A BIOCHEMICAL AND GENETIC ANALYSIS OF THE BRACHMANN-DE LANGE SYNDROME

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This is to certify that the

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A BIOCHEMICAL AND GENETIC ANALYSIS OF THE BRACHMANN-DE LANGE SYNDROME

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ABSTRACT

A BIOCHEMICAL AND GENETIC ANALYSIS OF THE BRACHMANN-DE LANGE SYNDROME

by William L. Daniel

This research was designed to define the Brachmann-De Lange Syndrome biochemically and clinically and to attempt the elucidation of the genetic basis of the syndrome.

Serum carbohydrates, lipids, amino acids, and keto acids were separated by thin layer chromatography and quantitated either by elution or by the spot area technique. Urinary amino acids were assayed by thin layer chromatography and elution. Serum proteins were fractionated by Cohn Method 10 and further separated by polyacrylamide disc electrophoresis (Cohn et al., 1950). The electropherograms were quantitated with a scanning photodensitometer using white light. The activities of the serum transaminases were determined by measuring the absorbancy of the keto acid phenylhydrazones. Leukocyte glutamic dehydrogenase activities were assayed by measuring the increase in absorbancy at 340 millimicrons.

Serum glutamic acid, alpha-keto glutaric acid, and serum glutamic oxalacetic transaminase were elevated in

the patients. Generalized hypoaminoaciduria and hypogammaglobulinemia were also present. No leukocyte NAD-linked glutamic dehydrogenase activity could be demonstrated. The hyperglutamicacidemia, hypogammaglobulinemia, and hypoaminoaciduria were previously reported (Ptacek et al., 1963).

Both parents of each patient had high levels of glutamic acid and somewhat reduced activities of leukocyte NAD-linked glutamic dehydrogenase. Alpha-keto glutaric acid, gammaglobulin, and serum glutamic oxalacetic transaminase activity were normal in the parents.

Monozygous twin girls (nineteen out of nineteen blood group antigens concordant) were included in this sample. One twin appears to be free of the syndrome. The "normal" twin did not have demonstrable leukocyte NAD-linked glutamic dehydrogenase activity. Both serum glutamic acid and alpha-keto glutaric acid were elevated, but serum glutamic oxalacetic transaminase activity was normal. Her glutamic acid level was lower and her keto acid level higher than those in her affected sister.

No environmental factors which could have caused the syndrome were found in these families. Pedigree data failed to reveal any further information of genetic importance.

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A BIOCHEMICAL AND GENETIC ANALYSIS OF THE BRACHMANN-DE LANGE SYNDROME

By

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INTRODUCTION

Brachmann described the first case of a syndrome characterized by profound growth and mental retardation and congenital malformations in 1916 (Brachmann, 1916). Cornelia De Lange, a Dutch pediatrician, followed with a second case report in 1933 (De Lange, 1933). De Lange assigned the name "Typus Degenerativus Amsteldamensis" to the syndrome. Later workers found this name cumbersome and called the condition the "De Lange Syndrome" or the "Cornelia De Lange Syndrome" (Jervis and Stimson, 1963; Ptacek et al., 1963). Ptacek rediscovered the Brachmann paper and appended Brachmann's name to complete the presently accepted form.

The Brachmann-De Lange Syndrome (BDL) displays a variety of congenital abnormalities which include pronounced hirsutism, bone defects, heart abnormalities, and severe retardation of mental and physical development. The hirsutism is manifest in bushy eyebrows meeting in the midline (synophris), long eyelashes, excessive hair growth on the lower lumbar region of the back, and generalized infantile lanugo. Bone involvement is largely restricted to the extremities, and its severity ranges from slightly incurved fifth fingers (clinodactyly) and proximally

located thumbs to absence of digits and bones of the lower arm. The proximally located thumb has been found to be due to a short metacarpal bone (Kurlander and DeMeyer, 1967). The lower extremities do not appear to be as severely affected. There is an abnormality of the ankle which appears to displace the arches slightly laterally causing difficulty in walking. X-ray studies have shown a retarded osseous maturation to be responsible for the defect. Other osseous manifestations include elbow abnormalities which prevent its full extension, micrognatha or small jaw, and a flattened occipital bone.

Investigators have mentioned simian creases, abnormal axial triradii, and lack of dermal ridges on the soles and palms (Ptacek et al., 1963; Opitz et al., 1965). The simian creases are most common.

The patients' facial features are frequently diagnostic. In addition to the previously mentioned eyebrows and lashes, the nose is foreshortened and the nostrils flared. Their ears are situated at the angle of the jaw, and their lips are downturned at the ends.

The majority of patients have an I. Q. less than 25; however, McIntyre has described one patient who is only mildly retarded (McIntyre and Eisen, 1965). Their oral communication is generally restricted to a low hoarse cry until the patients are five or older. Their vocabulary never surpasses a few words or sentences.

The visceral involvement of the syndrome is striking. The internal organs are usually non-palpable, and, in the majority of cases, the genitalia are immature. Systolic murmurs are frequent (Jervis et al., 1963; Ptacek et al., 1963; McIntyre et al., 1965; Schlesinger et al., 1963). Autopsies have revealed non-crepitant lungs, immature glomeruli, microcephalic brains, anomalous systemic drainage, defective heart valves and gonads in the primitive streak stage (Ptacek et al., 1963; Schlesinger et al., 1963; Noe, 1964; Hart et al., 1965).

Diverse endocrine anomalies have been mentioned, although none appear to be common to a majority of the patients examined. Autopsies have demonstrated absence of basophilic cells in the pituitary, hypoplasia of the thymus and the adrenal gland, and absence of the Zona Fasciculata of the adrenal gland. Björklöf and Brundelet (1965) sectioned the hypophysis and discovered a cyst in the mid-section of the pituitary. It was subsequently suggested that the BDL resulted from a cyst of Rathke's Cleft. No other data have accumulated to support this hypothesis.

Necropsies have exhibited several neural ramifications of the syndrome. Poor myelinization in various regions of the brain is frequently observed. De Lange reported malformations in the gyri and absence of the Sulcus of Rolandi (De Lange, 1933). Cortical cerebral atrophy was mentioned by Schlesinger et al. (1963). Hart et al. (1965)

observed an abnormal sulcal pattern, wide gyri, and generalized reduction of ganglion cells in the cerebral cortex in one of his patients.

The developmental histories of the patients include a series of crises which frequently end in early death. The birth weight is usually less than six pounds and frequently under five pounds, yet the majority of pregnancies are full term. The patients are cyanotic at birth, have difficulty feeding, and are frequently susceptible to upper respiratory infections. These intestinal obstructions, and peritonitis are the most common causes of death during infancy.

The growth rate of the BDL patients is retarded, and the deviation from normalcy increases with increasing age (Ptacek et al., 1963). The final stature of the patients is that of a dwarf, seldom surpassing fifty-four inches.

Biochemical data are highly incomplete. Adrenocortical function and blood levels of cholesterol, protein bound iodine, potassium, sodium, and chloride are normal (Schlesinger et al., 1963). There is no elevation of the urinary phenylketones or mucopolysaccharides (Noe, 1964). Blood sugar, calcium, phosphorus, and alkaline phosphatase fall within the normal accepted range (Hart et al., 1965). Ptacek et al. (1963) reported hyperglutamicacidemia, generalized hypoaminoaciduria, and hypogammaglobulinemia in their patients. The hyperglutamicacidemia and hypoaminoaciduria have been confirmed by McIntyre et al. (1965). Serum

glutamic-oxalacetic transaminase was normal in Ptacek's sample. Silver detected a high level of serum lipid phosphorus in his patient (Silver, 1964).

Genetic studies have been fragmentary. Both an autosomal recessive inheritance and an autosomal dominant inheritance with reduced penetrance have been suggested (Ptacek et al., 1963; Opitz et al., 1965). Borghi, Ghiusti, and Bigozzi concluded that the BDL Syndrome was the result of dominant, recessive, and accessory gene interaction (Borghi et al., 1954). No environmental factor was apparent in the published cases. No parental or maternal age effect has been observed. The syndrome has been found in most European populations and in Caucasians and Negroes in the United States. Recent studies have revealed sibships with several affected children or relatives. Two sets of identical twins have been found to be concordant for the syndrome (Opitz et al., 1965). Nine miscarriages were detected in published family histories (186 pregnancies) (Opitz et al., 1965). No consanguinity for the parents has been detected.

Cytogenetic studies have been contradictory. Acentric fragments, a B/G translocation, and partial trisomy for a region on an A group chromosome resulting from a translocation of that region to a G chromosome have been observed (Jervis et al., 1963; Massimo et al., 1964; Geudeke et al., 1963; Ford, 1964; Falek et al., 1966). Opitz

criticizes the partial trisomy and suggests that the patients may have a syndrome closely resembling but not identical to the BDL Syndrome (Opitz and Smith, 1966). The majority of karyotypes reported have been normal.

This research was conducted to collect data that would permit an unambiguous diagnosis of the syndrome based upon clinical observations and biochemical criteria. The clinical and biochemical information was also used in an attempt to elucidate the heredity of the syndrome and its probable cause.

METHODS AND MATERIALS

The state homes for the mentally retarded were screened for patients exhibiting the characteristics of the Brachmann-de Lange Syndrome. The medical records were checked to exclude extraneous syndromes from the sample. The final sample consisted of five Caucasian males and one Negro female. All of them were between ten and sixteen years of age. Two of these patients had been included in the study by Hart et al. (1965). A sixth patient died shortly before the present study was begun; however, the results of the autopsy and other clinical information were available. One of the boys expired during the course of the study; however, an autopsy was unable to be performed. The parents of each child were contacted via the medical superintendents, and interviewed personally regarding their family histories and the developmental history of their affected child. Permission was obtained to collect blood and urine specimens from each of their children as well as themselves. The parents were questioned informally about the history of the pregnancy leading to the birth of the affected child. Information was obtained concerning drugs taken prior to or during pregnancy by the mother or by the father preceding conception, possible exposure to radiation,

and contact of the mother with infectious diseases during the pregnancy. Data were collected pertaining to miscarriages and stillbirths in the family and among the relatives, possible occurrence of another affected child in the pedigree, and the appearance of individual components of the syndrome in the parents, sibs, and relatives. Information regarding the presence of other forms of mental retardation or mental illness was obtained. Further data such as birth order, age of parents, possible consanguinity, and other genetic information were collected. Whenever possible the responses of the parents were verified by other relatives.

Controls were selected from within the three institutions in which the patients resided. The controls were chosen at random from patients of similar age and weight who exhibited no known biochemical disorders. Parents of children who were being admitted to the institution and who had no known biochemical disorders were used as controls for the BDL parents.

Clinical Analyses

Fasting blood and twenty-four hour urine samples were used for the biochemical and clinical tests. The following tests were performed to confirm scattered reports in the literature. Serum glucose was determined by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1945). Serum phospholipids were assayed according to Youngburg and

Youngburg (1930). Serum cholesterol was measured by the method of Crawford (1958). Total serum amino acid nitrogen was determined according to Danielson (1933). Urinary creatinine was assayed by the method of Folin (1914).

Urinary Amino Acids

Two hundred fifty microliters of twenty-four hour urine samples were chromatographed on Whatman 3mm paper and developed in two dimensions using n-butanol: methanol: acetic acid: water (500:500:3:250) and n-butanol: acetone: diethylamine: 29% ammonium hydroxide: water (450:450:45: 0.75:228) respectively. The dried chromatograms were sprayed with 0.5% ninhydrin in n-butanol, heated at 100° C., and the individual spots eluted with one ml. of 50% aqueous ethanol. The optical density was determined spectrophotometrically at 540 millimicrons. Urinary amino acids were expressed as milligrams of amino acid nitrogen per mg. creatinine.

Serum Amino Acids

Serum proteins were precipitated from 1 ml. of serum with 1 ml. of 10% trichloroacetic acid and centrifuged. The supernatant was heated in a boiling water bath for fifteen minutes to reduce the acidity. Standard 0.1% solutions of the serum amino acids were treated similarly. One hundred microliters of serum extract were spotted on a 0.3 mm. layer of Whatman CC-41 cellulose powder (available from Reeve Angel Corporation, Clifton, New Jersey).

The powder slurry (10 g.: 20 ml. water) was layered on 20 cm. x 20 cm. glass plates with a calibrated spreading device (A. H. Thomas, Philadelphia) and allowed to dry at room temperature. Ten microliters of each standard were spotted on each of two plates. One plate was developed unidimensionally in chloroform: methanol: 4% NH_4OH (2:2:1) and the other in n-butanol: ethanol: 0.5 N ammonium hydroxide (3:1:1). The serum extracts were chromatographed two dimensionally in these solvents and the plates were sprayed with a ninhydrin solution consisting of the following:

Solution A

50 ml. 0.2% ninhydrin in anhydrous ethanol

10 ml. glacial acetic acid

2 ml. collidine

Solution B

1% cupric nitrate in anhydrous ethanol

Fifty parts of A were mixed with three parts of B immediately before use (Moffat et al., 1965). The plates were heated over a hot plate at 100° C., and the spots circled. The amino acids were scraped from the plates, eluted from the powder with one ml. of 50% aqueous ethanol, and centrifuged. The supernatant was decanted into a cuvette, and the optical density read at 540 millimicrons in a spectrophotometer. A blank was prepared by scraping a similar quantity of powder from a region of the plate over which the solvents

had passed and treating it similarly. Standard curves were prepared using known amounts of amino acids.

Serum Keto Acids

Serum alpha-keto acids were isolated as their 2,4-dinitrophenylhydrazones by a method modified from that of Seligson and Kvamme (Seligson et al., 1952; Kvamme et al., 1954). Two and one-half milliliters of serum were used, and 2.5 ml. of 10% trichloroacetic acid were substituted for meta-phosphoric acid. The supernatant was diluted ten times with distilled water, and 0.2 ml. of 0.5% 2,4-dinitrophenylhydrazine in 6N HCl was added. The reaction was allowed to proceed for thirty minutes. The reaction mixture was extracted three times with 1.5 ml. of chloroform: ethanol (4:1). The combined solvent layers were extracted with 1.5 ml. of 1N Na_2CO_3, and the solvent was discarded. The Na_2CO_3 solution of the dinitrophenylhydrazones was washed with 1.0 ml. of $CHCl_3$:EtOH (4:1) and acidified at 0-4° C. with 0.5 ml. of 6N HCl. The solution was extracted three times with $CHCl_3$:EtOH (4:1) using 1.0, 0.5 and 0.5 ml. respectively. The aqueous layer was discarded and the combined extracts were evaporated to dryness at 0 to 4 degrees in a gentle steam of air. The dinitrophenylhydrazones were redissolved in 0.1 ml. of $CHCl_3$:EtOH (4:1) and spotted on plates coated with a 0.3 mm. layer of cellulose powder slurry (Whatman CC-41, 1 part powder to 2 parts water, available from Reeve Angel Corporation, Clifton, New Jersey). The chromatogram

was developed with n-butanol: ethanol: 0.5N ammonium hydroxide (70:10:20). An ultraviolet lamp was used to locate the spots (sensitivity 0.01-0.1 micrograms). The spots were circled, scraped from the plates, treated with 1.0 ml. of 1N NaOH, and shaken for ten minutes. After centrifuging, the supernatant was placed in a cuvette and read at 455 millimicrons (sensitivity 0.2 micromoles).

Serum Lipids

Serum lipids were extracted with chloroform: methanol (4:1). One hundred-fifty microliters of the extract were spotted on plates coated with a 0.3 mm. layer of SG-41 (Reeve Angel) and chromatographed with chloroform: methanol: water (65:24:4) (Wagner et al., 1961). The plates were sprayed with 50% H_2SO_4 and heated over a hot plate set at 100° C.

Serum Carbohydrates

Serum sugars from the trichloroacetic acid extract were separated by thin layer chromatography. Fifty microliters of extract were spotted on Whatman CC-41 (0.3 mm. layers) cellulose powder and developed unidimensionally in ethyl acetate: pyridine: water (2:1:2). The plates were dried and rechromatographed in the same direction with the same solvent system. The reducing sugars were detected by carefully immersing the plates in a solution of silver nitrate in acetone. The solution was prepared by dissolving 0.122 grams of silver nitrate in 1 ml. of water and

adding the solution to 200 ml. of acetone. The fine precipitate which formed was redissolved with a minimum amount of water (Sherwood and Jermyn, 1965). The plates were removed from the bath, dried and sprayed with 0.5N NaOh in ethanol. The reducing sugars were indicated by dark brown spots on a light brown background. The amounts of the sugars were determined by a spot area technique. Known quantities of sugars were spotted, dipped and sprayed. The spots were traced on $mm.^2$ graph paper, and their areas determined. The areas were plotted on semilog paper (areas along the linear axis, weights along the log axis) (Randerath, 1965). Linearity was observed to hold from one to forty micrograms of glucose.

Serum Protein Analyses

Serum albumins, alpha-globulins, beta-globulins, and gamma-globulins were measured by the phosphate turbidimetric method using standard phosphate solutions prepared by Hycel, Inc. (Moran, 1963).

Plasma protein analysis involved immunoelectrophoresis, acrylamide disc electrophoresis, and Cohn fractionation followed by disc electrophoresis. Immunoelectrophoresis was performed by the micro method of Scheidegger (1955). Prepared antibodies were obtained from Hyland Laboratories (Hyland Laboratories, 4501 Colorado Blvd., Los Angeles, Calif.). The electrophoretic medium consisted of two grams of Difco agar noble, fifty milliliters of borate buffer

(pH 8.2), fifty milliliters of distilled water, and ten milligrams of sodium azide. The electrode vessel buffer was prepared with 3.092 grams boric acid, 1 ml. 8N NaOH, and 3 ml. 1N HCl. The pH of this solution was 8.4. The electrode buffer was diluted 75:25 with distilled water to give the gel buffer, pH 8.2. The agar mixture was heated to 100 degrees on a hot plate until dissolved. Three milliliters of solution were applied to each microscope slide and allowed to solidify. Antigen wells and the slit for the antiserum were prepared with a cork device consisting of two parallel double edged razor blades and two glass capillary tubes.

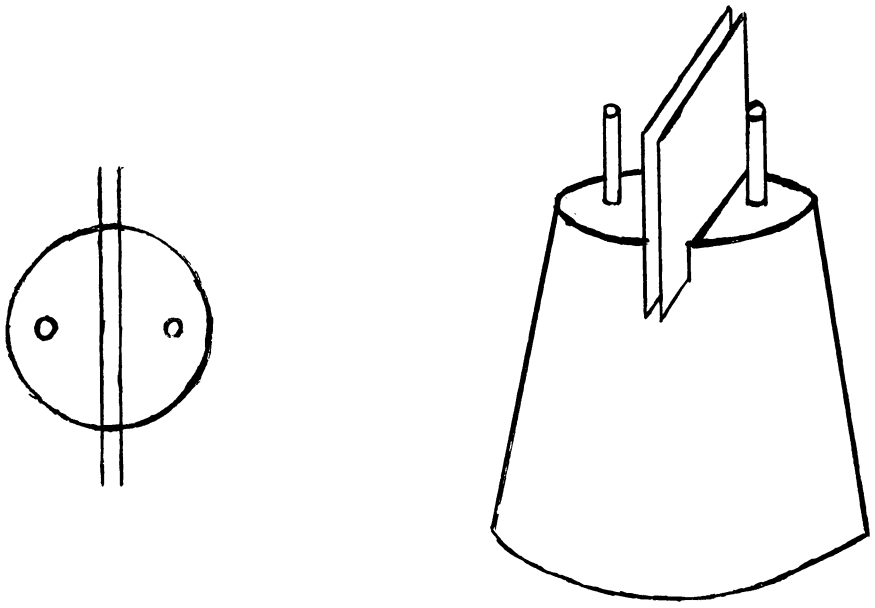


Figure 1.--Slot and Well Cutter for Immunoelectrophoresis.

The antigen wells are 1 mm. in diameter and situated 3 mm. from the antiserum trough. The dimensions of the antiserum trough are 1.0 mm. x 45 mm. The antigen-well plugs were removed by suction with a Pasteur pipette connected to an aspirator. One lambda of plasma was introduced to each antigen well such that patient and control were run on opposite sides of the antiserum trough. The wells were sealed with cooled liquid agar. A potential gradient of 45 volts was applied to each slide for 45 minutes. The electrophoresis apparatus is shown in Figure 2.

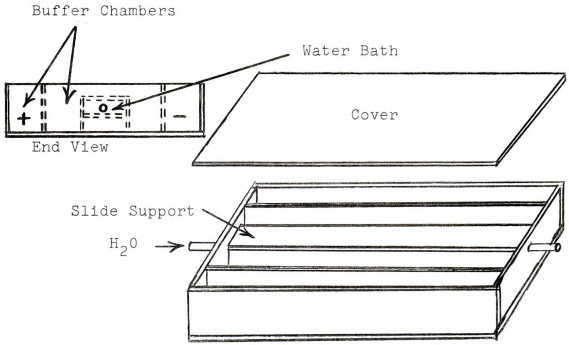


Figure 2.--Immunoelectrophoresis Apparatus

The electrode and inner buffer chambers were connected by Whatman 3mm filter paper bridges. Buffer solution was conveyed to the microscope slides by Whatman 3mm filter paper bridges by an agar bridge of the same consistency as that on the slides. Following the electrophoresis the antiserum trough plug was carefully removed with a razor blade. Five one-hundredths milliliter of antiserum was applied uniformly along the groove with a 100 lambda pipette. The optimal concentration of antiserum was determined by trial and error. The slides were placed on a level surface in a moist chamber (glass dishes containing a vial of water and covered with cellophane), and placed in an incubator set at 18° C. The precipitin lines were fully developed after 36 hours. The slides were washed for 36 hours in three changes of 0.8% NaCl and dried under filter paper. The dried slides were fixed in 2% acetic acid for five minutes, stained in 0.5% Buffalo Black 10B (Amidoschwarz 10B) in methanol-glacial acetic acid 9:1 for five to ten minutes, and washed three times in MeOH:HOAc (9:1) for fifteen minutes each (Scheidegger, 1955). The resultant immunoelectropherograms are stable indefinitely. Total human antiserum and specific human protein antiserums were used and are listed below:

Anti-Human Serum
Anti-Human Ceruloplasmin
Anti-Human Alpha-2-Haptoglobin
Anti-Human Beta Lipoprotein
Anti-Human Beta 1C/Beta 1A Globulin
Anti-Human Gamma G

Anti-Human Immunoglobulins
Anti-Human Transferrin
Anti-Human Alpha-2-Globulin
Anti-Human Gamma M
Anti-Human Gamma A
Anti-Human Orosomucoid

The Cohn alcohol fractionation technique (method 10) was modified to separate serum, leukocyte, and erythrocyte proteins (Cohn et al., 1950). The same modification was used for each series of proteins in order to permit possible comparisons between them. Ten milliliters of a solution consisting of 250 ml. of 95% ethanol and 2.5 ml. of sodium acetate (0.4×10^{-6} g. per ml.) per liter were added to 2.5 ml. of serum at 0° C. The solution was added gradually over a period of one minute. The reaction mixture was adjusted to pH 5.8-5.9 with 0.1 N HCl. (In this step and all succeeding steps, 0.5 ml. of reaction mixture was diluted to 2.5 ml. with 0.02M NaCl. The pH of the diluted mixture was adjusted to the desired pH. The number of drops of 0.1N HCl or 0.1N NaOH required to adjust the pH to the desired range was then multiplied by a factor which was determined by trial and error, and the requisite number of drops added to the master tubes.) The suspension was stirred fifteen minutes at -5° C. and centrifuged at 4000 r.p.m. for thirty minutes at -5° C. The pellet contained fractions I, II, and III; and the supernatant contained fractions IV, V, and VI. All manipulations from this point on were carried out at -5° C. One milliliter of a solution containing 100 ml. 95% ethanol and 27.4 g. zinc acetate

dihydrate per liter was added to the decanted supernatants. The protein suspensions were allowed to stand for thirty minutes, centrifuged thirty minutes, and the supernatants decanted. The supernatants contained Fraction VI. The volumes were measured and 1 ml. was withdrawn for a biuret determination. Seventeen and one-half milliliters of a solution composed of 160 ml. 95% ethanol, 2.6 g. barium acetate, 20 ml. 1M sodium acetate, and 7.28 ml. 1M acetic acid per liter were added to the precipitates; the pH was adjusted to 5.5-5.6; and the suspension was stirred for one hour. The tubes were centrifuged for thirty minutes and the supernatants (Fraction V) decanted. The volumes were measured, and 1 ml. withdrawn for protein determinations. Two and one-half milliliters of a solution consisting of 160 ml. 95% ethanol, 0.10 g. zinc acetate dihydrate, and 50.0 ml. 1M sodium acetate per liter were added to the pellets, and the pH was adjusted to 6.1-6.2. The suspensions were stirred for one hour, centrifuged thirty minutes, and the supernatants (Fractions IV-6+7) decanted. The volumes were measured and 1 ml. withdrawn for biuret determinations. Precipitate IV-1 was resuspended in 2 ml. of 0.15M sodium chloride, and the proteins were brought into solution with 10 drops of 0.5M sodium citrate. The volumes were measured, and 1 ml. withdrawn for protein determinations.

Precipitates I+II+III were stirred into a paste and resuspended in 5 ml. of a solution containing 150 ml. 95%

ethanol, 2.0 ml. 1M sodium acetate, 1.40 ml. 1M acetic acid, and 45.0 g. glycine per liter. (This solution will be referred to as solution 7.) The pH was adjusted to 5.5. The reaction mixtures were stirred for fifteen minutes, centrifuged thirty minutes, and the supernatants decanted. The volumes were measured, and 1 ml. withdrawn for biuret determinations. This fraction was designated fraction II. Precipitate I+III was stirred into a paste, and 10 ml. of a solution composed of 160 ml. 95% ethanol, 45 g. glycine, 2.5 ml. of solution 7, 3.2 ml. of 0.5M disodium phosphate, and 2.4 ml. of monosodium phosphate per liter added. The pH was adjusted to 6.8-6.9. The suspension was stirred for one hour, centrifuged forty-five minutes, and the supernatants (fraction III-0) decanted. The volumes were measured, and 1 ml. withdrawn for protein determinations. The precipitates were stirred into a paste and treated with 2.5 ml. of a solution consisting of 160 ml. 95% ethanol, 1.2 ml. 1M citric acid, and 120 ml. 1M sodium citrate per liter. The protein suspensions were adjusted to pH 7.1-7.2, stirred for one hour, centrifuged thirty minutes, and the supernatants (fraction III-1,2) decanted. Their volumes were measured, and 1 ml. withdrawn for biuret determinations. Precipitate I+III-3 was redissolved in 2 ml. of 0.02M sodium citrate. The volumes were measured, and 1 ml. withdrawn for protein determinations.

The solutions in the preceding paragraphs were prepared fresh for each set of fractionations.

The biuret determinations were performed as follows. One milliliter of the fraction was diluted to 2 ml. with 0.8% sodium chloride. Two milliliters of the dilution were added to 5 ml. of biuret working reagent. The stock biuret solution contained 11.25 g. of sodium potassium tartrate, 100 ml. 0.2N sodium hydroxide, 3.75 g. copper sulfate pentahydrate, and 1.25 g. potassium iodide per 250 ml. The working solution was derived from the stock reagent by diluting it one to five with 0.2N sodium hydroxide (Weichselbaum, 1965). The reaction mixtures were placed in a water bath at 37° C. for ten minutes, cooled five minutes, and read in a spectrophotometer at 552.5 millimicrons. A standard curve was constructed using aliquots from a standard serum. The serum was standardized by microkjeldahl total nitrogen and non-protein nitrogen techniques (Wong, 1923; Folin, 1919). The average of four determinations on a pooled serum sample was used for the standard value.

Total sera and the serum fractions were subjected to disc electrophoresis in polyacrylamide gels. The gel column included a 7% gel, 5% gel, stacking gel, and sample gel (Fig. 3). The Gel compositions were adapted from Davis (1962).

The gel compositions are presented in Table 1.

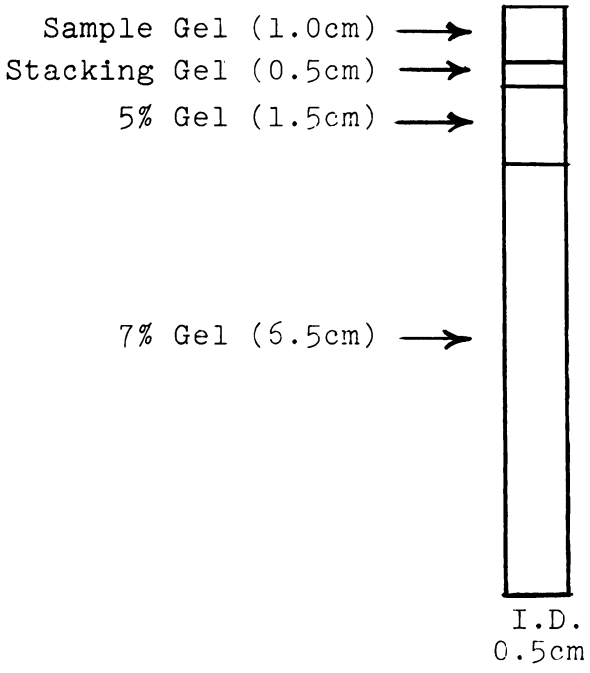


Figure 3.--Polyacrylamide Gel Columns

The tubes were made from flint glass tubing and placed vertically in rubber caps (available from Canalco). The gel solutions were introduced sequentially using a 10 cc. disposable syringe with a 21 gauge needle. The 7% gel was layered first followed by a 2 mm. layer of distilled water added drop by drop down the side of the glass tube with the same syringe. The gels (in caps) were placed upright in a test tube rack, put in the dark, and allowed to polymerize for thirty minutes. The water layer was removed by inverting the tube and gently blotting the water on paper toweling. The 5% gel was then layered on the 7% gel and the above

TABLE 1.--Stock and working solutions for disc acrylamide gels.

pH 8.3

I. Stock Solutions

A.	1N HCl	48 ml.	B.	1N HCl	approx. 48 ml.
	TRIS*	36.6 g.		TRIS	5.98 g.
	TEMED[X]	0.23 ml.		TEMED	0.46 ml.
	H_2O to pH 8.9	100 ml.		H_2O to	100 ml.
				Adjust pH to 6.7 with 1N HCl.	
C.	Acrylamide[X]	28.0 g.	D.	Acrylamide	10.0 g.
	BIS[X].	0.735 g.		BIS	2.5 g.
	H_2O to	100 ml.		H_2O to	100 ml.
E.	Riboflavin	4 mg.	F.	Sucrose	40 g.
	H_2O to	100 ml.		H_2O to	100 ml.
G.	Acrylamide	10.0 g.			
	BIS	0.368 g.			
	H_2O to	50 ml.			

II. Working Solutions

7% Gel	5% Gel	Sample and Stacking Gels
1 part A	1 part A	1 part B
2 parts C	2 parts G	2 parts D
1 part H_2O	1 part H_2O	1 part E
4 parts 0.14% Ammonium Persulfate (A. P.)	4 parts A. P.	4 parts A. P.

III. Buffer

TRIS	6.0 g.	The buffer was diluted 1 to 10 before use
Glycine	28.8 g.	
H_2O to 1 liter pH 8.3		

*Sigma 7-9, Sigma Chemical Company, St. Louis, Mo.

TEMED: N,N,N',N'-Tetramethylethylenediamine

BIS: N,N'-Methylenebisacrylamide

[X]Available from Canal Industrial Corp. (Canalco), Rockville, Md.

procedure repeated. After the water was removed, the stacking gel was introduced to the tube, overlayed with water, and the gels allowed to polymerize in fluorescent light. This was accomplished by standing the tubes approximately three inches from a desk type fluorescent lamp. Polymerization time was approximately fifteen minutes. The water layer was removed, and the sample layered on the stacking gel with a ten lambda micropipet. Ten ml. of serum, 10 ml. of serum fraction IV-1, or 30 ml. of the other serum fractions were applied to the column. The sample gel was gently squirted against the side of the tube such that the sample was mixed and trapped within it. This gel was overlayed with water and allowed to polymerize in fluorescent light. Polymerization time was approximately thirty minutes. The length of the sample gel had to be increased proportionally with increased sample volume, approximately 0.5 mm. per ten lambdas of volume. Completion of polymerization was signified by the appearance of a distinct line at the gel-water interface. This was accompanied by the disappearance of the yellow color and appearance of a slight cloudiness in the riboflavin-catalyzed gels.

The tubes were removed from the base caps and inserted, sample gels uppermost, into silicone stoppers (Canalco) such that they protruded slightly above the stopper. The balance of the tubes was filled with diluted buffer. The electrophoresis apparatus was constructed from

pine, glass tubing, rubber bands, platinum wire (upper electrodes), a pyrex baking dish, copper wire, one-holed rubber stoppers, and galvanized clothesline (lower electrode).

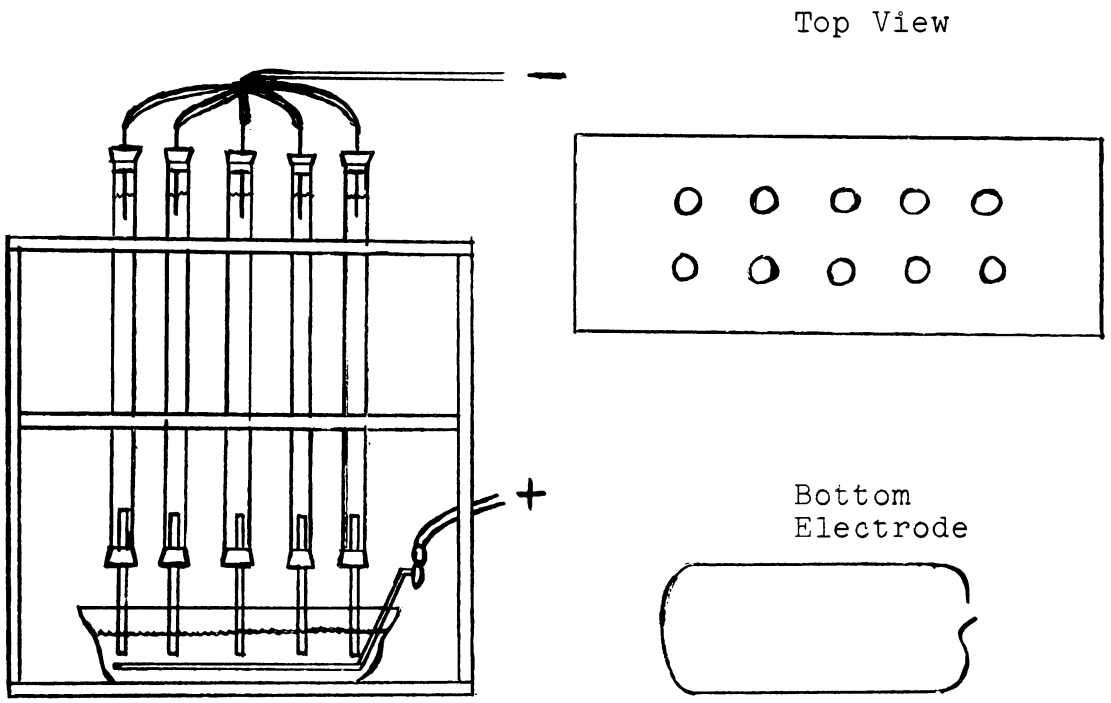


Figure 4.--Disc Acrylamide Electrophoresis Apparatus.

The silicone stoppers were inserted into the bottoms of the buffer tubes, and diluted buffer poured down the glass rod and the buffer tube walls to a point one-inch from the top. This buffer contained a few drops of 0.5% aqueous Bromphenol Blue as a tracking dye. Diluted buffer was poured into the baking dish, and the electrophoresis

circuit completed. The tracking dye, which marks the buffer front, was allowed to approach a point 1 cm. from the lower end of the arylamide tube.

The gels were gently removed from the tubes with a 10 cc. syringe equipped with a 21 gauge needle and filled with buffer. The needle was slowly inserted between the gel and the tube wall while applying a gradual pressure on the syringe plunger. The tube was then slowly turned (pressed tightly against the needle), and a steady gentle pressure maintained on the plunger. This procedure introduced a film of buffer between the gel and the tube. The tube was inverted, and the technique repeated. The gel then fell free into a container filled with buffer.

The gels were stained individually in test tubes with 0.5% Buffalo Black in 7% acetic acid for one hour or longer. Destaining was accomplished electrophoretically in a bath of 7% acetic acid. The gels were preserved in stoppered test tubes containing 7% acetic acid. The protein bands were quantitated on a Densicord scanning photodensitometer (Photovolt, N. Y., N. Y.) using white light. The curves were automatically integrated, and the protein content calculated from the percentages (area under peak A/total area) and the total protein in the fraction (biuret determination).

Leukocyte and Erythrocyte Proteins

This procedure was repeated on erythrocyte and leukocyte extracts. Ten lambdas of erythrocyte fraction VI or thirty lambdas of the remaining erythrocyte fractions were introduced to the gel columns. Forty lambdas of leukocyte extract were employed in the electrophoresis.

Leukocytes were isolated from 10 ml. of heparinized blood by allowing the blood to stand at room temperature for approximately one hour. The erythrocytes sedimented out leaving the leukocytes suspended in the plasma. The leukocytes were cultured three days in a medium consisting of 10 ml. TC-199 (Difco Laboratories, Detroit, Michigan) to which 0.1 cc. of Penicillin/100 ml. (300,000 units/cc.) and 0.025 cc. of Streptomycin/100 ml. (1 g./2.5 cc.) had been added, 2.5 ml. of serum, and four or five drops of Bacto-phytohemagglutinin (P) (the latter may be obtained from Difco).

The leukocytes were spun down in a clinical centrifuge washed in 0.85% sodium chloride six times, suspended in 0.5 ml. of saline and sonicated fifteen seconds at the maximum setting. The extracts were electrophoresed in the pH 8.3 buffer and gel system mentioned previously, and at pH 4.5 according to Reisfeld et al. (1962). The compositions of the stock and working solutions for the latter system are presented in Table 2. The arrangement of the gels is identical to that in Figure 3.

TABLE 2.--Solutions for disc Acrylamide electrophoresis pH 4.5.

I. Stock Solutions

A.	1N KOH	48 ml.	B.	1N KOH	48 ml.
	Glacial Acetic Acid (HOAc)	17.2 ml.		HOAc	2.87 ml.
	TEMED	4.0 ml.		TEMED	0.46 ml.
	H_2O to	100 ml.		H_2O to	100 ml.
	pH 4.3			pH 6.8	
C.	Acrylamide	10 g.	D.	Acrylamide	10 g.
	BIS	2.5 g.		BIS	0.37 g.
	H_2O to	100 ml.		H_2O to	50 ml.
E.	Riboflavin	4.0 mg.	F.	Acrylamide	30 g.
	H_2O to	100 ml.		BIS	0.8 g.
				H_2O to	100 ml.
G.	Sucrose	40 g.			
	H_2O to	100 ml.			

II. Working Solutions

7% Gel	5% Gel	Sample and Stacking Gels
1 part A	1 part A	1 part B
2 parts F	2 parts D	2 parts C
1 part H_2O	1 part H_2O	1 part E
4 parts 0.28% A.P.*	4 parts 0.28% A.P.	4 parts G

III. Buffers

Beta Alanine	31.2 g.	The buffer was diluted one to ten with distilled water before use
HOAc	8.0 ml.	
H_2O to	1000 ml.	
pH 4.5		

*A.P. = Ammonium Persulfate.

The erythrocytes were washed six times in 0.85% saline. The supernatant following the final centrifugation was decanted, and the cells sonicated at the maximum setting for fifteen seconds. The erythrocyte extracts were electrophoresed in the pH 8.3 buffer and gel system.

Serum Enzyme Activities

Activities of the serum enzymes associated with glutamic acid were measured by colorimetric procedures. Serum glutamic-oxalacetic transaminase and serum glutamic-pyruvic transaminase were assayed by the methods of Reitman and Frankel (1957). The procedures were modified by halving the quantities of serum and reagents they used. A serum blank was prepared for each sample by omitting the incubation period and adding 2,4-dinitrophenylhydrazine to 0.1 ml. of serum and 0.5 ml. 0.85% sodium chloride immediately. The reagent blank was modified to include 0.6 ml. of distilled water, 0.5 ml. of 2,4-dinitrophenylhydrazine, and 5 ml. of 0.4N sodium hydroxide. In both cases, standard curves were prepared as suggested to give results comparable to those obtained by the ultraviolet method (Karmen et al., 1955). All tubes were read at 505 millimicrons.

Serum glutaminase was determined by a method modified from the L-asparaginase technique of Mashburn and Wriston and the L-glutaminase procedure of Meister (Mashburn et al., 1963; Meister, 1955). The substrate contained 0.08M L-glutamine in 0.05M Tris buffer, pH 8.6. Six-tenths

TABLE 3.--Solutions for deriving SGOT standard curve.

ml. Standard	ml. Water	Unit Equivalent
0.0	2.2	0
0.1	2.1	20
0.2	2.0	55
0.3	1.9	95
0.4	1.8	148
0.5	1.7	216

1 ml. of 2,4-dinitrophenylhydrazine and 10 ml. of 0.4N sodium hydroxide were added to each tube as outlined in the above method.

milliliter of serum was added to 1.7 ml. of substrate and incubated at 37° C. for thirty minutes. One-tenth milliliter of 1.5M trichloracetic acid (TCA) was added to stop the reaction, and the mixture was centrifuged for five minutes. Serum blanks were prepared by adding TCA before the enzyme to 1.7 ml. of substrate and centrifuging. Five-tenths milliliter of the supernatant was combined with 7.0 ml. of water and 1.0 ml. of Nessler's Reagent. A reagent blank was prepared by adding 1.0 ml. Nessler's Reagent to 8.7 ml. of water. After ten minutes, the color was read at 480 millimicrons. An ammonium sulfate solution containing 14 micrograms of nitrogen per 0.5 ml. was used as a standard. Activity was expressed as:

$$\frac{\text{micrograms N liberated/mg. serum protein/min}}{14}$$

Leukocyte Enzyme Analyses

Peripheral leukocytes from 10 cc. of blood were isolated, and their extracts prepared as outlined about (Leukocyte and Erythrocyte Proteins). Leukocyte L-glutamic dehydrogenase activity was measured by the procedure of Strecker (1955). The NADP-specific enzyme was tested by substituting 0.1 ml. of NADP solution (3 micromoles/ml. water) for the NAD specified by Strecker. In both the NAD-specific analysis and the NADP-specific analysis, 0.3 ml. of leukocyte extract was substituted for the 0.1 ml. of purified enzyme.

RESULTS

Biochemical Studies

Silver's report of an elevation in serum phospholipids was not upheld in this sample (Table 4). The patient mean was actually lower than the control mean, although the difference was not significant. It is interesting that one of the controls had a value exceeding that quoted in Silver's paper (12.8 mg/100 ml.) (Silver, 1964).

The data contained in Table 4 fail to show deviations by serum amino acid nitrogen, glucose, and cholesterol from control ranges. Control 3 and patient 3, both of whom had glucose levels below 50 mg.% are now deceased.

Ptacek et al. reported a low gamma globulin level in his patients (Ptacek et al., 1963). The phosphate buffer fractionation failed to demonstrate significant differences between patient and control gamma globulin concentrations (Table 5). Other evidence tends to support Ptacek's finding and will be reported below. Serum albumins, alpha globulins, and beta globulins fell within the control ranges.

Thin layer chromatography displayed fifteen amino acids. Positive identification was hampered somewhat by trailing caused by the excess acid, however, gradual heating

TABLE 4.--General clinical tests.

		Phospholipids (mg%)	A. A. Nitrogen (mg%)	Glucose (mg%)	Cholesterol (mg%)
Control	1	9.7	7.0	76.0	192
	2	10.3	9.1	52.7	165
	3	13.7	7.3	43.7	200
	4	9.7	8.0	72.1	170
	5	9.7	9.7	62.6	250
BDL	1	8.4	5.8	70.6	208
	2	8.5	4.4	54.5	244
	3	8.6	8.5	47.0	220
	4	11.4	10.0	71.5	180
	5	9.2	8.2	83.8	204
Accepted Range		9-11[a]	5-8[a]	50-90[b]	150-250[a]
$\bar{X}_C \pm SE_C$		10.6 ± 0.8	8.4 ± 0.5	61.4 ± 6.0	195 ± 15
$\bar{X}_P \pm SE_P$		9.2 ± 0.6	7.4 ± 1.0	65.5 ± 13.1	211 ± 10
F (Crit. Reg.)[c] (0.05;4,4)		0.104, 9.604			
F[d]		1.919	0.277	1.043	2.095
t (C. R.) (0.05;8)		-2.306, 2.306			
t		1.458	0.881	-0.462	-0.870

[a]These values were taken from Hawk's Physiological Chemistry, 14th ed., B. L. Oser, ed., New York: McGraw-Hill, 1965, pp. 977-979.

[b]J. E. Middleton and W. J. Griffiths, Brit. Med. J., 2:1525, 1957.

[c]The pairs of numbers represent the upper and lower bounds, respectively, of the critical region.

[d]Test on homogeneity of variances.

TABLE 5.--Serum protein levels: Hycel precipitation method.[1]

		Total	Albumins	Globulins		
				Alpha	Beta	Gamma
Control	1	6.03	3.42	0.88	0.78	0.96
	2	7.04	4.84	0.70	1.06	0.46
	3	7.55	4.88	1.24	0.68	0.73
	4	7.10	4.50	1.51	0.33	0.75
	5	8.22	5.15	1.29	0.53	1.23
BDL	1	7.60	4.76	1.29	0.59	0.97
	2	7.50	4.52	1.44	0.71	0.82
	3	5.40	2.96	1.14	0.67	0.63
	4	6.50	3.92	0.83	0.69	1.06
	5	8.80	5.32	1.18	1.07	1.22
$\bar{X}_C \pm SE_C$		7.19 ± 0.36	4.56 ± 0.30	1.12 ± 0.15	0.68 ± 0.13	0.83 ± 0.13
$\bar{X}_P \pm SE_P$		7.16 ± 0.57	4.30 ± 0.40	1.18 ± 0.10	0.75 ± 0.08	0.94 ± 0.10
F C. R. (0.05;4,4)		0.104, 9.604				
F		0.392	0.568	2.115	2.286	1.600
t C. R. (0.05;8)		-2.306, 2.306				
t		0.044	0.516	-0.335	-0.470	-0.670

[1]Values in table are expressed in gram percent.

of the plates resulted in the appearance of the amino acids as small spots. The rate of color development, variability of amino acid-ninhydrin colors, and differences in R_f values facilitated their identification. Glutamic acid and glutamine were confirmed by adding a known amount of their standard solutions to a serum amino acid extract and chromatographing the mixture in the two solvents. The respective spots were more intense than those of the other amino acids. The amino acid levels obtained by the elution technique were slightly higher than those mentioned by White et al. (1964) (Table 6). However, the total amino acid levels fell well within their quoted levels. Fourteen of the fifteen amino acids did not show a significant deviation from control ranges; however, glutamic acid exhibited an elevation which was significant at the 2% level. This confirms the discovery of Ptacek et al. (1963).

The hyperglutamicacidemia prompted an investigation of the serum enzymes which are involved in its synthesis and degradation. The data for serum glutaminase, serum glutamic oxalacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) are presented in Table 7. There were significant differences between control and patient SGOT activities; however, differences between the respective glutaminase and SGPT activities were not significant.

TABLE 6.--Serum amino acids.[a]

		Gly	Lys	Cys	Ser	Thr	Glu-HN_2	Ala	Glu	Val	Pro	Try	Ileu	Met	Leu	Phe
Control	1	3.8	2.4	1.2	2.0	2.2	7.0	3.8	3.0	1.0	2.5	0.9	0.9	0.4	3.1	0.4
	2	2.6	2.4	1.6	1.6	0.9	7.0	2.4	3.8	3.2	2.6	2.2	0.4	2.4	3.6	2.6
	3	1.0	1.9	1.4	3.1	1.5	6.9	3.2	2.2	2.1	1.9	0.5	1.2	1.2	2.5	0.9
	4	1.2	2.0	2.0	4.2	1.4	8.9	4.1	1.9	1.8	3.5	1.1	0.6	1.0	2.1	1.5
BDL	1	2.8	1.8	1.2	1.4	1.2	10.4	4.8	6.4	1.1	3.0	0.4	1.0	1.1	3.0	1.0
	2	1.0	1.4	2.2	2.7	1.5	6.6	3.6	5.2	1.8	4.1	0.6	0.9	2.6	2.7	0.2
	3	1.2	2.4	2.5	3.3	0.9	10.4	5.2	9.4	1.9	2.9	2.8	2.0	0.9	1.9	1.5
	4	0.6	1.2	1.0	1.8	0.6	4.9	0.3	4.0	1.3	1.5	1.0	0.4	2.2	2.5	1.5
	5	0.8	1.8	2.6	3.8	3.8	7.8	4.8	7.0	2.4	2.4	2.6	2.4	1.4	3.8	3.0
$\bar{X}_C \pm SE_C$		2.2 0.7	2.2 0.1	1.6 0.2	2.7 0.6	1.5 0.3	7.4 0.5	3.4 0.4	2.7 0.4	2.0 0.5	2.6 0.3	1.2 0.4	0.8 0.2	1.2 0.4	2.8 0.3	1.4 0.5
$\bar{X}_P \pm SE_P$		1.3 0.4	1.7 0.2	1.9 0.4	2.6 0.4	1.6 0.6	8.0 1.08	3.7 0.9	6.4 0.9	1.7 0.2	2.8 0.4	1.3 0.4	1.3 0.4	1.6 0.3	2.8 0.3	1.4 0.5
t		1.233	1.923	-.625	.139	-.145	-.545	-.273	-3.364[b]	.625	-.357	-.172	-1.136	-.769	0	0

[a]These values are expressed as milligrams per 100 ml. serum.

[b]The t value for Glutamic Acid is significant at the 0.02 level. The probability of observing a t value as small as or less than -3.49 is 0.01.

TABLE 7.--Serum enzyme activities.

		Glutaminase[a] (Units x10^3)	SGOT[b] (Units)	SGPT[b] (Units)
Control	1	2.82	28.2	14.0
	2	2.02	36.0	17.9
	3	1.23	18.0	14.0
	4	1.17	8.5	2.0
	5	1.39	11.0	6.0
	6	1.72	41.0	35.5
	7	0.70	8.0	14.4
	8	0.66	28.5	14.1
	9	1.75	11.0	46.5
	10	1.00	15.0	7.5
	11	1.54	45.2	33.2
	12	1.54	31.0	12.0
	13	1.84	40.5	12.5
	14	1.40	34.5	18.0
BDL	1	0.64	67.0	13.0
	2	1.97	71.5	18.0
	3	1.92	55.0	22.0
	4	----	61.0	22.0
	5	----	112.0	----
	6	----	110.0	----
$\bar{X}_C \pm SE_C$		1.48 ± 0.15	25.4 ± 3.5	17.7 ± 3.3
$\bar{X}_P \pm SE_P$		1.51 ± 0.44	79.4 ± 10.2	18.8 ± 2.1
F		0.5467	0.2776	8.366
t		-0.080	-6.375*	-0.172

[a]One unit is defined: $\frac{\text{mcg. N/mg. serum protein/min.}}{14}$

[b]One unit is defined as 0.022 mcg. product/min./ml. serum which is comparable to the rate of decrease in the absorbance of $NADH_2$ at 340 millimicrons/min./ml. serum.

*Significant at the 0.01 level.

The activities of leukocyte NAD-linked and NADP-linked glutamic dehydrogenase are listed in Table 8. The NAD-linked dehydrogenase was deficient in the patients. No activity could be detected in four of the five patients. The activity of the NADP-linked enzyme did not differ significantly between patients and controls.

The elevation in serum glutamic oxalacetic transaminase suggested that a corresponding increase might be found in the serum keto acids. Thin layer chromatography revealed four spots which were found to be oxalacetate, alpha-keto glutarate, pyruvate, and a spot which contained alpha-keto isovalerate, alpha-keto isocaproate, and aceto-acetate. These substances are listed in order of increasing R_f. Two points should be stressed at this time. The standard keto acid phenylhydrazones must be prepared fresh. If allowed to stand in organic solvents, decarboxylation occurs resulting in several secondary spots which increase in intensity with increasing time. The excess acid causes significant trailing between oxalacetate and pyruvate. A spot of uniform size was scraped from this area at a point corresponding with the R_f of the alpha-keto glutarate standard. The observations contained in Table 9 do not show a trend between the respective levels of alpha-keto glutarate and either oxalacetate or pyruvate. The patients appear to have a higher concentration of alpha-keto glutarate. The other keto acids fell within control ranges.

TABLE 8.--Specific activities of the leukocyte glutamic dehydrogenases.

	NAD-GD[a] (units/min./mg.)*	NADP-GD[b] (units/min./mg.)*
Patients		
1	0.0	22.5
2	0.2	24.0
3	0.0	17.3
4	0.0	42.3
5	0.0	27.1
Controls		
1	46.5	25.0
2	36.1	64.3
3	15.2	75.6
4	25.4	14.1
5	65.9	36.0
6	28.4	45.8
7	43.4	30.1
8	22.0	56.0
9	12.7	15.3
$\bar{X}_P \pm SE_P$	0.04 ± 0.04	26.6 ± 4.2
$\bar{X}_C \pm SE_C$	32.8 ± 5.7	40.2 ± 7.2
t	----	1.315
U	0**	-----

[a]NAD-GD = NAD-linked glutamic dehydrogenase.

[b]NADP-GD = NADP-linked glutamic dehydrogenase.

*One unit is defined as a change in optical density of 0.001 under the conditions specified by Strecker (1955).

**Significant at the 0.002 level.

TABLE 9.--Serum keto acids (mg%).

		Ox	AKG	Pyr	AKIV AKIC AcAc
Control	1	0.97	0.57	4.23	1.50
	2	3.02	0.89	5.31	1.31
	3	2.12	0.96	2.35	2.14
	4	0.63	0.10	1.56	1.83
	5	1.56	0.34	0.62	1.85
	6	1.00	0.60	0.98	1.98
	7	0.88	0.27	1.42	1.60
	8	0.89	0.40	1.26	2.21
BDL	1	0.98	2.23	2.89	2.46
	2	0.94	2.57	2.95	1.68
	3	1.47	2.02	2.12	1.58
	4	----	1.69	----	----
	5	----	2.16	----	----
$\bar{X}_C \pm SE_C$		1.38 ± 0.29	0.52 ± 0.11	2.22 ± 0.59	1.80 ± 0.11
$\bar{X}_P \pm SE_P$		1.13 ± 0.17	2.13 ± 0.14	2.65 ± 0.27	1.91 ± 0.28
F		7.6016	0.8712	13.1382	0.4242
t		+0.505	-9.200*	-0.420	-0.454

*Significant at the 0.01 level.

Ox = Oxalacetic Acid; AKG = Alpha-Keto Glutaric Acid; Pyr = Pyruvic Acid; AcAc = Acetoacetic Acid; AKIV = Alpha-Keto Isovaleric Acid; AKIC = Alpha-Keto Isocaproic Acid.

Serum carbohydrates (Table 10) failed to display any abnormal trends as measured by the thin layer chromatography and silver nitrate dipping technique. The values for glucose obtained by this method agreed favorably with those determined by the Nelson-Somogyi method (Table 4). Compound "X" remains unidentified. No standards agreed with its R_f. Glucosamine was unique due to its tendency to exhibit a white area in the center of its spot after the silver nitrate and sodium hydroxide treatment.

Serum lipids of the Brachmann-de Lange patients qualitatively resembled those of the controls. Six major lipid fractions were obtained by thin layer chromatography. Palmitic and stearic acid (R_f = 0.81), oleic and linoleic acid (R_f = 0.73), cholesterol (0.66), lecithin (0.16), and phosphatidyl inositide and cephalin (0.10) were positively identified by using suitable standards. The sixth spot which had an R_f of 0.64 remained unknown. This may have been sphingomyelin. The lipid bands were too light to permit their quantitative estimation by photodensitometry. The spot area technique was not reliable as a quantitative procedure due to the tendency of some compounds, especially the unsaturated fatty acids, to deviate markedly from a linear relationship between concentration and the logarithm of the spot area. One of the controls exhibited two additional components with R_f 0.67 and R_f 0.19 respectively. One of the patients also presented two additional spots

TABLE 10.--Serum carbohydrates (mg%).

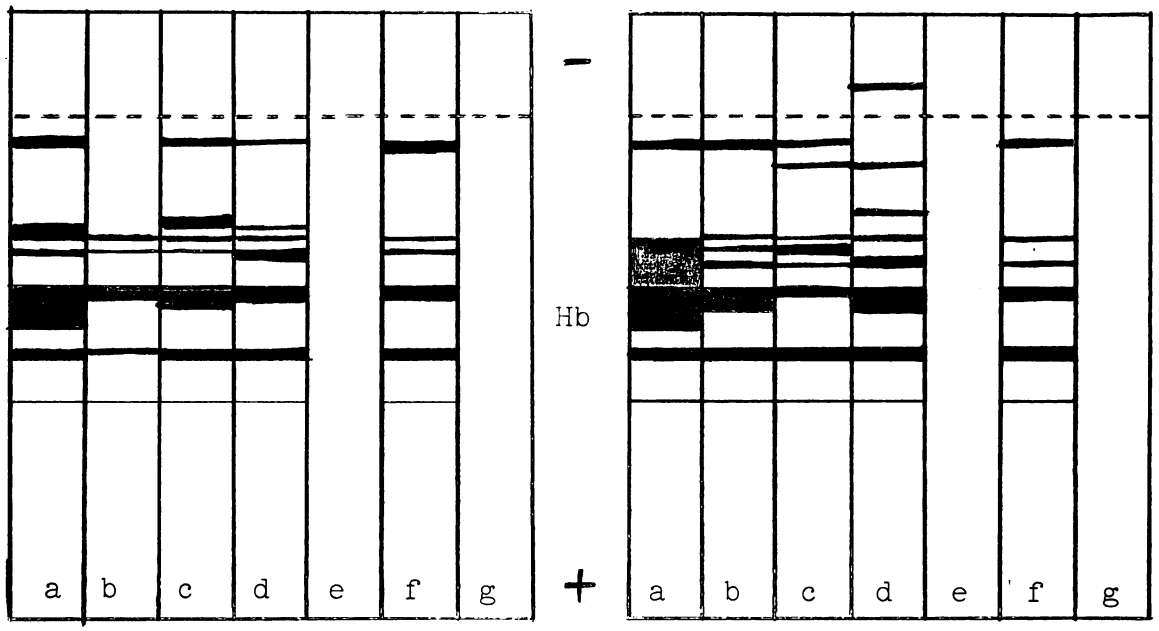
		Glucose	Fructose	Glucosamine	X
Control	1	80.0	2.0	46.6	0.6
	2	46.5	1.0	56.2	1.0
	3	48.5	7.2	85.2	4.0
BDL	1	66.5	2.5	42.6	2.4
	2	49.5	3.4	62.0	3.2
	3	48.0	4.8	91.0	2.0
	4	62.5	1.0	63.0	0.0
$\bar{X}_C \pm SE_C$		58.3±10.9	3.4±1.9	62.7±11.6	1.9±1.1
$\bar{X}_P \pm SE_P$		56.6± 4.6	2.9±0.8	64.6±10.0	1.9±0.7
t		0.467	0.255	-0.1297	-0.0356

with R_f 0.49 and R_f 0.40. The latter formed a sky-blue color after treatment with sulfuric acid and heat. The color soon changed to rose, a behavior characteristic of several steroidal hormones.

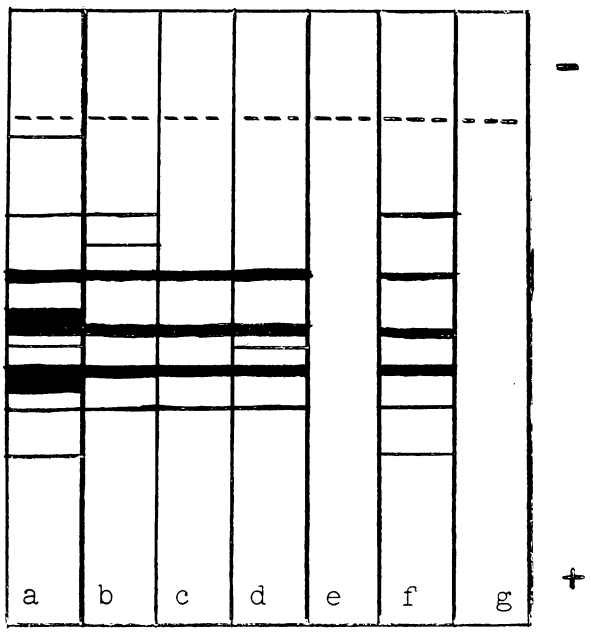
Erythrocyte Proteins

Electrophoresis of the fractionated erythrocyte proteins isolated a maximum of ten and a minimum of five bands. Two general patterns were detected in the control and patient samples (Figure 5). The third phenotype illustrated in Figure 5 is a hemoglobin heterozygote. This individual was an American Negro, possibly implicating Hemoglobin S as the identity of the variant. The data in Table 11 were obtained by summating the fraction values for each peak. The peak numbers (increasing from anode to cathode) indicate the bands in the total protein gel which is a composite of the fractions illustrated in Figure 5. There were no significant differences between patient and control means for any of the bands observed. There is a bias toward lower concentrations of hemoglobin and higher concentrations of non-hemoglobin proteins due to an inability of the integrator to account for the high quantity of hemoglobin (95% of the red cell proteins).

Immunoelectrophoresis of the serum proteins displayed precipitin lines similar to those of the controls. However, there was a quantitative difference between patient and control proteins precipitated by anti-human gamma G antiserum.



Control 1 Control 2



Control 3

a = VI

b = V

c = I + III - 1,2,3

d = IV-1

e = IV-6 + 7

f = II

g = III - 0

Hb = Hemoglobin

Figure 5.--Profiles of Erythrocyte Proteins

TABLE 11.--Erythrocyte protein concentrations.

Peak	BDL-1	BDL-2	BDL-3	BDL-4	C-1	C-2	C-3[a]	C-4	C-5	C-6	C-7	$\bar{X}_C \pm SE_C$	$\bar{X}_P \pm SE_P$	t
1	0.7	0.7	4.1	2.8	0.7	4.3	0.3			4.3	2.7	1.8±0.7	2.1±0.8	-2.017
2	14.4	4.7	23.8	16.0	9.8	4.3	0.9	8.4	6.2	19.9	9.8	8.5±0.7	14.7±3.9	P(U≤7)= .278*
3	120.0	100.2	172.5	164.0	70.1	131.3	94.4	183.6	152.9	207.5	144.7	140.6±18.0	139.2±17.4	+0.053
4	448.6	452.4	526.0	536.3	741.4	486.6	189.8	356.5	392.8	557.8	392.3	497.6±50.8	490.8±23.4	+0.096
5	210.3	264.1	186.3	154.2		98.6	198.9	156.0	206.3	207.7	215.8	177.0±22.2	203.7±23.2	- .824
6	100.4	73.5	109.7	48.2	37.5	33.7	166.9	77.7	86.9	81.8	82.1	67.5±8.4	83.0±13.9	-1.019
7	19.8	20.9		16.2	10.9		72.6	30.6				20.8±9.8	19.0±1.4	+0.235
8			5.7		6.0		7.2	6.8		4.4	2.8	5.4±1.1	5.8±0.2	-0.064
9			1.9					2.8		2.8	1.9	2.5±0.3		
10			0.6					0.1		2.1				

[a]Hemoglobin heterozygote. Peaks 1,2,3,8,9, and 10 are comparable to others. The sum of peaks 4,5, and 6 approximate peak 4 of the other phenotypes. Peak 7 appears to correspond to peak 6 of the others.

*Mann-Whitney "U" test used because of variance heterogeneity.

The low gamma G protein in the patients was verified by the Ouchterlony technique (Ouchterlony, 1964). Since this technique does not lend itself readily to quantitative procedures, the combination of polyacrylamide disc electrophoresis with the Cohn alcohol fractionation was employed to resolve the plasma proteins into a maximum number of subgroups. The disc method separated the serum proteins into an average of 26 groups which are illustrated in Figure 6. The individual at the far right died one week after the sample was taken. The dark band near the anode is albumin. The band marked "Transferrin" contains but is not totally transferrin. Gamma globulin occupies the majority of the 5% gel.

Two protein groups deviated significantly from the control sample (Table 12). The numbering system is similar to that for the erythrocyte proteins. The reference gel was a composite constructed from the individuals presented in Figure 6. Albumin is peak 1; 17 is that peak immediately trailing the transferrin group; peak 37 is the major gamma globulin peak. The significance in peak 17 is questionable. The alpha error was set at one in twenty. There are eighteen "t" tests in Table 12. The deviation in Peak 37 is significant at the 1% level. This peak contains the gamma globulins, which are selectively precipitated into Cohn Fraction II. This evidence appears to support Ptacek's discovery of low gamma globulin levels in his

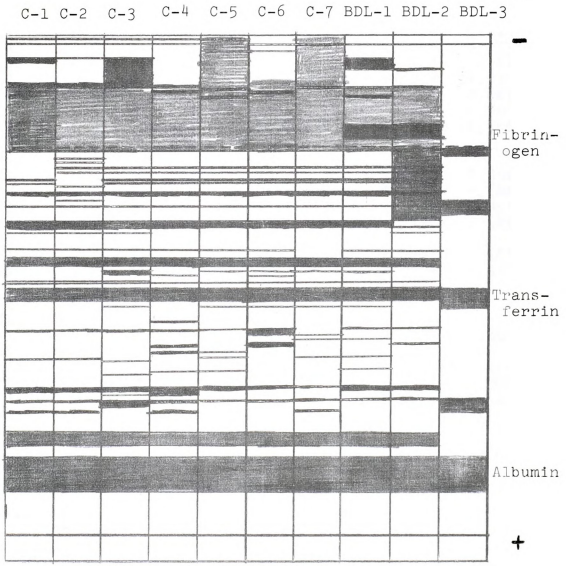


Figure 6.--Profiles of Total Serum Proteins. pH 8.3

TABLE 12.--Disc profiles of total serum proteins (g.%).

Peak	$\bar{X}_C \pm SE_C$	$\bar{X}_P \pm SE_P$	t	F
1	4.58 ± 0.26	4.40 ± 0.25	+0.415	1.380
2	0.211 ± 0.014	0.207 ± 0.040	+0.129	0.250
3	0.058 ± 0.010	0.082 ± 0.038	-0.100	0.214
4	0.056 ± 0.010	0.078 ± 0.022	-1.100	0.333
6	0.081 ± 0.010	0.075 ± 0.013	+0.353	0.158
10	0.052 ± 0.020	0.050 ± 0.007	+0.074	14.000
14	0.064 ± 0.010	0.060 ± 0.016	+0.235	0.667
16	0.390 ± 0.014	0.396 ± 0.030	-0.200	0.500
17	0.030 ± 0.006	0.056 ± 0.013	-2.600*	0.500
18	0.024 ± 0.002	[a]		
20	0.045 ± 0.014			
21	0.113 ± 0.020	0.071 ± 0.013	+1.273	5.800
22	0.060 ± 0.018	0.028 ± 0.009	+1.067	9.600
23	0.068 ± 0.014	0.034 ± 0.019	+1.545	0.857
24	0.096 ± 0.018	0.124 ± 0.040	-0.778	0.545
25	0.059 ± 0.010			
27	0.099 ± 0.018	0.094 ± 0.032	+0.135	1.100
28	0.055 ± 0.004	0.045 ± 0.025	+0.714	0.077
29	0.101 ± 0.008			
30	0.059 ± 0.022			
31	0.124 ± 0.035			
35	0.206 ± 0.048	0.233 ± 0.026	-0.346	7.810
36		0.411 ± 0.083		
37	0.276 ± 0.070	0.232 ± 0.043	+4.000**	6.074
41	0.182 ± 0.040	0.105 ± 0.022	+1.375	5.000
42	0.148 ± 0.029	0.102 ± 0.004	P(U≤2)=0.200	52.000*
44	0.056 ± 0.011	0.061 ± 0.000		
45	0.032 ± 0.009			

*These values are significant at the 5% level.

**This value is significant at the 1% level.

[a]A space in this table denotes that only one observation is available for this protein group.

patients and to contradict the results of the phosphate buffer fractionation. This problem will be dealt with below.

The electropherograms of the modified Cohn fractions are illustrated in Figures 7-13. In each figure the lower end of the gel is oriented toward the anode. With few exceptions, none of the fractions contain proteins unique to that fraction. This appears to be due to both the trailing of a given protein from one fraction to another and to the fact that a given band in the total gel (far left) may consist of several proteins of similar charge and molecular size but different solubilities. The former is demonstrated by albumin which is found in seven of the eight fractions (Figure 14). However, albumin is also a mixture of proteins of similar shape, size, and charge; and it is possible that some of these may differ from the others in solubility which will also contribute to their trailing from fraction to fraction. Examples of the demonstration of the heterogeneity of a band with respect to the solubilities of its components may be found in those bands indicated with an X in Figure 14. These proteins appear to selectively aggregate in a given fraction and to be totally absent from others.

The fact that zinc, barium, and sodium are used in the fractionation raises the possibility that these ions may combine with specific molecular groups and thereby

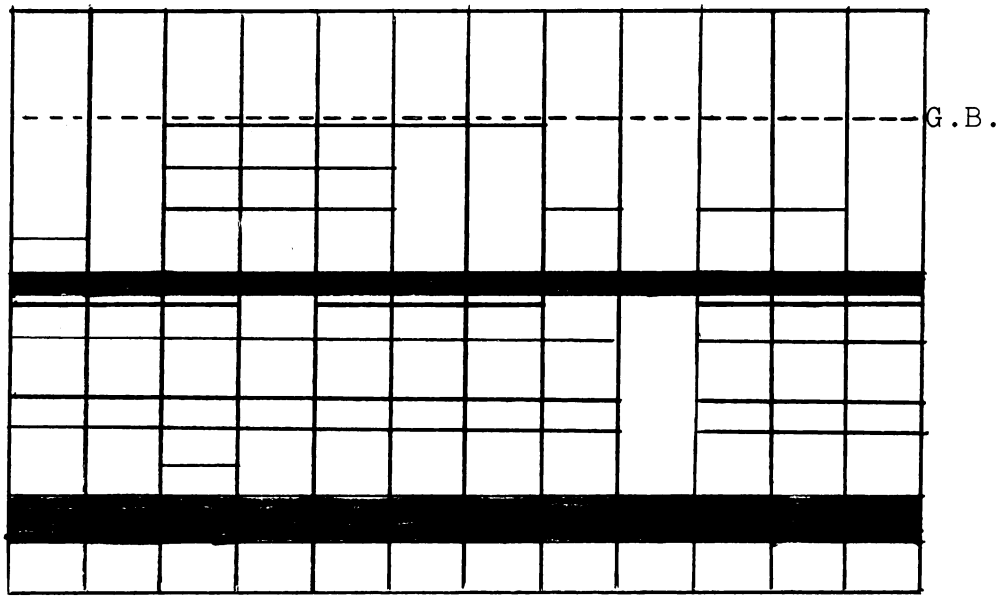


Figure 7.--Cohn Fraction V.

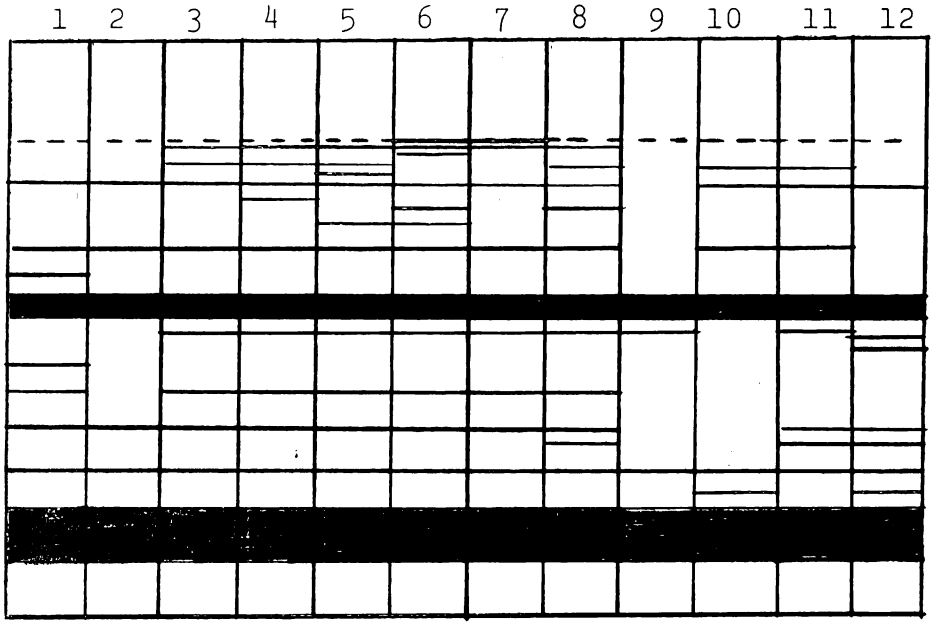


Figure 8.--Cohn Fraction IV-6+7.

1,3,4,5,6,7,8,9,10 are controls; 2,11, and 12 are patients.

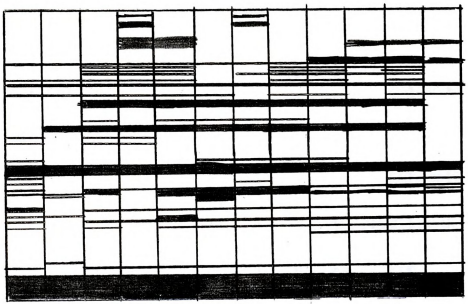


Figure 9.--Cohn Fraction IV-1.

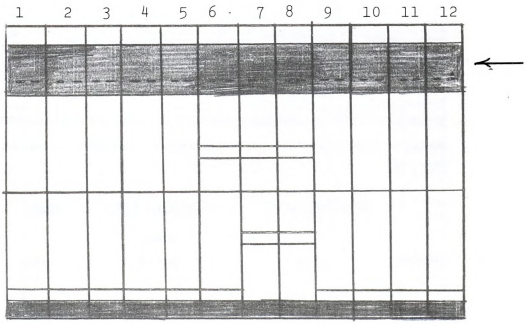


Figure 10.--Cohn Fraction II-

1,3,4,5,6,7,8,9 and 10 are controls; 2,11, and 12 are patients.

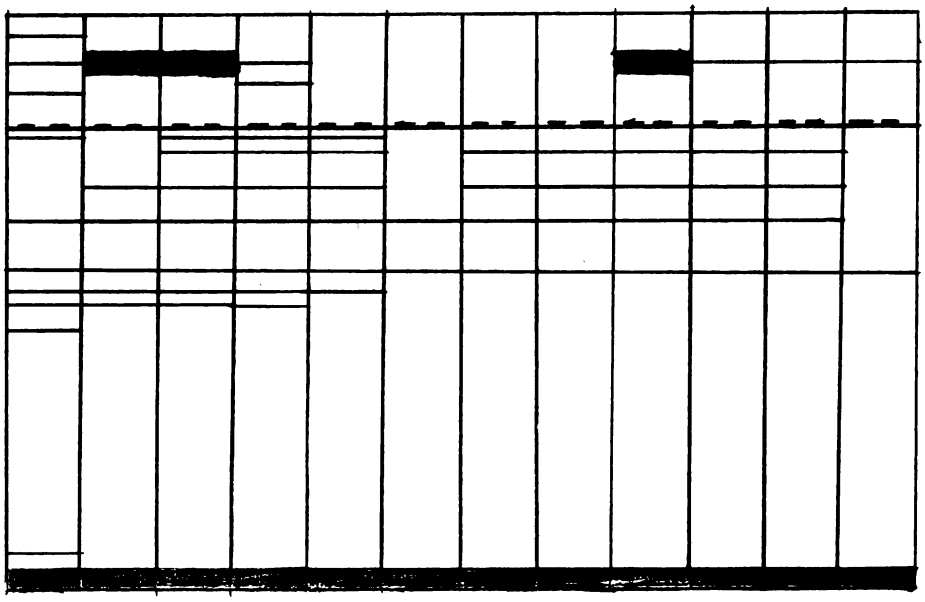


Figure 11.--Cohn Fraction III-0.

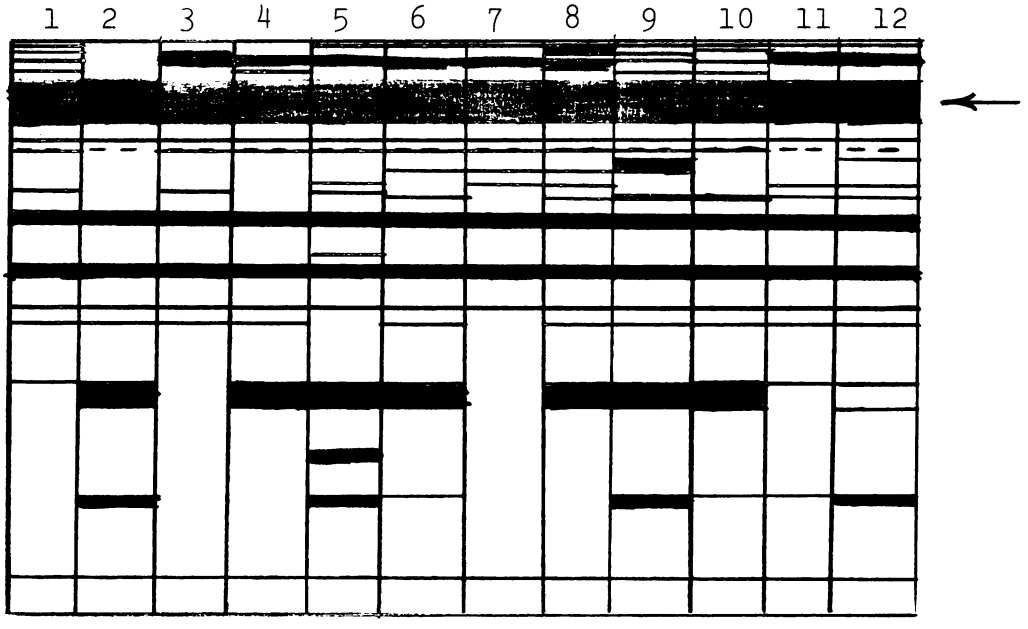


Figure 12.--Cohn Fraction III-1,2.

1,3,4,5,6,7,8,9, and 10 are controls; 2,11, and 12 are patients.

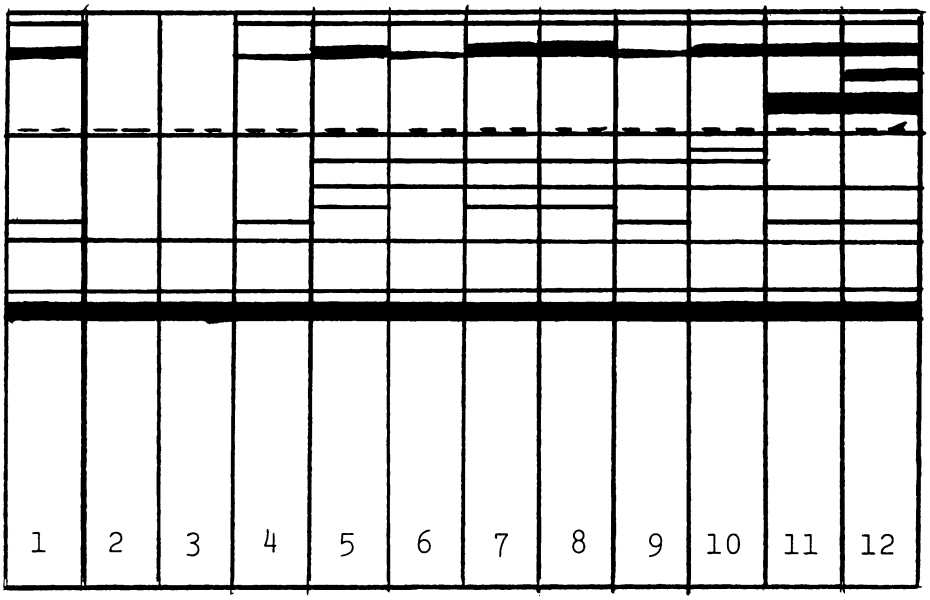
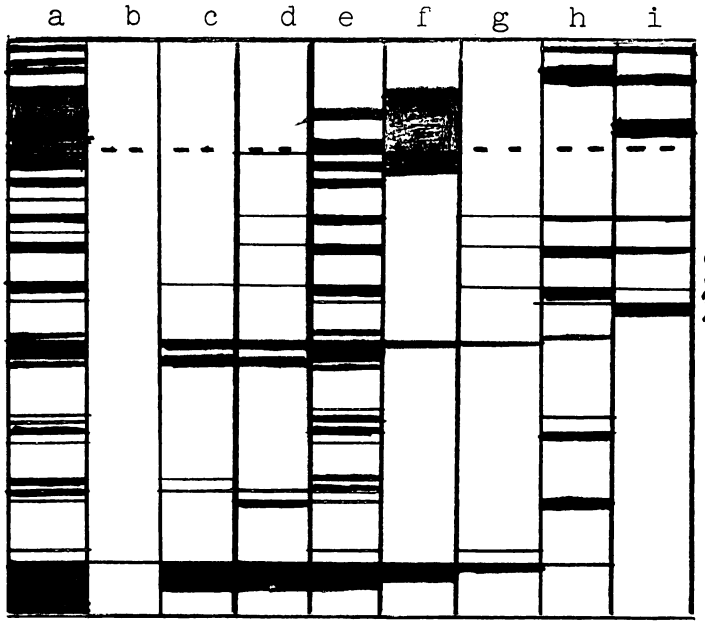


Figure 13.--Cohn Fraction I+III-3.



Fibrinogen

a = Total
b = VI
c = V
d = IV-6+7
e = IV-1
f = II
g = III-0
h = III-1,2
i = I + III - 3

Figure 14.--Profile of Brachmann-de Lange Patient T. G.

alter both the configuration and the net molecular charge of the protein. Any reversible binding of an ion with a negatively charged group (such as the carboxylate groups of glutamate and aspartate) does not contribute greatly to these alterations since the volume of the alcohol solution containing the protein is smaller than that of the surrounding buffer such that the ion may diffuse from the group to which it was bound. The negative pH of the buffer system also leads to a counteraction of the effect of these ions by removing hydrogen ions from other groups resulting in a predominantly net negative protein charge. This latter phenomenon will, in part, neutralize the irreversible bonding of the zinc and barium ions to certain protein groups. Specific coupled reactions involving enzymes and various dyes will eventually help determine the identity of the components of a given band but will not exclude the possibility that other proteins may also reside within them. The same may be said for the use of specific antisera as a tool for protein identification.

Fractions II and III-1,2 contain information that may explain the disparity between the gamma globulin levels measured by the densitometer method (total protein gels) and the phosphate fractionation procedure. Figures 10 and 12 contain the gels under consideration. The arrows indicate the zone in question. The data are presented in Table 13.

TABLE 13.--Variations in Fraction II and Fraction III-1,2.

	Control $\bar{X} \pm$ SE (g.%)	BDL $X \pm$ SE (G.%)
Peak 37 (Fraction II)	0.287 ± 0.05	0.119 ± 0.012
Peak "X" (Fraction III-1,2)	0.42 ± 0.014	0.197 ± 0.108
Peak 37	$P(U \leq 3) = 0.10$	F = 45.3*
Peak "X"	t = -2.39*	F = 0.0511

*These values are significant at the 5% level.

There appears to be a compensation for the low gamma globulin concentrations in the patients by an increase in the levels of protein in the same zone in Fraction III-1,2. Unfortunately the differences between the control and Brachmann-de Lange means are not highly significant. The problem seems to stem from variance heterogeneity between the respective samples. This was significant at the 5% level for Peak 37 and bordered on significance at that level for Peak "X" in Fraction III-1,2. (The term "X" is used to differentiate the protein in Fraction III-1,2 from Peak 37 in Fraction II although both appear to have identical mobilities.) Larger sample sizes may resolve this problem.

Clinical Observations

L. W. is a Negro girl who was born illegitimately in December of 1950. She has a low hairline, lanugo covering her forehead, dense hair along the spine in the lower lumbar

area, synophris, and long eyelashes. Her nose is small and has flaring nostrils. The lips are downturned at the ends. Her cry is typical of the syndrome. L. W. has one finger on her right hand and a thumb and two rudimentary fingers on her left hand. L's birthweight was three pounds twelve ounces. The pregnancy was full term. At the present time she is able to walk, but cannot talk.

M. B. is a ten and one-half year old Caucasian boy. He has micrognatha and micromelia of the hands and feet. M. B. has bilateral simian creases, clinodactyly of the fifth digits, and proximally placed thumbs. His testes are undescended, and the penis is small with phimosis (tight foreskin). This patient has a low hairline, lanugo on the forehead, synophris and long matted lashes. There is long lanugo along the lower spine. He does not speak, and vocalizes with a low hoarse cry. M. B.'s birthweight was five pounds eleven ounces.

F. S. was a Caucasian male born in November of 1952. His birthweight was four pounds four ounces. He was six weeks premature. His hair pattern resembled those of the preceding patients. He had undescended testicles; however, the genitalia were otherwise normal. F. S.'s spleen, liver and kidneys were not palpable, and there was little subcutaneous fat. The hands and feet were small. The right hand possessed the first, third and fifth digits and their metacarpal bones, but the other bones were absent. The

left ulna was missing, and the left hand had the first and third digits and their metacarpals. The remaining bones of the left hand were missing. There was contracture of both elbows, and cutaneous webbing extended across the right anticubital space. F. S. had a cardiac murmur. His head was microcephalic, and has a flattened occipital bone. The ears were low set, and the eyes exhibited a convergent strabismus. F. S. died from upper respiratory illness April, 1967.

A. J. is a twelve and one-half year old Caucasian male. He was a full term baby, and his birthweight was five pounds twelve ounces. His hair pattern resembles those of the previous patients. He has clinodactyly of the fifth digits, proximally placed thumbs, and bilateral simian creases. There is flexion contracture of the elbow. The bones of the wrists and ankles are fused. His ears are set at the angle of the jaw, and his nose is small and the nostrils flared. His testes are small, but the penis is normal.

T. G. is the oldest patient in this sample, nineteen and one-quarter years of age. He was a full term baby, and his birthweight was five pounds two ounces. He is a Caucasian male. He has excessive hair along the spine, and his facies are typical of the syndrome. Clinodactyly of the fifth digits and proximally placed thumbs are present. There are no simian creases. He originally had an extra

finger rudiment on each hand. These were non-osseous and their growth was prevented by tying them off with a string. Liver, spleen, and thyroid are not palpable. His left testis was undescended at fourteen years of age, but both testes are now present in the scrotum. He has displaced arches and walks on his toes. His neck is short and thick. His nose is short and the nostrils are flared. He rarely vocalizes, but, when he does so, he emits a low interrupted gutteral sound. T. G. has a systolic murmur.

K. Bu. is a two year old Caucasian girl. She presents the various components of the syndrome with the exception of the clinodactyly. K. Bu. has an identical twin sister who exhibits a slight synophris but no mental retardation. The remainder of her family is normal. There have been two infant deaths among the relatives. The causes of death and the ages of the children at death were not known.

D. S. died at three years of age from lobar pneumonia. He was thirty-one inches in length and weighed eighteen pounds three ounces. He was a premature baby, and weighed four pounds ten ounces at birth. He had a short neck, synophris, long eyelashes, and extensive hair along the midline of his back. His testicles were undescended. The left hand had one finger and a thumb, and the right hand resembled a claw with what were described as two thumbs, an index finger, and fifth digit. His hearing was questionable, and he did not talk.

Family Studies

An attempt was made to demonstrate possible environmental factors that may be involved in the causation of the syndrome. No environmental agents were found.

No evidence of consanguinity was present in any of the families studied (the pedigrees may be found in Appendix I). Each patient was unique to his kindred with one possible exception. M. B.'s paternal second cousin was severely retarded. He was kept in the home and died twelve years before this study. The information available stated that he could not talk or feed himself. He could not recognize anyone. His sound was described as an "interrupted gutteral noise." No pictures were available for comparison with the patient. Local doctors diagnosed him as having Down's Syndrome. It might be mentioned that two of the patients in the present sample were originally classified as Down's Syndrome. Of further interest in the pedigree of M. B. is the presence of clinodactyly in a maternal uncle and great-grandmother and the occurrence of synophris in one of the paternal relatives. The patient's maternal grandmother had a miscarriage at six weeks. His mother's first pregnancy also ended in miscarriage during the same period.

The paternal great-grandmother of A. J. had two nephews or nieces (records are not available to confirm sex) who were in Canadian mental hospitals. The type of mental illness was unknown. His paternal great-aunt was a patient

at Pontiac State Hospital and was said to have moderate idiopathic mental deficiency. The maternal grandmother had two cousins who were committed to mental hospitals (diagnosis unknown), and the maternal uncle, a patient at Lapeer State Home and Pontiac State Hospital was described as having a "passive aggressive personality." No pedigree for this patient was prepared due to lack of parental cooperation.

L. W. was an illegitimate child whose father was unavailable. Her maternal uncle had bilateral club feet; however, the other maternal relatives are normal. No pedigree for this patient is available due to inability to contact the mother.

The parents of F. S. could not be located. Medical records stated that his two older siblings and relatives were normal. The father had a heart murmur.

The paternal grandmother of T. G. was a manic depressive psychotic. T. G.'s paternal aunt's (II-16) fourth pregnancy ended in a miscarriage at four months. The patient's maternal aunt's (II-6) second and third pregnancies miscarried in the second month of prenatal development.

A cousin of D. S.'s paternal grandfather was hydrocephalic. Three maternal relatives were retarded; however, no information on the type of retardation was available.

The clinical picture of these patients closely matches that described in the literature. All had synophris,

clinodactyly of the little finger, heart murmurs, cutaneous syndactyly of the second and third toes, long eyelashes, long lumbar hair, generalized hirsutism, and ankle abnormalities. Immature genitalia were common, and three patients exhibited claw hands. T. G. had non-osseous sixth digits whose further growth was prevented by tying them off with a string. Their thumbs were proximally placed and osseous maturation was retarded.

The developmental histories of the patients followed similar patterns. The pregnancies were uneventful with the exception of F. S. The history of his pregnancy mentions frequent vomiting and uterine bleeding during the pregnancy, neither of which occurred during the pregnancies of his siblings. The average birth weight of the patients was five pounds, although only two were premature. The weights ranged from three pounds twelve ounces to five pounds twelve ounces. The early postnatal histories list episodes of nausea, cyanosis, and a general inability to feed or suck. Upper respiratory failure and pneumonia were frequently contacted during this period.

A. J. continues to have cyanotic seizures during which he collapses, appears lifeless, becomes blue, blanches and eventually falls asleep. M. B. was hospitalized at six months for a period of forty-five days for a heart condition and respiratory failure. The developmental history of T. G. is unusual in that the patient, although suffering

periodically from respiratory disease and vomiting episodes, progressed to the point where he could say three or four words. He showed a subsequent regression of development, however, such that he does not presently talk. T. G. has also developed peptic ulcers.

D. S. was a premature baby. His postnatal history was complicated by gastro-intestinal abnormalities and several corrective surgical operations. At birth the colon was malrotated and, along with the appendix was displaced to the left side of the abdomen. An annular pancreas and intestinal obstruction were also reported at this time. The patient succumbed at the age of 3 1/2 years from lobar pneumonia. Autopsy findings listed generalized gross immaturity of the internal organs, a solid, noncrepitant lung, reduced panniculus and pectoral muscles, undescended testes, calcification of the vertebrae and "old degenerative changes" in the skeletal muscle fibers. The patient had hypoproteinemia with half of the normal amount of albumin. Alpha-2 globulin was elevated. Alpha-1, beta, and gamma globulins were normal.

Chromosomal studies on M. B., L. W., and F. S. did not reveal any abnormalities. D. S. possessed an "extra long" arm on the second autosomes.

Other researchers (see introduction) have mentioned gross osseous abnormalities of the hands and arms and simian creases in their patients. Three of the six patients

in this study had severe hand and arm manifestations, and two of the six had bilateral simian creases. All but one of the patients had dermal ridges on their hands and feet. This contradicts Ptacek's report (1963). All had low set ears, severe growth and mental retardation, prominent mandibular symphyses, anteversion of the nostrils, and contracture of the elbows. Systolic murmurs were present in two of the patients. The average age of the Brachmann-de Lange patients in this sample was 14.7 years (range 10.5-19.2). The ages, weights, and heights are given in Table 14.

TABLE 14.--Age, weight, and height of BDL patients.

	Age (years)	Weight (pounds)	Height (inches)
M. B.	10.5	21	38
A. J.	12.5	37	44
F. S.*	14.5	30	40
L. W.	16.5	40	35
T. G.	19.2	79	56.8

*Deceased 4-67.

Biochemical Studies

The results of the biochemical tests performed on the parents and siblings are presented in Table 15, and the comparison of the control, patient, and family means

TABLE 15.--Glutamic acid, alpha-keto glutaric acid, SGOT, NAD-GD, and protein levels in the parents and siblings.

	GA (mg%)	AKG (mg%)	SGOT (Units)	NAD-GD (Units/min./ mg.)	GG (g%)	Protein "X" (g%)
R. G.*	2.8	0.76	37.5		0.292	0.180
P. G.	4.9	0.29	47.0		0.228	0.124
M. G.**	4.7	0.59	48.0		0.204	0.236
M. G.	1.0	0.92	12.5		0.332	0.096
J. G.	1.4	0.87	43.0		0.236	0.552
G. B.*	4.5	1.05	18.5		0.172	0.208
B. B.**	4.0	0.65	37.0		0.376	0.184
D. B.	2.8	0.49	49.3		0.432	0.232
B. B.	1.2	0.55	37.8		0.372	0.280
Mr. Bu.*	2.8	0.92	12.5	7.4	0.498	0.098
Mrs. Bu.	2.6	0.91	9.0	9.2	0.624	0.145
L. Bu.[a]	3.9	2.41	13.3	0.0	0.451	0.048
K. S.*	2.4	0.35	37.5	11.0	0.328	0.051
L. S.**	3.3	0.51	46.0	3.3		
R. We.*	2.9	0.41	22.1	18.6		
C. We.**	2.5	0.53	16.5	15.0		
G. We.	2.8	0.71	9.0	23.7		
L. We.	1.4	0.68	32.1	44.6		
$\bar{X}_F \pm SE_F$	2.9±0.3	0.76±0.10	29.4±3.5	14.8±4.5	0.350±0.036	0.187±0.036

*Father **Mother

[a]Identical twin sister of K. Bu., a patient.

are listed in Table 17. Table 16 contains additional data for glutamic acid, alpha-keto glutaric acid, and serum glutamic oxalacetic transaminase (SGOT). The parental controls were selected from parents, twenty to forty years of age, of patients being committed to Lapeer State Home. The parents had no known biochemical disorders. The Scheffé analysis demonstrated significant differences between patient and control, patient and family, and family and control means for glutamic acid. A similar pattern was observed for alpha-keto glutaric acid. SGOT levels differed significantly between patient and control, patient and family, but not family and control means. The patients appear to have low levels of gamma globulin; however, the differences between their levels and those of either the controls or their relatives were not statistically significant. Both patient and family means for Protein "X" were significantly higher than that of the controls, but they did not differ from each other. Although the family mean for leukocyte NAD-linked glutamic dehydrogenase appears to be lower than that of the controls, the difference is not statistically significant.

L. B., the twin sister of K. B. lacked the NAD-glutamic dehydrogenase and had high levels of glutamic acid and alpha-keto glutarate, but her SGOT activity was normal.

TABLE 16.--Serum glutamate, serum alpha-keto glutarate, and SGOT.

	GA (mg%)	AKG (mg%)	SGOT (Units)
A. Patient Controls			
1	0.40	0.89	
2	1.05	0.40	
3	1.85	0.57	
4	0.70	0.96	
5	1.00	0.23	Refer to
6	1.00	0.10	Table 7
7	0.95	0.38	
8	1.00	0.60	
9	1.80	0.23	
10	1.85	0.22	
11	1.80	0.34	
12	1.00	0.27	
$\bar{X}_A \pm SE_A$	1.20 ± 0.14	0.43 ± 0.08	25.4 ± 3.5
B. Parental Controls			
1	1.30	0.85	31.8
2	1.70	0.68	17.5
3	1.95	0.48	23.1
4	1.45	0.66	16.5
5	1.02	0.40	25.0
6	1.09	0.57	27.5
7	0.90	0.79	40.0
8	0.45	0.51	27.0
9	0.67	0.71	40.0
10	1.12	1.07	25.3
$\bar{X}_B \pm SE_B$	1.16 ± 0.14	0.67 ± 0.06	27.4 ± 2.5
t	0.196	-7.717**	-0.424

**Significant at the 0.01 level.

TABLE 17.--Comparison of control, patient, and family means.[1]

	GA (mg%)	AKG (mg%)	SGOT (Units)	GG[4] (g%)	Protein "X" (g%)	NAD-GD (Units)
$\bar{X}_A \pm SE_A$	1.20 ± 0.14	0.43 ± 0.08	25.4 ± 3.5	0.287 ± 0.050	0.042 ± 0.014	32.8 ± 5.7
$\bar{X}_B \pm SE_B$	1.16 ± 0.14	0.67 ± 0.06	27.4 ± 2.5			
$\bar{X}_P \pm SE_P$	5.69 ± 0.65	2.28 ± 0.18	78.2 ± 8.7	0.119 ± 0.012	0.197 ± 0.108	0.04 ± 0.04
$\bar{X}_F \pm SE_F$	2.88 ± 0.28	0.76 ± 0.10	29.4 ± 3.5	0.350 ± 0.036	0.187 ± 0.036	14.8 ± 4.5
F[2]	34.3983[c]	35.2799[c]	23.7310[c]			
H[3]				7.108[a]	9.563[c]	13.863[c]
Scheffe						
$\bar{X}_P - \bar{X}_A$	c	c	c		c	c
$\bar{X}_P - \bar{X}_F$	c	c	c			
$\bar{X}_F - \bar{X}_{A+B}$	c	c			c	

[a]Significant at the 0.05 level. [b]Significant at the 0.02 level. [c]Significant at the 0.01 level.

[1]$\bar{X}_A$ = mean of patient controls; $\bar{X}_B$ = mean of parental controls; $\bar{X}_P$ = mean of patients; $\bar{X}_F$ = mean of families of patients (siblings and parents); SE = standard error; $\bar{X}_{A+B}$ is the weighted mean of A and B.

[2]F = One-way analysis of variance.

[3]H = Kruskal-Wallace non-parametric one-way analysis of variance. [4]Gamma Globulin.

DISCUSSION

No familial evidence was obtained in support of a hereditary cause of this syndrome. The patients with one doubtful exception were unique in their respective families. No consanguinity was present in any of the families, and there was only scant evidence for the appearance of parts of the syndrome in other family members. There were eight miscarriages in 106 pregnancies in the families included in this study or a frequency of 0.075. Previous reports list nine miscarriages in 186 pregnancies or a frequency of 0.048. Both frequencies are very low. No environmental cause was detected in this sample; however, this does not exclude the possibility that one may exist.

Opitz discounts two published cases of consanguinity (Optiz et al., 1965). In the first, the maternal grandparents were related, but not those of the patient. In the second report the parents came from a highly inbred population in Italy. Seven sibships have been reported which have more than one affected sibling. Two sets of monozygous twins, each twin exhibiting the snydrome, have also been reported. Three pairs of first cousin sibships are known which contain at least one affected child in

each sibship (Opitz et al., 1965). The Opitz paper also includes numerous cases in which there are appearances of portions of the syndrome present in other family members. These facts tend to indicate that a hereditary factor is involved in the syndrome.

It was previously mentioned that L. Bu. and K. Bu. were identical twins. They were concordant for nineteen out of nineteen blood antigens tested. The appearance of the syndrome in one identical twin and not in the other creates some difficulty for the hypothesis that the syndrome has a genetic basis. The problem may be one of penetrance, the lack of expression of the syndrome by an individual possessing the genes determining the conditions which lead to the syndrome. This problem will be discussed below.

The high incidence of the Brachmann-de Lange (BDL) Syndrome in first cousins with no consanguinity present in the pedigree does not support recessive inheritance unless the recessive gene is relatively common in the population. The probability that two individuals heterozygous for the recessive allele will mary two unrelated persons carrying the same allele is very small (if it is assumed that the BDL Syndrome is rare). The occurrence of the BDL Syndrome in many races is also not characteristic of the majority of rare recessives. It is possible that the condition is due to an autosomal dominant with low penetrance.

The biochemical data seem to implicate the NAD-linked glutamic dehydrogenase (NAD-GD) deficiency as a possible factor leading to the syndrome. This enzyme removes the alpha amino group from glutamic acid forming alpha-keto glutaric acid and ammonia. There is a similar enzyme, NADP-linked glutamic dehydrogenase (NADP-GD) which also catalyzes this type of reaction but uses nicotinamide adenine dinucleotide phosphate as the hydrogen acceptor. Both enzymes are widely distributed in tissues. A deficiency of NAD-GD could lead to an accumulation of glutamic acid. This hypothesis would require that NAD-GD is the principle means for degrading glutamic acid and that NADP-GD does not compensate for the lack of NAD-GD activity. The data in Table 8 actually show slightly lower levels of NADP-GD in the patients.

A defect in NAD-GD would explain the glutamic acid elevation. However, such a defect might lead to a decreased level of alpha-keto glutaric acid. In fact, a higher level of this keto acid was observed in the patients. Glutamic oxalacetic transaminase could remove some of the excess glutamic acid by converting it to alpha-keto glutaric acid, resulting in the observed elevation of the keto acid. This enzyme catalyzes the following reaction.

glutamate + oxalacetate $\rightleftharpoons$ alpha-keto glutarate + aspartate

No elevation in aspartic acid was observed in the serum. There is no obvious reason why glutamic oxalacetic

transaminase and not glutamic pyruvic transaminase would be favored as a method for disposing of the excess glutamate. There is a precedent for this type of behavior. During myocardial infarcation serum glutamic oxalacetic transaminase (SGOT) ranges from 100 to 200 units per milliliter while serum glutamic pyruvic transaminase (SGPT) remains within normal limits. The reverse is true for liver damage where SGPT rises more rapidly and attains a higher level than SGOT (Oser, 1965). These trends are not understood. The level of any constituent in the blood is subject to the relative rates of its production and degradation in the cells, the rate at which it is introduced to the blood stream by those cells, and its rate of removal by the kidney and other tissues. Liver biopsy material may help clarify the transaminase observations.

The following hypothesis may be suggested as a possible cause of the Brachmann-de Lange Syndrome. The primary biochemical lesion occurs at the level of NAD-GD. Glutamic acid accumulates in the tissues, including the brain and kidney, leading to the observed mental retardation and hypoaminoaciduria (Appendix II). SGOT, which is elevated in the patients, removes some of the glutamic acid by converting it to alpha-keto glutaric acid which is also elevated. Why this keto acid is not removed via the citric acid cycle is not known. Attempts to measure the activity of leukocyte alpha-keto glutarate dehydrogenase were not

successful. It is unlikely that an enzyme defect in this pathway could be tolerated by man, although some mutant microorganism with a defect in this enzyme have been raised on highly supplemented media.

Glutamic acid is intimately involved in brain metabolism (Tsukada et al., 1966). An elevation in this amino acid could result in mental retardation by creating a biochemical imbalance in the interconversion of glutamine, glutaric acid, and gamma-aminobutyric acid (GABA). Glutamine is an important storage form of cellular ammonia. Glutamic acid is converted to GABA by glutamic decarboxylase in an irreversible reaction. Glutamic acid has been found to be an excitatory compound while GABA is a depressant. It is possible that GABA may actually be responsible for the observed retardation.

The renal involvement first cited by Ptacek et al. (1963) may also be due to high intracellular levels of glutamic acid. Ptacek et al. have suggested that the hyperglutamicacidemia is due to faulty tubular excretion. Recent studies have shown that there are two systems involved in amino acid transport (Rosenburg et al., 1967; Scriver et al., 1967). One is specific for a given amino acid while the other carries a family of closely related amino acids. Because of this duality, it is unlikely that one amino acid could arrest the transport of all the others by this route. Glutamic acid could interact with the excretion of the other

amino acids by collaborating in some way with glutamine. Glutamine has been shown to be important in the excretion of ammonia by the kidney tubules. During ammonium chloride induced acidosis, the ammonia is combined with glutamic acid at the interface of the blood and the membrane of the cells of the renal tubules. At the cell-lumen interface the reverse reaction occurs such that glutamic acid is released, and the ammonia is liberated into the lumen where it subsequently combines with a hydrogen ion and a chloride ion to form ammonium chloride. Two glutaminase enzymes have been found to be involved in this reaction (Meister, 1956). An allele may result in a lack or reduced efficiency of one of these enzymes which would result in an accumulation of glutamic acid. One objection to this hypothesis is the fact that certain aspects of the syndrome appear long before the kidneys begin to function in the fetus.

The patients also appear to have lower levels of gamma globulin than the controls. The number of patients used in this assay was too small to attain statistical significance. However, the deficiency was demonstrable by both the Ouchterlony double diffusion technique and by scanning stained acrylamide gels. Others have also mentioned a hypogammaglobulinemia in their patients (Ptacek et al., 1963). The elevation in Peak 37 of Fraction III-1,2 does not appear to be connected to the syndrome. Inspection

of Table 15 reveals a concentration of high levels of this family of proteins in two families. The others have protein concentrations which approach those of the controls.

The number of bands obtained by the Cohn-acrylamide procedure should be considered with caution. Brewer (1967) has demonstrated artifact production by ammonium persulfate. The persulfate produced radicals which interacted with tryptophan and tyrosine residues which were made accessible by the action or urea on disulfide bonds in other regions of the protein molecules. Since no urea was used in this research, only free sulfhydral groups need to be considered. Ott et al. (1963) have reported polymer formation in "pure" proteins by passing them through acrylamide. Upon inspection, their diagrams failed to show any extraneous bands other than the series of haptoglobin complexes which have been reported elsewhere. No criteria of purity for their protein solutions were mentioned by the authors.

All of the parents of the patients had high serum levels of glutamic acid. Four out of eight of the patient's siblings also exhibited hyperglutamicacidemia. Alpha-keto glutarate and serum glutamic oxalacetic transaminase (SGOT) did not show any noticeable pattern. Leukocyte NAD-linked glutamic dehydrogenase (NAD-GD) appeared to be low in all of the parents and absent in one of the three siblings tested. The distributions of the glutamic acid and NAD-GD levels may be explained by assigning a single allele for

normal enzyme activity and an allele leading to production of defective enzyme or absence of enzyme to each of the parents and to those siblings with hyperglutamicacidemia and low enzyme activity. Siblings with normal levels of glutamic acid and normal enzyme activity do not appear to have the defective allele. The patients and L. Bu. appear to have a double dose of the allele which results in absence of enzyme activity.

L. Bu., the identical twin sister of K. Bu., had no NAD-GD activity. Her SGOT activity was normal, and her serum glutamic acid level was lower than that of her affected sister. L. Bu.'s serum alpha-keto glutaric acid concentration was approximately twice that of K. Bu., and higher than those of any of the other patients. It is possible that L. Bu. has relieved the glutamic acid stress, perhaps by means of the SGOT route.

The biochemical evidence supports a hypothesis which assigns a double dose of a gene to the patients which dictates the synthesis of a defective enzyme or which fails to dictate the synthesis of any enzyme. Their parents and some of their siblings appear to carry a single dose of this allele. Family studies based upon presence or absence of the BDL Syndrome or the appearance of its various components tend to discredit a recessive mode of inheritance and to favor an autosomal dominant with weak penetrance. Resolution of this apparent contradiction will require more comprehensive studies.

The primary effect of the gene, if one assumes that a gene is involved, is the disruption of the normal sequence of developmental events. The initial time of action appears to occur during the second fetal month. Initiation of bone development in the arms and hands occurs at this time. Ossification of the phalanges proceeds from the second to the fourth fetal month (Patten, 1946). The primordial hair follicles appear in the eyebrows, eyelids, lips, chin, and scalp during the third prenatal month (Patten, 1946). The follicles cover the body during the fourth prenatal month, and lanugo is noticeable the following month (Allan, 1960). The bone maturation is retarded while hair growth is enhanced in the BDL Syndrome. Little is known of the biochemical processes or control mechanisms that are involved in these processes. Investigators have recently shown that the placenta will concentrate amino acids on the fetal side of the membranes several fold over that on the maternal side (Kerr et al., 1966). This would enhance the disruption of normal developmental processes if glutamic acid were, in fact, involved.

SUMMARY

Biochemical analyses revealed a hyperglutamicacidemia, generalized hypoaminoaciduria, and an apparent hypogammaglobulinemia in patients with the Brachmann-de Lange Syndrome. Serum alpha-keto glutaric acid and serum glutamic oxalacetic tranaminase (SGOT) were elevated. No leukocyte NAD-linked glutamic dehydrogenase (NAD-GD) activity could be demonstrated in these patients. SGOT, gammaglobulin, and alpha-ketoglutarate were normal in the parents and siblings of the patients. An identical twin sister of one of the patients who appeared to be free of the syndrome had lower levels of glutamic acid and SGOT, and a higher level of alpha-ketoglutarate. She had no NAD-GD activity. Both parents of each of the patients had elevated serum glutamic acid levels. Family pedigrees failed to yield information of genetic importance.

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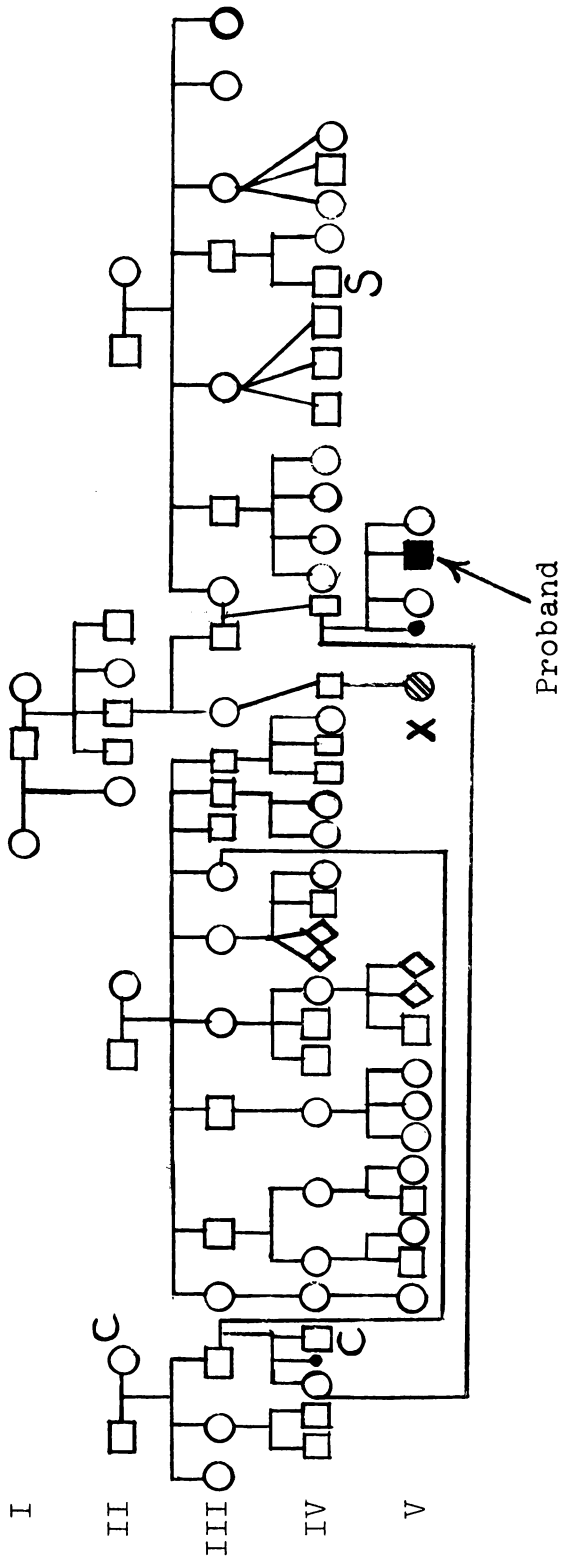
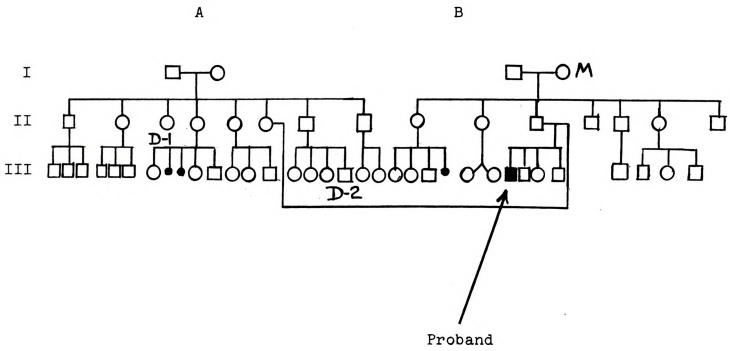
APPENDICES

APPENDIX I

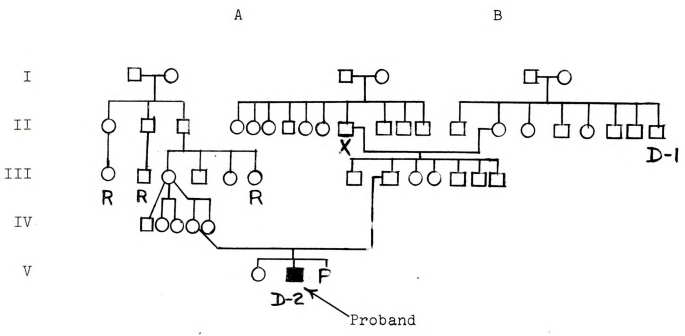
Pedigree of M. B.





Key

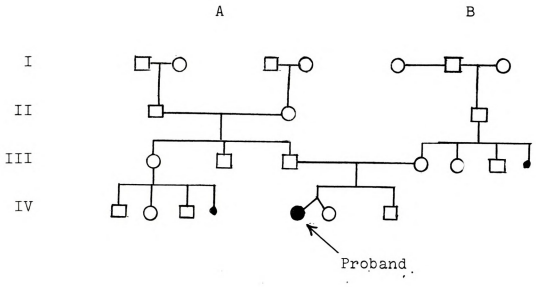
	Identical twins, zygosity determined on the basis of physical appearances only.
M	Manic depressive psychosis.
D-1	Deceased, pneumonia at 6 months.
D-2	Deceased, umbilical cord strangulation at birth.



Key

R	Retarded, type unknown.
X	His cousin died at the age of 5, hydrocephaly.
D-1	Died of pneumonia at age 5.
D-2	Deceased, lobar pneumonia
P	Pregnant

Pedigree of L. Bu.



Key

Miscarriage or infant death, type unknown.
Monozygous twins, concordant for 19 out of 19 blood antigens tested.

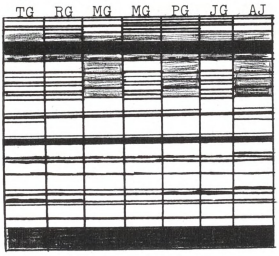


Figure 15.--Total Protein Profiles of BDL Family.

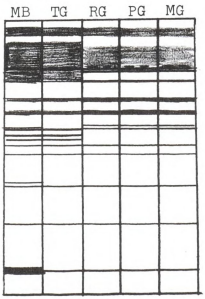


Figure 16.--Fraction III-1,2.

APPENDIX II

The pH 8.3 electrophoretic system revealed one band that had a mobility intermediate between those of albumin and transferrin. The pH 4.5 system displayed three bands. There were no differences between patients and controls. The leukocytes did not proliferate sufficiently to yield adequate protein for these analyses.

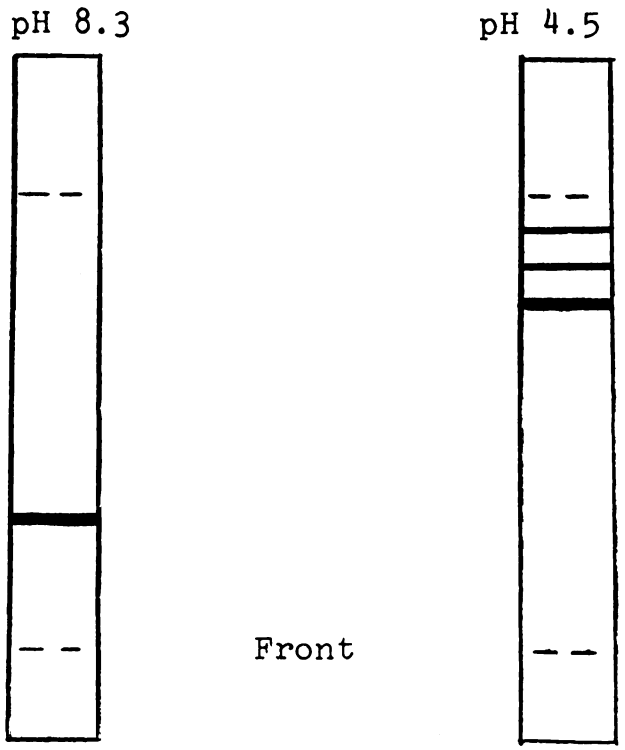


Figure 17.--Electrophoresis of Leukocyte Extracts.

Two patients who presented several traits of the Brachmann-De Lange Syndrome were tested for enzyme

activity, amino and keto acid levels, and protein concentrations.

Patient M who showed a greater resemblance to the BDL patients that patient G did not have an elevated SGOT activity but did tend to show a tendency to follow the remaining BDL pattern. It is further evident from this data that Peak 37 in Fraction III-1,2 is not connected to the syndrome.

TABLE II-1.--Enzyme, protein, amino and keto acid levels in two fringe cases.

	SGOT (Units)	GA (mg%)	AKG (mg%)	GG (g%)	P."X" (g%)
M	25.0	3.1	1.68	0.100	0.620
G	19.0	0.8	0.85	0.140	0.332
A. J.*	79.2		2.02	0.168	0.352

*Patient retest

The urinary amino acids were quantitated by thin layer chromatography and elution for A. J. Earlier paper chromatograms which were not quantitated exhibited a visible decrease in intensity of the spots of the patients in comparison to those of the controls.

TABLE II-2.--Urinalysis of creatinine and total amino acids.

	mgAA/24 hr.	mg. Creatine/24 hr.	mg. AA/mg. Creatinine
A. J.	99.7	190.0	0.52

This value borders the lower range quoted by Carver (0.46-0.95) for patients three to ten years old.[1] Since the thin layer technique gives values which are slightly higher than those obtained by column chromatography, this value is probably a high estimate.

[1]Carver, M., and Paska, R., Ion-exchange chromatography of urinary amino acids. I. Normal Children, Clin. Chim. Acta, 6:721, 1961.