moving component might therefore have been expected. It seems likely that this increased negative mobility is somehow correlated with the small structural changes produced in the molecule on exhaustive amidination, as discussed above. These changes may in turn alter the anion binding capacity of the protein molecule. Chervenka and Wilcox (1956) noted that guanidinated chymotrypsinogen showed an enhanced negative electrophoretic mobility at *p*H 8.7; Cobain and Wilcox (private communication, 1962) conclude from equilibrium dialysis measurements that the guanidinated protein binds more phosphate anions than unmodified protein.

Comparison of the ultracentrifuge patterns for 98% amidinated and unmodified BSA (Fig. 8a,b) shows a somewhat larger amount of faster-sedimenting material in the former. This difference disappeared, however, on treatment of the amidinated BSA with cysteine, and only one homogeneous peak was revealed upon subsequent ultracentrifugation (Fig. 8c). The faster-sedimenting material apparently resulted from some dimerization, possibly through the linking of two sulfhydryl groups by means of a bivalent metal cation. The latter may have been present as a trace impurity in the ethyl acetimidate hydrochloride reagent. Aside from this trivial difference, however, no significant change between fully amidinated and native protein is evident ultracentrifugally. Within experimental error the sedimentation constant is unchanged; thus, the structural alterations in exhaustively amidinated proteins are apparently not large enough to produce any effect on the frictional coefficients.

When the changes consequent to the amidination of proteins are examined, the truly outstanding feature—from the point of view of chemical modification—is that practically no sign of conformational alteration appears up to the point of about 90% amidination. Only with exhaustive amidination are even small effects observed. There are few, if any, other examples of so complete a protein modification with such minimal secondary structural effects. That the net charge and charge distribution on the protein molecule are left essentially unchanged by the amidination reaction is no doubt of primary importance in maintaining the native conformation of the protein. The additional bulk added to the molecule by the amidine groups probably has little structural effect, since the ϵ-amino groups of lysine which are amidinated are very likely on the exterior surface of the native protein molecule (Kendrew *et al.*, 1961).

Amidination and Immune Activity.—The results of the study of amidinated antibodies lead to these summary conclusions:

1. Extensive amidination produces remarkably few detectable effects on the biological activity of these antibodies.

2. The evidence is against the critical involvement of lysine in the reactive sites of any of the antibodies examined.

Had amidination occurred at a lysine residue residing in or intimately related to the active region of an antibody molecule, a substantial reduction in binding capacity should have been observed. This conclusion is derived from the vast accumulation of evidence on the striking sensitivity of antibody activity to even small differences in the size, properties, or steric configuration of antigenic determinants. The classic studies of Landsteiner (1947) showed that azobenzenearsonate conjugated antigens are not precipitated by antisera to azobenzenesulfonate or azobenzoate. Here the charge on each of the haptenic groups is identical, so that the extraordinary specificity can only be rationalized as reflecting very stringent steric requirements for a specific antigen-antibody bond.

In Figure 12, the small differences of size among these haptens are compared with the much larger alter-

[3] Hunter and Ludwig (1962) showed that the *N*-terminal amino groups of insulin vary in their relative reactivity toward imidoester reagents; the *N*-terminal phenylalanyl residue, for example, is decidedly less reactive toward methyl acetimidate at *p*H 8.5 to 9.25 than either the *N*-terminal glycyl residue or the ϵ-amino lysyl residue of insulin. Perhaps the single *N*-terminal aspartyl residue of BSA also reacts relatively slowly. The degree of α-amino modification in our studies, however, was not further investigated.

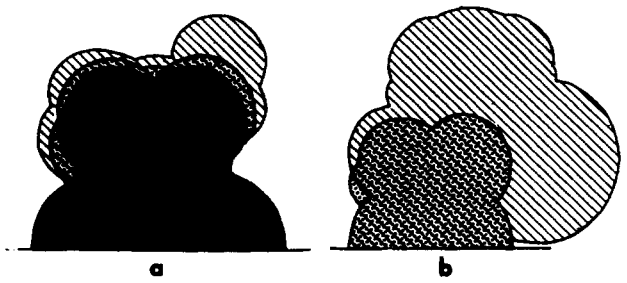


FIG. 12.—These superimposed two-dimensional projections of molecular models represent the spatial requirements of several functional groups. (*a*) The solid area represents benzoate, and is also common to the benzenesulfonate and benzenearsonate antigenic determinants. (Only a section of the aromatic nucleus is shown.) The dotted portion represents the additional area required by benzenesulfonate. The lined area indicates the requirements of benzenearsonate beyond those of the other two determinants. (*b*) The dotted area is the projection of the ϵ-ammonium group of lysine (with the terminal methylene group of lysine indicated). The lined portion represents the added area due to the projection of the acetamidine function of ϵ-acetamidine lysine. Bond distances, bond angles, and van der Waals radii are based on data appearing in: "Tables of Interatomic Distances and Configuration in Molecules and Ions," London, The Chemical Society, 1958; and in Pauling (1940). Scale: 1 cm = 1 A.

ation at a combining site that must accompany the acetamidination of a hypothetical critical lysine residue. In the case of the anionic haptens, the carboxylate anion is planar, whereas the sulfonate and arsonate groups are presumably tetrahedral. The ammonium-to-acetamidinium conversion changes a small, tetrahedral ionic group to a much larger, planar ionic group, with a methyl substituent to be accommodated. For such a change at an antibody reactive site to leave binding capacity intact, there would have to be considerable freedom of motion for the affected lysine side-chain, and ample space to contain the added amidine group atoms in a configurational orientation and proximity necessary for strong interaction with a complementary antigenic function. But if such flexibility is permitted for the presumed lysine side-chain, it is difficult to see why the closely similar anionic haptens would be distinguished by antibody in the first place.

To carry the argument further, let us consider the results obtained in the hapten systems surveyed by the method of equilibrium dialysis. In none of them did even total amidination of antibody produce a drastic reduction in binding capacity (Table III). Although it was not possible, given the amounts of pure antibody available, to establish complete equilibrium binding curves for each individual antihapten preparation, enough was known or determined about each system to make possible comparative equilibrium dialysis assays at points where any substantial deviation in binding energy from that of a control would have been clearly discernible.

To illustrate this, we may consider how a hypothetical loss in binding energy would have expressed itself in the $\frac{r \times 10^{-4}}{c}$ values found for the anti-benzenearsonate antibody preparation (Table III). In the case of the control, $\frac{r \times 10^{-4}}{c} = 6.0$, $r = 1.28$, and $c = 2.1 \times 10^{-5}$. The intrinsic association constant, K_i, for this point on the binding curve can be calculated from equation (1) with $n = 2$. K_i is found to be approximately 8.4×10^4. This represents a $\Delta F°$ value at 25° for the reaction of about −6.7 kcal/mole. If it is assumed that elimination of the contribution to binding by a group in the reactive site increased $\Delta F°$ by only 1 kcal/mole, the new value of K_i would be 1.5×10^4. At an equilibrium concentration of free hapten where $c = 2 \times 10^{-5}$, r would now equal about 0.46 moles of hapten bound per mole of antibody. The new $\frac{r \times 10^{-4}}{c}$ value would be approximately 2.3; the activity, on the scale used in Table III, would be only about 38.

The conversion of a hypothetical critical lysine ϵ-ammonium group to an acetamidinium group should have increased $\Delta F°$ of hapten-antibody binding by considerably more than 1 kcal/mole. This follows from the observations that (*a*) the difference in $\Delta F°$ for the binding of anti-*p*-(*p'*-azophenylazo)-benzoate antibody to benzoate ion as compared to *p*-aminobenzenearsonate ion is at least 2.5 kcal/mole (Nisonoff and Pressman, 1957); and (*b*) the steric change involved in the ammonium-acetamidinium conversion is considerably greater (Fig. 12) than that in the benzoate-benzenearsonate substitution. Thus, the effects of exhaustive amidination on the specific hapten-binding affinities of anti-benzenearsonate, anti-D-benzoylaminophenyl acetate, and anti-β-lactoside antibodies are so small as to rule out the presence of a critical lysine residue in the active sites of any of these antibodies.

In the case of the anti-DNP system, the titration used to assess binding capacity was considerably less sensitive than equilibrium dialysis. Since the assay was performed at 10^{-5} M antibody concentrations, and since the dissociation constant for ϵ-*N*-DNP lysine is about 10^{-8}, only a substantial decrease in binding affinity would have been detected. A change in $\Delta F°$ from −11 kcal/mole (Velick *et al.*, 1960) to about −7 kcal/mole would have resulted in the presence of an appreciable amount of free hapten in equilibrium with bound ϵ-*N*-DNP lysine at a concentration of 10^{-5} M anti-DNP antibody. Such a change, whether due to specific or nonspecific effects induced by amidination, would have been reflected in a measurable decrease in r value. Since the experimental r values obtained with completely modified and native anti-DNP antibody were identical, it can be concluded that total amidination produced no change in $\Delta F°$ greater than about 3 kcal/mole.

Precipitin results for 89% amidinated anti-benzenearsonate (Fig. 9), 75% amidinated anti-SIII (Table IV), and 65% amidinated anti-BSA (Fig. 10) antibodies show only slight losses, if any, in precipitating capacity. The significance of this finding should be considered in light of the well-known sensitivity of antibody precipitating capacity to changes in antibody structure. In the precipitin reaction of even totally amidinated anti-benzenearsonate with the trivalent R'$_3$-resorcinol hapten, more than half of the anti-benzenearsonate antibody activity is retained. This is quite striking since only 20% acetylation of amino groups eliminates precipitating capacity in the somewhat analogous antibody, anti-benzoate (Nisonoff and Pressman, 1958).

Total amidination of the lysine residues of anti-SIII antibody still permitted the large polymeric antigen to precipitate 25–50% as much modified as unmodified antibody (Table IV). In fact, this is a conservative estimate, since the substantial turbidity observed in the supernatants of the specific precipitates implies the presence of complexes with not quite large enough a three-dimensional framework for precipitation.

Only in the case of anti-BSA antibody, an antibody directed to a native protein antigen, was the effect of complete amidination of the lysine residues marked.

No precipitating capacity was retained by fully amidinated anti-BSA antibody. Nevertheless, the electrophoresis results show that fully amidinated pure anti-BSA antibody is completely bound to fully amidinated BSA in excess antigen; the patterns clearly show the presence of antigen-antibody complexes, and there is no peak attributable to free modified antibody (Fig. 11). Electrophoresis was conducted at *p*H 9.5 in order to avoid problems of insolubility with the two totally modified proteins. Quantitative information concerning the effect of amidination on the equilibrium association constant for the reaction could not be obtained from one such experiment—it would be necessary first to assess quantitatively any decrease in equilibrium constant in the native antigen-antibody system due to the relatively high *p*H. Clearly, however, appreciable antibody binding capacity is retained by anti-BSA antibody even after total amidination of the free amino groups, and although precipitating capacity of the antibody is lost.

The precipitating capacity of anti-BSA antibody is lost only in the range of exhaustive amidination in which nonspecific effects on protein structure are produced, as is indicated by the physical studies. The differences in precipitation characteristics of completely amidinated anti-BSA, anti-benzenearsonate, and anti-SIII antibodies do not necessarily mean that antibody activity is inherently more sensitive to "nonspecific" effects in one case than in the others. Probably the difference in precipitin activities has no relation to unique structural or chemical properties of the antibody molecules themselves, but is rather related to the different properties of the protein, the hapten, and the polysaccharide used as antigens in these different systems. The precipitating capacity of an antibody must depend to some significant extent on the solubility characteristics of the antigen molecule. The same intrinsic binding affinity in the anti-BSA and anti-benzenearsonate cases might result in a precipitating framework in the latter system, but not in the former; the anti-benzenearsonate framework, built around a relatively small hapten, consists primarily of antibody γ-globulin and even small aggregates may exceed the limits of solubility; BSA, however, is a large soluble antigen, and a bigger framework, including more antibody molecules, might be necessary to overcome its solubilizing influence.

With the three antibodies anti-DNP, anti-SIII, and anti-BSA, the results obtained show that no lysine residue of ordinary chemical reactivity is critically involved in the active sites. On the other hand, the possibility exists that in these cases a lysine ϵ-amino group of abnormally low reactivity toward ethyl acetimidate might be present in these sites, and might remain partially unmodified even after exhaustive modification of all other amino groups on the antibody molecules. This possibility appears, however, to be highly unlikely. The active site of an antibody must be at or near the external surface of the molecule in order to be accessible to haptens, and it is difficult to reconcile this accessibility of the active site with inaccessibility or repulsion of a small molecule such as ethyl acetimidate. Any small effects of exhaustive amidination on antibody activity are more likely to be explained by the small structural changes in the antibody molecule which attend complete modification of the amino groups.

In undertaking the present investigation, it was considered that antigens bearing a prominent negatively charged group would be of special interest, since in systems of this type the antibody reactive site might more probably contain a complementary positively charged ϵ-ammonium lysine residue. Clearly, however, the effects of amidination did not distinguish antibody to negatively charged antigenic determinants (anti-benzenearsonate, anti-D-benzoylaminophenyl acetate, anti-SIII) from antibody to the neutral hapten (anti-β-lactoside), or from antibody to the strongly polar hapten (anti-DNP).

In light-scattering studies of the effect of *p*H on the equilibrium constant characterizing the association of anti-benzenearsonate antibody and a specific bivalent hapten (Epstein and Singer, 1958), it was found that the results followed a single-group titration curve. That is, the results could be explained quantitatively by the hypothesis that a single critical group of $pK_a = 9.9$ was present in each antibody reactive site. Chemical evidence was adduced to indicate that this critical group was an ϵ-ammonium group of a lysine residue. We now consider this chemical evidence to have been inadequate and to be superseded by the results and conclusions in this paper. The influence of *p*H on the hapten-anti-benzenearsonate equilibrium constant must therefore be attributed to other factors, among which might be: (*a*) the critical involvement of a single tyrosine hydroxyl group, $pK \sim 10$, in each anti-benzenearsonate reactive site; chemical evidence exists for this hypothesis (Pressman and Sternberger, 1951; Nisonoff and Pressman, 1958; Grossberg *et al.*, 1962); or (*b*) the reversible unfolding or distortion of the active sites of the antibody molecule with increase in *p*H (*cf.* Jirgensons, 1952, 1954; Phelps and Cann, 1957). This unfolding might be triggered by the ionization of a single group either inside or outside the active site, and hence the dependence of equilibrium constant on *p*H might appear to follow a single group titration curve. Further evidence is needed to decide which of these alternatives, or whether some other possibility, correctly explains the results. If a positively charged group is indeed present in the reactive site of anti-benzenearsonate molecules, and the imidazolium group of histidine (Epstein and Singer, 1958) and the ϵ-ammonium of lysine are eliminated, the only remaining possibility is the guanidinium group of arginine. At present, it is difficult to devise chemical modification studies or physical chemical studies to test this possibility.

The observation that a very high degree of antibody activity is retained upon exhaustive amidination of antibodies offers perhaps the most dramatic evidence of the absence of serious nonspecific structural modifications in protein molecules resulting from the amidination reaction.

The uniquely mild conditions and effects that characterize it suggest that this reaction may find many useful applications in protein chemistry.

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Developmental Changes of Mammalian Lactic Dehydrogenases

I. H. FINE, N. O. KAPLAN, AND D. KUFTINEC*

From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts

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Evidence is presented supporting the view that the five electrophoretic forms of lactic dehydrogenase found in animals represent two distinct types of enzymes (M and H) with three intermediate hybrids. Data indicating that the hybrids are formed at random are given. Evidence is also given showing that, when the electrophoretic patterns do not follow a binomial distribution, more than one cell type is involved. In the rat the embryonic form of lactic dehydrogenase is the M form; during development and maturation in rat heart, there is a shift from M type of lactic dehydrogenase units to H type. Data have also been obtained indicating that the rabbit and bovine embryonic forms are the M type of lactic dehydrogenase. In contrast, the human embryonic type is the H form. Great variations in the composition of the two types of lactic dehydrogenase in the livers of adult mammals as well as in other animals have been found. The significance of the two distinct lactic dehydrogenases and the possible factors that control the changes in development are discussed.

In recent reports, we presented evidence indicating that two types of lactic dehydrogenase (LDH) exist in most animals (Cahn *et al.*, 1962b; Kaplan and Ciotti, 1961a). These two types appear to be controlled by separate genes and are different in their amino acid compositions, catalytic characteristics, physical properties, and immunologic reactions. We have designated one type as M, since it occurs largely in skeletal muscle, and the second, which is usually present in cardiac muscle, as H. Both types appear to exist as tetramers consisting of identical polypeptide units. We have designated these aggregate forms as M_4 and H_4. In addition to the two homologous forms, hybrids between the two types also containing four units have been identified as intermediate electrophoretic entities. We have designated these hybrids M_3H_1, M_2H_2, and M_1H_3. The form M_3H_1 contains three M subunits and one H unit, M_2H_2 two units of each type, and M_1H_3 one M peptide chain and three of the H type. These hybrids have intermediate immunologic, catalytic, and physical properties.[1]

We have previously reported that the lactic dehydrogenase of the heart of the newborn rat was different from that of the adult. This difference was deter-

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