GENETIC STUDIES OF ACID α-GLUCOSIDASE IN MAN

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1. Introduction

1.1. Physical characteristics of acid alpha-glucosidase

Mammalian lysosomal alpha-glucosidases hydrolyze both 1→4- and 1→6- glucosidic bonds found in their natural substrates glycogen, maltose and isomaltose. In addition, the enzymes are also capable of hydrolyzing the artificially derived substrates 4-methylumbelliferyl-alpha-D-glucoside (MUG) and 6-bromo 2-naphthyl-alpha-D-glucopyranoside (Swallow et al., 1975).

At least two genetically distinct forms of human alpha-glucosidase (alpha-D-glucoside glucohydrolase E.C.3.2.1.20) activity have been recognized. Acid alpha-glucosidase, GAA, has a pH optimum in the acid range,and is coded for by a locus assigned to chromosome 17q21→qter (Solomon et al., 1979; D'Ancona et al., 1979; Weil et al., 1979), whereas neutral alpha-glucosidases have a neutral pH optimum and the gene locus for neutral alpha-glucosidase C has been assigned to chromosome 15 (Martiniuk et al., 1980).

Human liver GAA is a glycoprotein (Belen'ky et al., 1979) with a molecular weight of 97,000-103,000 (Auricchio et al., 1967; Swallow et al., 1975; Belen'ky et al., 1979). Data on the subunit structure of GAA are conflicting. The GAA of bovine liver appears to have four subunits of similar molecular weight (25,000) held together by noncovalent bonds (Bruni et al., 1970). However, Belen'ky and

Rosenfeld (1975) suggested that GAA from human liver consists of at least three structurally distinct subunits with different N terminal amino acids and different molecular weights as estimated by sodium dodecyl sulfate (SDS) electrophoresis.

Swallow et al.,(1975) were able to demonstrate that the GAA locus is polymorphic in man, using affinity electrophoresis, where the electrophoretic separation of isozyme components depends on differences in affinity of the components for the support medium rather than molecular size or charge. After starch gel electrophoresis three phenotypes, GAA1, GAA2 and GAA1-2 were observed and attributed to two alleles GAA*1 and GAA*2 with frequencies of 0.97 and 0.03 respectively in Europeans. A third rare allele, GAA*3, has been reported to occur in Malaysians of Indian ancestry (Teng and Tan, 1979).

Column isoelectric focusing of human placental samples revealed that the isoelectric point, pI, of the GAA1 isozyme, pH 4.52, was slightly more acidic than that of the GAA2 isozyme, pH 4.58, (Swallow et al., 1975). Belen'kii et al., (1975) determined the pI of GAA from adult human liver to be between 4.58 and 4.60 while Fisher et al., (1977) using human kidney and fetal liver found the pI to be 4.61 (4.45-4.78 range). Enzyme phenotypes were not determined in these studies.

1.2. Polymorphism

A genetic polymorphism exists when the individual members of a population can be classified into two or more distinct groups each of which occurs more frequently than can be explained by recurrent mutation. In practice, loci have been considered to be polymorphic when the frequency of the second most common allele is 0.01 or greater, implying that 2% of the population will be heterozygous at the locus. Harris, (1977) has revised the definition of polymorphism to describe those situations in which a number of alleles add up to a frequency of 0.01, yet the frequency of the most common allele is not greater than 0.99. Specific activity stains together with gel electrophoresis, which utilizes migration of proteins in an electric field to detect small differences in charge and shape, has been used extensively to study genetic polymorphisms. At least 25% of the loci which determine enzyme structure are polymorphic and the average level of heterozygosity per gene locus detectable by conventional unidimensional electrophoresis has been estimated to be in the range of 7-10% in man and other vertebrates (Harris and Hopkinson, 1972). It is likely these data are an underestimate of the true incidence of polymorphism and the average heterozygosity per locus, as electrophoresis can only detect certain types of molecular differences. Since the genetic code is degenerate, some single base change mutations will alter a codon specifying a certain amino acid to another codon specifying the same

amino acid. A mutation of this kind does not produce a structural change in the protein and hence will not be detected electrophoretically.

Theoretically only about one third of the possible protein variants due to single amino acid substitutions are expected to show a change in charge which would be detectable by a change in electrophoretic mobility (Harris, 1977). Electrophoresis will not detect protein variants produced by two different amino acid substitutions in a polypeptide that result in the same net change in electrophoretic mobility. At some loci, variants which actually occur may not have been detected because of the inadequacy of the technique, such as in the case of placental alkaline phosphatase where two buffer systems at different pH's distinguish six phenotypes (Robson and Harris, 1965) while the previous system at one pH distinguished only three phenotypes (Boyer, 1961). Alleles which result in a complete loss or marked reduction in enzymic activity may also go undetected with electrophoretic techniques.

Some common allelic variants that are not detectable by standard electrophoretic techniques can be differentiated by isoelectric focusing (IEF). IEF separates proteins according to their pI , the pH at which they have a net charge of zero, while electrophoretic separations depend on comparing the relative mobility of proteins at a particular pH, usually somewhat removed from their pI's. In the case of phosphoglucomutase-1 (PGM1) after starch gel electrophoresis three

common phentoypes attributed to two common alleles are observed (Spencer et al., 1964) whereas after IEF ten phenotypes attributed to four common alleles are observed (Bark et al., 1976). Also, additional phenotypes of transferrin, (TF) (Kuhnl & Spielman, 1978) and group-specific protein (GC) (Constans et al., 1978) that are not detectable after electrophoresis have been identified after IEF.

1.3. Gene mapping

Several different approaches have been used for mapping human genes. The most widely used method involves interspecific somatic cell hybrids, but this method is not generally satisfactory for mapping several classes of genetic markers such as blood group antigens, gene products of highly differentiated cell types and, more importantly, diseases of unknown molecular defect that can only be diagnosed by clinical criteria. Therefore, other approaches such as in situ hybridization, gene dosage effects and exclusion mapping in persons with aneuploidy, as well as classical linkage analysis are important. Ferguson-Smith et al.,(1973) were the first to use deletion mapping for assigning a gene locus to a specific chromosome. In this case a person with an unbalanced autosomal translocation involving a deletion of a segment of chromosome 2 had apparently only one allele at the acid phosphatase (ACP1) locus indicating this locus was within the deleted

segment. Conversely, in many cases of autosomal deletions heterozygosity at a number of genetic marker loci can be demonstrated thereby excluding the location of the structural gene from the deleted segment (Ferguson-Smith, 1975).

Linkage is the occurence of two gene loci sufficiently close together on a chromosome such that their alleles do not assort independently. The distance between two loci on a chromosome can be measured indirectly by the recombination that occurs between them: the further apart loci are, the more likely it is that recombination will occur, with random assortment giving 50 percent recombination. However, loci can be sufficiently far apart on the same chromosome that they do not show linkage. Robson et al.,(1973) and Cook et al., (1974) demonstrated linkage between Rh and PGM1, but not between PGM1 and Fy or between Rh and Fy although all of these loci are on chromosome 1. If one of the loci in a linkage group can be assigned to a specific chromosome then it is possible to map the linkage group to that chromosome. This approach has been used in obtaining the data resulting in the assignment of 34 loci to specific chromosomes by the time of Human Gene Mapping 5 (1979).

Linkage analysis of human data using the lod score method of Morton (1955) makes use of simultaneously segregating markers. The lod score method consists of comparing the likelihood of obtaining the marker data on the assumption of various recombination values between two loci as compared with a recombination value of 0.50, i.e. independent

assortment. Data from different pedigrees can be combined by adding together the lod scores calculated for each pedigree, as many families and several studies are often required to provide evidence for or against linkage. When polymorphic loci are used as test loci, data can be collected in a more efficient manner because more families are informative. Lod scores of less than -2.0 are sufficient reason to reject linkage while a lod score of greater than +3.0 is a reliable criterion for accepting linkage. The recombination fraction estimated from the largest lod score is usually higher for females than for males. Renwick and Schulze (1965) were the first to demonstrate that in data for nail-patella:ABO linkage the female recombination fraction was greater than that in the male (the 95% probability limits of the female:male ratio were 3.6:1 and 0.84:1). In the Lutheran:Secretor data the recombination fraction in women was approximately 1.75 times that in men (Cook, 1965).

1.4. Pompe disease

Pompe disease [glycogen storage disease type II (GSD type II)] which has an autosomal recessive pattern of inheritance (Sidbury, 1967; Loonen, 1979) is due to a deficiency of lysosomal GAA activity (Hers, 1963). At least three different types of Pompe disease are distinguishable by clinical features. The classical or infantile type

of GSD type II is characterized by massive cardiomegaly, severe hypotonia, hepatomegaly and muscular weakness which may be present at birth or develop during the first weeks of life. Most infants die of cardiorespiratory failure by the age of one year (di Sant Agnese et al., 1950). Both cross-reacting material (CRM) positive patients, in whom a catalytically inactive enzyme protein is synthesized and CRM negative patients in whom no enzyme protein is detected have been described (deBarsey et al., 1972; Koster and Slee, 1977; Beratis et al., 1978). The late infantile or juvenile form of the disease begins with slowly progressive muscular weakness during the first years of life and patients die during the first or second decade (Zellweger et al., 1965; Smith et al., 1967; Swaiman et al., 1968). Cardiomegaly may be present in some of these patients. In the adult type the clinical manifestations are limited to skeletal muscle. There is a generalized weakness and wasting of the muscles in the second to third decade with death usually occurring in the forties (Hudgson et al., 1968; Engel, 1970; Karpati et al., 1977). Reuser et al.,(1978) have detected some residual activity of GAA (7-22%) in fibroblasts from patients with the adult type, but not from patients with the infantile type of GSD type II. The severity of clinical manifestations and the amount of residual activity in fibroblasts appear to be correlated.

Assays using the fluorogenic substrate, MUG, have been used in the postnatal diagnosis of GAA deficiency (Salafsky and Nadler, 1973; Dreyfus and Poenaru, 1980). Swallow et al.,(1975), did not find any

differences in the kinetic properties of the GAA1 and GAA2 isozymes (from crude placental extracts) using MUG or maltose as substrates, but it is possible that differences might exist if macromolecular substrates were used. Beratis et al.,(1980) used a partially purified preparation of GAA from a GAA1-2 placenta, and found that the GAA2 had reduced catalytic activity for glycogen. However, there was no difference observed between the GAA1 and GAA2 isozymes when MUG or maltose were used as substrates.

1.5. Aims

The purpose of this thesis is to help further the understanding of the genetically determined qualitative, as well as quantitative variation of GAA. The specific aims are as follows:

1. To determine if the small difference in the pI of the GAA1 and GAA2 isozymes observed after column IEF was demonstrable by flatbed polyacrylamide gel IEF.

2. To use flatbed polyacrylamide gel IEF to assess the amount of qualitative variation of GAA in a sample from a Canadian population.

3. To test if the variation detected by the flatbed polyacrylamide gel

IEF technique was genetically determined.

4. To determine if alleles at the GAA locus segregated independently of alleles at a series of marker loci.

5. To determine if quantitative variation of GAA was associated with qualitative variation.

6. To determine if the level of GAA activity was affected by the anticoagulant that had been used when the blood samples were collected.

2. Methods and materials

2.1. Samples

2.1.1. Tissue samples

Pieces of placentae (collected from healthy full term deliveries over a four month period) and post mortem tissue were homogenized in an equal volume of deionized water using a Polytron homogenizer (Brinkman, Luzern, Switzerland). Debris was removed by centrifugation at 17,000 x g. for 20 min. and the supernatant stored at -70°C until used .

2.1.2. White blood cells

Blood samples were obtained from volunteers as well as members of families as part of an ongoing research project designed to map human genes by family studies. The buffy coat from anticoagulated venous blood was removed after centrifugation at 1640 x g. for 5 min. after which most of the erythrocytes were removed by hypotonic shock. White blood cell (wbc) pellets collected by centrifugation were lysed by freezing and thawing four times in a small volume of deionized water (approx. 0.4 ml/10 ml of whole blood) and stored at -30°C until examined.

2.1.3. Fibroblasts

Fibroblast cell strains obtained from The Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, New Jersey were cultured in McCoy's 5A modified medium with 10% fetal calf serum and antibiotics. The cultures were passaged until there were sufficient cells for analysis. When the cell cultures were confluent, cells were harvested by trypsinization and then centrifuged at 3015 x g. for 5 min. to remove the trypsin. A small volume of deionized water (approx. 0.15 ml per 150 mm plate of cells) was added to the cell pellet and

after freezing and thawing four times the lysate was centrifuged at 17,000 x g. for 10 min. to remove cell debris.

Four Pompe cell strains were examined: GM244 from a 5 mo. old female with GSD type II, GM248 from a 4 mo. old male with GSD type II, GM1935 from a 30 yr. old female with late onset GSD type II, GM443 from a 30 yr. old male with late onset GSD type II (approx. 23% normal GAA activity), as well as two control cell strains: GM23 from a 31 yr. old female and GM41 from an apparently normal 3 mo. old male.

2.2. Qualitative analysis

2.2.1. Neuraminidase treatment

Samples were treated with neuraminidase prior to starch gel electrophoresis and IEF. 40 ul of wbc lysate and 10 ul of neuraminidase (Sigma Type V, No. N 2876 from Cl. perfringens, 1 U/3 ml 10 mM Na_2HPO_4/ citric acid buffer pH 4.8) or 50 ul of tissue extract or fibroblast lysate and 10 ul of neuraminidase (1 U/0.5 ml buffer) were incubated in sealed culture tubes overnight at 4°C or for one hour at 37°C.

2.2.2. Starch gel electrophoresis

Horizontal starch gel electrophoresis was carried out using either the Tris/citrate pH 5.8 system or a phosphate buffer system at pH 6.5 (0.2 M Na_2HPO_4/NaH_2PO_4 bridge buffer, 0.01 M Na_2HPO_4/NaH_2PO_4 gel buffer) as described by Swallow et al., 1975. Gels were prepared with 11% hydrolyzed starch (Connaught Laboratories, Toronto, Canada) and were electrophoresed at 4°C.

2.2.3. Isoelectric focusing

The LKB Multiphor apparatus and LKB 2103 power supply were used for all IEF experiments. Thin layer acrylamide gels containing 2 ml of 29.1% (w/v) acrylamide, 2 ml of 0.9% (w/v) N,N'-methylene bisacrylamide, 7.5 ml deionized water, 0.1 ml pH 3.5-10 LKB ampholyte, 0.5 ml pH 4-6 LKB ampholyte and 0.1 ml of 40 mg% (w/v) riboflavin were poured between glass plates 12.5 cm x 12.5 cm separated by a thin gasket (<1mm). After polymerization under fluorescent light, gels were refrigerated approximately 20 min. to facilitate removal of one of the glass plates. Gels were routinely wrapped in plastic film and stored at 4°C until used (less than one week). Samples were applied to the gel 2-3 cm from the cathodal end using 6 mm x 10 mm strips of Whatman #17 chromatography paper. Cathodal and anodal electrode solutions were

1M NaOH and 1 M H_3PO_4 respectively. Isoelectric focusing was carried out at a constant power of 12.5 W, maximum voltage of 1200 V for 2.5 hours at a constant temperature of 16°C. Chromatography papers were removed for the last half hour of the running time.

Determination of the pH gradient was carried out either by using a surface electrode or by elution of the ampholines. The edges of the IEF gel were stained to locate the GAA isozymes and the gel was cut horizontally into 5 mm strips in the area corresponding to the isozymes. The strips of gel were eluted in 1 ml of deionized water overnight and the pH of the eluate determined.

Thin layer gels measuring 25 cm x 12.5 cm were cast in the same manner for IEF gels across the length to expand the gradient to facilitate separation of the isozymes. Gels were prefocused for 1/2 hour at a power of 15 W, maximum voltage of 1400 V at a constant temperature of 16°C. Samples were applied 5 cm from the cathodal end and focusing was carried out for an additional 2.5 hours. Chromatography papers used for sample application were removed for the last half hour of running time.

2.2.4. Detection of enzyme activity

GAA activity was detected after starch gel electrophoresis using a filter paper overlay technique with the substrate MUG (1 mg/ml

dissolved in a small amount of acetone and diluted with 0.2 M sodium acetate/acetic acid buffer pH 4.0; containing 100 mM KCl). Gels were incubated in a plastic box at 45°C and enzyme activity monitored under long wave UV light. If required, NH_4OH was used to intensify the bands of GAA activity.

After IEF GAA activity was detected in a similar manner except the MUG was heated with the buffer at 50°C for approximately 2 hours to increase the amount of substrate dissolved. Acetone was not used because its presence in the reaction mixture made the isozymes indistinct. Gels were incubated in a plastic box at 45°C for 20 min., after which concentrated NH_4OH solution was applied to the gels and the excess carefully blotted. The filter paper was removed and the bands of enzyme activity viewed under long wave UV light.

2.2.5. Photography

Gels were photographed under long wave UV light with a Polaroid MP-4 land camera using a green filter and Kodak Plus-X pan 4"x5" black and white sheet film.

2.3. Markers for linkage analysis

Red cell antigen typing was carried out by the Rh Laboratory of Winnipeg or the Canadian Red Cross in Winnipeg using standard techniques. Secretor status was inferred from Lewis phenotypes. Red blood cell and white blood cell enzyme phenotypes were determined in Dr. P.J.McAlpine's laboratory using the methods described by Harris and Hopkinson, (1976) with minor modifications. Serum proteins were analyzed in a number of different laboratories as part of a collaborative study. Dr. D.W. Cox of Hospital for Sick Children, Toronto analyzed samples for C3, PI, and GC. Dr. Arthur Steinburg of Case Western University, Cleveland analyzed samples for IGHG and IGKC, and Dr. E.R. Giblett, King County Central Blood Bank and School of Medicine, University of Washington, Seattle analyzed samples for C6 and ORM. HP, PLG and TF were analyzed in Dr. P.J. McAlpine's laboratory using the following methods: HP (Giblett, 1969), PLG (Raum et al., 1980) and TF (McAlpine, 1982). The number of markers tested helped to confirm that the family relationships were as stated.

2.4. Linkage analysis

GAA segregation data were tested for linkage using the Mark III program of Côté (1975) which is based on Edwards' marker algebra

(1972). This program utilizes the lod score method of Morton (1955) and calculates the $\log_{10}$ of the odds of obtaining the given results at specific values of the recombination fraction, θ, as compared with the probability of obtaining the same data when θ=0.50, i.e. there is independent segregation. For the data presented here lod scores were calculated at θ= 0.05, 0.10, 0.20, 0.30, and 0.40. Lod scores were separated into paternal, maternal, and intercross information and were summed separately. Recombinant and nonrecombinant counts were made from the phase known data.

2.5. Quantitative analysis

White blood cell lysates for assays were prepared from anticoagulated venous blood as described above. The anticoagulants ethylenediamine tetraacetic acid (EDTA), acid citrate dextrose (ACD), and heparin were used initially, but all subsequent samples were collected in heparin only. Just prior to analysis the wbc lysates were centrifuged in 1.5 ml Eppendorf microcentrifuge tubes at 3015 x g for 10 min. at 4°C. 200 ul of supernatant were diluted with 300 ul of 30 mM acetate buffer pH 4.0. This buffer was prepared from a stock solution of 0.3 M acetate pH 4.0 which was made using approximately 40 ml 1 M NaAc and brought to pH 4.0 with 1 M acetic acid, then diluting 3 volumes of buffer to 10 volumes with deionized water. Small aliquots

of the stock buffer were stored frozen. 50 ul of the diluted supernatant were pipetted into four glass test tubes (three tests and one blank). 100 ul of substrate mixture (4 mM MUG in 30 mM acetate buffer pH 4.0 heated for 2 hr. at 60°C to dissolve the MUG) were pipetted into the test samples which were incubated in a circulating water bath at 37°C for 1 hour. The reaction was stopped with 5 ml of 0.3 M glycine/NaOH buffer pH 10.6. Blanks were incubated for 1 hour at 37°C without substrate, after which 0.3 M glycine/NaOH buffer pH 10.6 and 100 ul of substrate mixture were added. Fluorescence was determined with a Turner flourometer (primary or emission filters 47B and 3 and secondary or excitation filters 10% and 760). A standard curve was constructed daily using 4-methylumbelliferone (0-3.0 mu moles). Protein concentrations on the diluted samples were determined according to the method of Lowry et al., (1951). Enzyme activity was expressed as mu moles of MUG hydrolyzed/ mg of protein/ hour(1 unit= 1 mu mole MUG hydrolyzed/ mg protein/ hour.

2.6. Nomenclature

Genetic notation is presented using the designations of the International System for Human Gene Nomenclature (Shows, et al.. 1979; Shows and M^{c}Alpine, 1982).

3. Results

3.1. Description of phenotypes

The isozyme patterns of the 201 placental samples observed after starch gel electrophoresis were the same as those described by Swallow et al., (1975), where the GAA1 showed a single isozyme which migrates slightly anodally, the GAA2 showed a more anodal isozyme, while the GAA1-2 showed both the GAA1 and GAA2 components (Fig. 1). Examination of the same samples by flatbed polyacrylamide gel IEF revealed an additional phenotype. 169 of the 189 samples (89.4%) that were typed GAA1 by starch gel electrophoresis showed a major band with a slightly weaker, more anodal, band of GAA activity after IEF and were designated as GAA1 (Table 1). The remaining 20 (10.6%) showed two major zones of GAA activity after IEF suggesting a heterozygous phenotype. One of these zones of activity corresponded to the 1 isozyme of the most common phenotypic class by both methods of analysis, whereas the second major isozyme which was cathodal to GAA1 has been designated GAA4. All placental samples that were GAA1-2 by starch gel electrophoresis showed a two banded pattern after IEF; one of these isozymes corresponded in pI to GAA1, while the second major zone of activity was cathodal to it, although anodal to the GAA4 isozyme. When the pH gradient of the gel was determined, the area to which GAA1 migrated (pH 4.90) appeared to

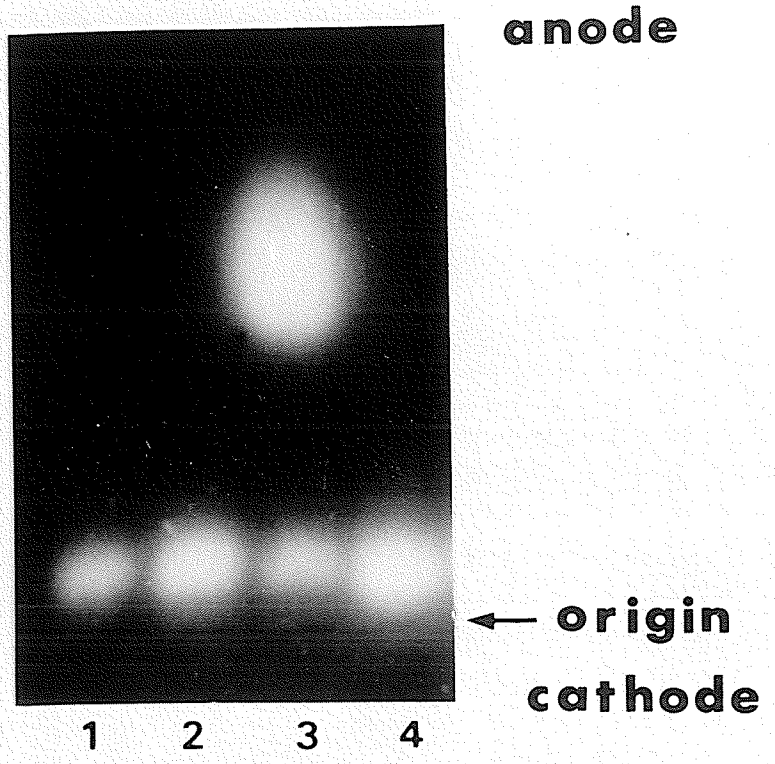

Fig. 1. Photograph of starch gel electrophoresis at pH 6.5 showing the patterns of GAA activity seen in placental extracts. Lanes 1, 2 and 4: GAA1; Lane 3: GAA1-2.

Table 1. GAA variants detected in a survey of placentae using starch gel electrophoresis and IEF.

		Phenotype by starch gel electrophoresis		
		1	1-2	Total
Phenotype by IEF	1	169	0	169
	1-4	20	0	20
	1-2	0	12	12
Total		189	12	201

be 0.05 pH units more acidic than the area to which GAA2 migrated (pH 4.95) and approximately 0.12 pH units more acidic than the area to which GAA4 migrated (pH 5.02)(Table 2).

When wbc lysates were examined by IEF, three additional phenotypes not observed in the placental samples were found: GAA2 and GAA4 which had been predicted on the basis of the analysis of the placental samples (Fig. 2) and an additional phenotype that has tentatively been called GAA1-5. The GAA1-5 showed a two banded pattern with one isozyme corresponding in pI to the GAA1 isozyme and the second major zone of activity being cathodal to the GAA4 isozyme. Only one wbc lysate from the population survey showed this phenotype and family studies were not possible. The GAA2-4 phenotype, although theoretically possible, was not observed. When there was any doubt about distinguishing the GAA1-4 and GAA4 phenotypes or the GAA1-2 and GAA2 phenotypes a 12.5 cm x 25 cm IEF gel was run across the length.

Repeated freezing and thawing of samples with heterozygous phenotypes resulted in the appearance of an additional isozyme of intermediate pI. When lysates of different tissues (brain, liver, adrenal, heart, lung, jejunum, spleen and kidney) obtained post mortem were analyzed by IEF all tissue extracts from the same individual showed the same phenotype.

Table 2. pI of GAA isozymes determined from three different flatbed polyacrylamide IEF gels.

Method used to determine pI	GAA1	GAA2	GAA4
surface electrode	4.90	4.95	5.00
strip method (trial #1)	4.65	4.72	4.81
strip method (trial #2)	5.14	5.18	5.24
mean	4.90	4.95	5.02

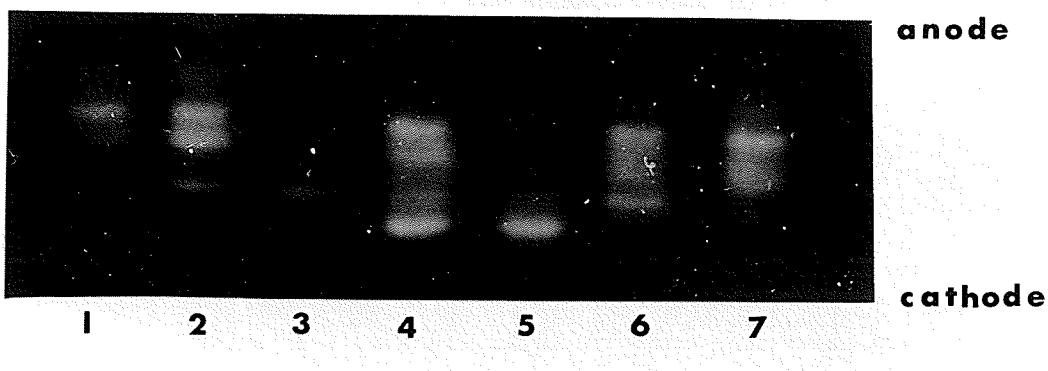

Fig. 2. Photograph of flat bed polyacrylamide gel IEF showing the patterns of GAA activity seen in wbc lysates from individuals of 5 different phenotypes. Lanes 1 and 7: GAA1; Lanes 2 and 6: GAA1-2; Lane 3: GAA2; Lane 4: GAA1-4; Lane 5: GAA4.

3.2. Population and family studies

Family data from this study (Table 3) indicate that six phenotypes are generated from homozygous and heterozygous combinations of three alleles at the GAA locus. GAA1, GAA2, and GAA4 represent individuals who are homozygous for the alleles designated GAA*1, GAA*2 and GAA*4 respectively, while the GAA1-2, GAA1-4 and GAA2-4 represent the heterozygous combinations indicated by the phenotypic nomenclature. Segregation ratios among the 207 offspring from the 61 matings examined were in keeping with Mendelian expectations.

Table 4 shows the distribution of GAA phenotypes in a sample of the general population selected from placentae and unrelated spouses of family members. Among the 633 individuals examined 528 (83.4%) were found to be GAA1, 69/633 (10.9%) were GAA1-4, 32/633 (5.1%) were GAA1-2, 2/633 (0.3%) were GAA4 and 2/633 (0.3%) were GAA2. From these data the frequencies of GAA*1, GAA*2 and GAA*4 alleles have been estimated to be 0.91, 0.03 and 0.06 respectively. The distribution of the phenotypes observed is not statistically different from that expected on the basis of Hardy-Weinberg equilibrium ($\chi^2 = 6.56$, $0.50 < p < 0.25$).

Table 3. Distribution of GAA phenotypes in 61 families.

Type of Mating	No. of Families	Children						Total
		1	1-2	2	1-4	2-4	4	
1x1	19	71	-	-	-	-	-	71
1x1-2	9	16	19	-	-	-	-	35
1x2	2	-	5	-	-	-	-	5
1x1-4	20	28	-	-	31	-	-	59
1-2x1-2	1	2	1	1	-	-	-	4
1-4x1-4	7	7	-	-	15	-	3	25
1-2x1-4	1	1	1	-	-	-	-	2
1x4	2	-	-	-	6	-	-	6
Totals	61	125	26	1	52	0	3	207

Table 4. The distribution of GAA phenotypes in placental and white blood cell extracts from a random sample of a Canadian population. (Expected distribution calculated from the gene frequencies: GAA*1 0.914; GAA*2 0.028; GAA*4 0.058.)

Phenotype	Observed		Expected	
	Number	Incidence	Number	Incidence
1	528	0.834	528.8	0.835
1-2	32	0.051	32.4	0.051
2	2	0.003	0.5	0.001
1-4	69	0.109	67.1	0.106
2-4	0	0.000	2.1	0.003
4	2	0.003	2.1	0.003
Total	633	1.000	633.00	0.999

3.3. Qualitative analysis of fibroblasts from GSD type II patients

Lysates prepared from fibroblasts of patients with infantile and adult onset GSD type II were examined by flatbed polyacrylamide gel IEF. The two cell strains from patients with the infantile onset form of the disease (GM248 and GM244) as well as one cell strain from a patient with the adult form of the diease (GM1935) showed no detectable GAA activity after IEF. One cell strain from a patient with adult onset GSD type II (GM443), which was reported to have 23% activity (The Human Genetic Mutant Cell Repository) appeared indistinguishable from GAA1 after IEF. Both control strains, GM23 and GM41 had appreciable GAA activity and both appeared to have the GAA1-2 phenotype.

3.4. Exclusion mapping of GAA

A patient has been described with the karyotype 46XY, r(17)(p13q25), which involves a deletion of the distal portion of the p and q arms, including q25→qter (Chudley et al., 1982). White blood cell lysates from the patient and his karyotypically normal parents, were examined by IEF (Fig. 3). Both the patient and his mother showed the GAA1-2 phenotype while the father showed the GAA1 phenotype. The patient apparently inherited the GAA*2 from his mother and the GAA*1 from his father. The demonstration of heterozygosity for a locus known

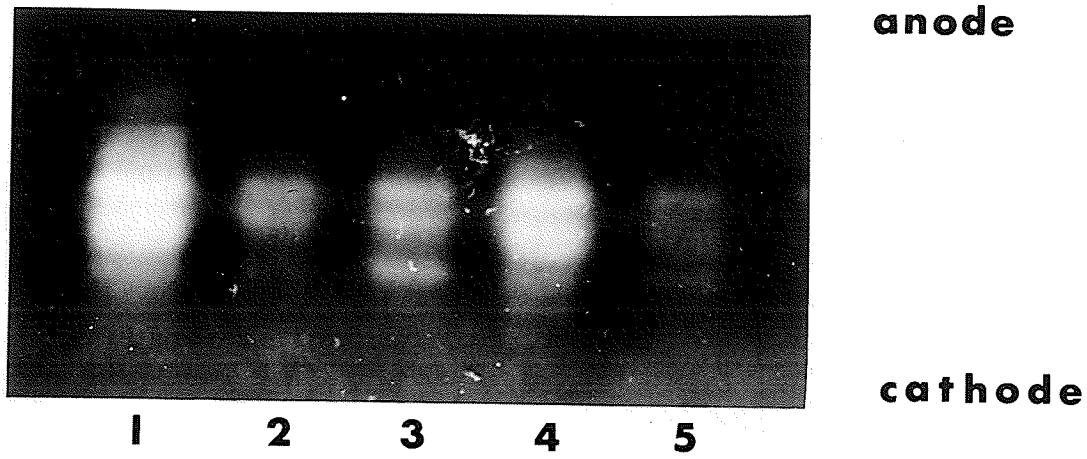

Fig. 3. Photograph of flat bed polyacrylamide gel IEF showing the GAA phenotypes of the r17 patient and his parents. Lane 1: GAA1 control; Lane 2: father of patient, GAA1; Lane 3: patient with r(17)(p13q25), GAA1-2; Lane 4: GAA1 control; Lane 5: mother of patient, GAA1-2.

to be assigned to chromosome 17 (Solomon *et al.*, 1979; D'Ancona *et al.*, 1979; Weil *et al.*, 1979) indicates that *GAA* cannot be in the region q25→qter of chromosome 17.

3.5. Linkage studies on the *GAA* locus

Paternal and maternal lod scores for *GAA* vs. the following loci: Colton blood group (*Co*),complement component-3 (*C3*), complement component-6 (*C6*), Diego blood group (*Di*), Dombrock blood group (*Do*), glutamic-oxaloacetic transaminase (mitochondrial) (*GOT2*), glutamic-pyruvic transaminase (*GPT*), immunoglobulin γ heavy chain (*IGHG*), immunoglobulin κ chain, constant region (*IGKC*), Kidd blood group (*Jk*), Lutheran blood group (*Lu*), orosomucoid (ORM), P blood group (P), alpha-1-antitrypsin (*PI*), plasminogen (*PLG*), ABH secretion (*Se*), and transferrin (*TF*) all of which were either unassigned or had provisional or tentative chromosomal assignments when this study was initiated are shown in Table 5. The categories of chromosomal assignments are based on those adopted by the International Workshops of Human Gene Mapping *viz.* provisional: data are provided by only one group of investigators, confirmed: data are provided by at least two independent groups of investigators and tentative: data have not reached formal levels of significance. Paternal lod scores for *GAA* and *C6*, *Di*, *Do*, *IGKC*, *Jk*, *PI*, *PLG*, *Se*, and *TF* were <-2.0 at $\theta=0.05$, thereby

Table 5. Lod scores for linkage between GAA and other loci.

Loci	Segregation Information	Number of Families	Number of Children	Assumed Recombination Fraction 0.05	0.10	0.20	0.30	0.40
Co	P*	1	3	-0.721	-0.444	-0.194	-0.076	-0.018
	M	1	2	-0.721	-0.444	-0.194	-0.076	-0.018
	T	2	5	-1.442	-0.888	-0.388	-0.152	-0.036
C3	P	3	11	0.045	0.211	0.238	0.146	0.044
	I	1	3	0.022	0.116	0.151	0.101	0.032
	T	4	14	0.067	0.327	0.389	0.247	0.076
C6	P	6	20	-2.275	-1.068	-0.180	0.065	0.073
	M	5	14	-4.905	-3.214	-1.642	-0.830	-0.328
	I	1	2	-0.718	-0.442	-0.193	-0.075	-0.018
	T	12	36	-7.898	-4.724	-2.015	-0.840	-0.273
Di	P	6	22	-3.511	-1.937	-0.621	-0.099	0.068
	M	3	9	-4.442	-2.985	-1.582	-0.818	-0.327
	I	1	5	-0.927	-0.458	-0.121	-0.023	-0.001
	T	10	36	-8.880	-5.380	-2.324	-0.940	-0.260
Do	P	5	24	-3.995	-2.535	-1.149	-0.457	-0.108
	M	2	4	-0.617	-0.360	-0.145	-0.053	-0.012
	I	1	5	-0.171	-0.113	-0.037	-0.007	0.000
	T	8	33	-4.783	-3.008	-1.331	-0.517	-0.120
GOT2	P	1	4	-0.464	-0.229	-0.060	-0.011	-0.001
GPT	P	4	19	-1.664	-0.888	-0.271	-0.058	-0.005
	M	8	30	-4.141	-2.110	-0.512	0.007	0.079
	I	4	13	-0.334	-0.002	0.086	0.044	0.032
	T	16	62	-6.139	-3.000	-0.697	-0.007	0.106
IGHG	P	2	6	-1.442	-0.888	-0.388	-0.152	-0.036
	M	5	17	-3.349	-2.010	-0.856	-0.356	-0.120
	T	7	23	-4.791	-2.898	-1.244	-0.508	-0.156

Table 5. (continued)

Loci	Segregation Information	Number of Families	Number of Children	Assumed Recombination Fraction 0.05	0.10	0.20	0.30	0.40
IGKC	P	3	13	-2.370	-1.350	-0.528	-0.215	-0.084
	M	2	6	-0.163	0.067	0.214	0.216	0.140
	T	5	19	-2.533	-1.283	-0.314	0.001	0.056
Jk	P	12	48	-5.744	-3.210	-1.170	-0.388	-0.080
	M	12	40	-9.094	-5.730	-2.659	-1.164	-0.362
	T	24	88	-14.838	-8.940	-3.829	-1.552	-0.442
Lu	P	1	8	-1.906	-1.116	-0.448	-0.163	-0.036
	M	3	6	-2.163	-1.332	-0.582	-0.228	-0.054
	T	4	14	-4.069	-2.448	-1.030	-0.391	-0.090
ORM	P	3	8	-1.184	-0.673	-0.254	-0.088	-0.019
	M	2	15	-1.093	-0.401	0.055	0.113	0.045
	I	1	5	0.987	0.773	0.377	0.100	0.007
	T	6	28	-1.290	-0.301	0.178	0.125	0.033
P	P	3	15	0.844	0.783	0.597	0.357	0.116
	M	5	25	-1.277	-0.567	-0.074	0.033	0.019
	I	1	2	-0.285	-0.207	-0.104	-0.043	-0.010
	T	9	42	-0.718	0.009	0.419	0.347	0.125
PI	P	4	20	-2.700	-1.222	-0.128	0.140	0.082
	M	4	8	-4.163	-2.729	-1.378	-0.672	-0.248
	T	8	28	-6.863	-3.951	-1.506	-0.532	-0.166
PLG	P	4	16	-3.090	-1.789	-0.702	-0.251	-0.055
	M	3	11	-2.349	-1.310	-0.458	-0.133	-0.022
	I	1	3	0.509	0.443	0.302	0.161	0.046
	T	8	30	-4.930	-2.656	-0.858	-0.223	-0.031
Se	P	4	16	-2.513	-1.243	-0.243	0.083	0.118
	M	1	3	-0.721	-0.444	-0.194	-0.076	-0.018
	I	1	5	-0.171	-0.113	-0.037	-0.007	0.000
	T	6	24	-3.405	-1.800	-0.474	0.000	0.100
TF	P	4	19	-2.256	-1.033	-0.135	0.090	0.055
	M	2	4	-2.721	-1.842	-0.990	-0.520	-0.212
	T	6	23	-4.977	-2.875	-1.125	-0.430	-0.157

*P = paternal, M = maternal, I = intercross, T = total.

indicating that close linkage between the GAA locus and any of these marker loci is unlikely (Fig. 4). In addition, Do and Jk can also be excluded from linkage with the GAA locus at values of $\theta < 0.10$ as the paternal lod scores were <-2.0 at this recombination fraction. Male segregation data for Co, GOT2, GPT, IGHG, Lu, and ORM have not reached formal levels of significance. Male C3 and P data are slightly positive, but more data will be required before there is enough information to accept or reject linkage with GAA.

3.6. Quantitative data

White blood cell samples from 52 unrelated people including 24 pregnant females were analyzed for GAA both qualitatively, by IEF, and quantitatively (Table 6). In the samples with GAA1 phenotypes from the non-pregnant group GAA activities were found to range from 20-200 units with a mean activity of 66.42 units and a standard deviation of $\pm$ 48.68 units. In the 11 samples with GAA1 phenotypes from the pregnant women GAA activities were found to range from 19-63 units with a mean activity of 35.45 units and a standard deviation of $\pm$ 16.12 units. In the six samples with GAA1-4 phenotypes from the non-pregnant group GAA activities were found to range from 14-106 units with a mean activity of 45.33 units and a standard deviation of $\pm$ 36.37 units. In the nine samples with GAA1-4 phenotypes from the pregnant women GAA activities

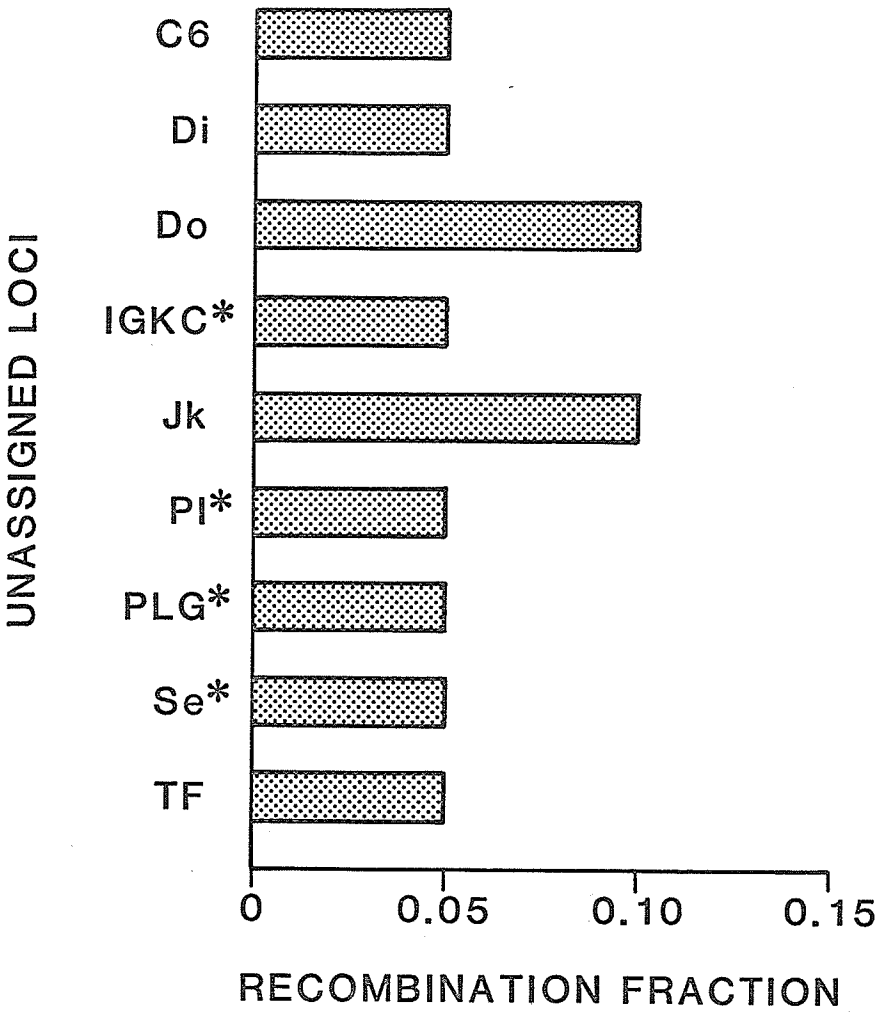

Fig. 4 Diagram showing loci excluded from linkage to GAA (lod score ≤ -2.00)

* These loci have since been assigned to chromosomes other than number 17.

Table 6. Distribution of GAA activity in a sample of 52 unrelated individuals of which 24 were pregnant females.

GAA phenotype	Pooled			Pregnant			Non-pregnant		
	no.	range	mean±SD	no.	range	mean±SD	no.	range	mean±SD
1	30	19-200	55.07±42.34	11	19-63	35.45±16.12	19	20-200	66.42±48.68
1-4	15	14-106	51.40±29.78	9	29-102	55.44±26.06	6	14-106	45.33±36.37
1-2	7	12-54	25.71±15.63	4	12-37	20.75±11.35	3	13-54	32.33±20.60

were found to range from 28-102 units with a mean activity of 55.44 units and a standard deviation of ± 26.06 units. In the three samples with GAA1-2 phenotypes from the non-pregnant group GAA activities were found to range from 13-54 units with a mean activity of 32.33 units and a standard deviation of ±20.60 units. In the four samples with GAA1-2 phenotypes from the pregnant women GAA activities were found to range from 12-37 units with a mean activity of 25.71 units and a standard deviation of ± 15.63 units. To determine if the data from the pregnant women could be pooled with the other data, a two tailed t-test was performed to compare the distributions of GAA activity in samples from pregnant women vs. the distributions of GAA activity in samples from non-pregnant people classified by phenotypic group. No statistically significant difference was found between the two groups at a 95% confidence interval (GAA1: t=2.543, df=24; GAA1-4: t=-0.588, df=8; GAA1-2: t=0.879, df=3) and thus the data were then pooled for further analysis. The variance seen in the distribution of activity of the samples from the pregnant women was notably less than that seen in the other samples (Table 6).

In the pooled data, the 30 samples with GAA1 phenotypes had GAA acivities ranging from 19-200 units with a mean of 55.06 and a standard deviation of ± 42.32 units (Fig. 5). In the 15 samples with GAA1-4 phenotypes, GAA activities were found to range from 14-106 units with a mean of 51.40 units and a standard deviation of ± 29.78 units. The seven samples with GAA1-2 phenotypes had GAA activities that ranged

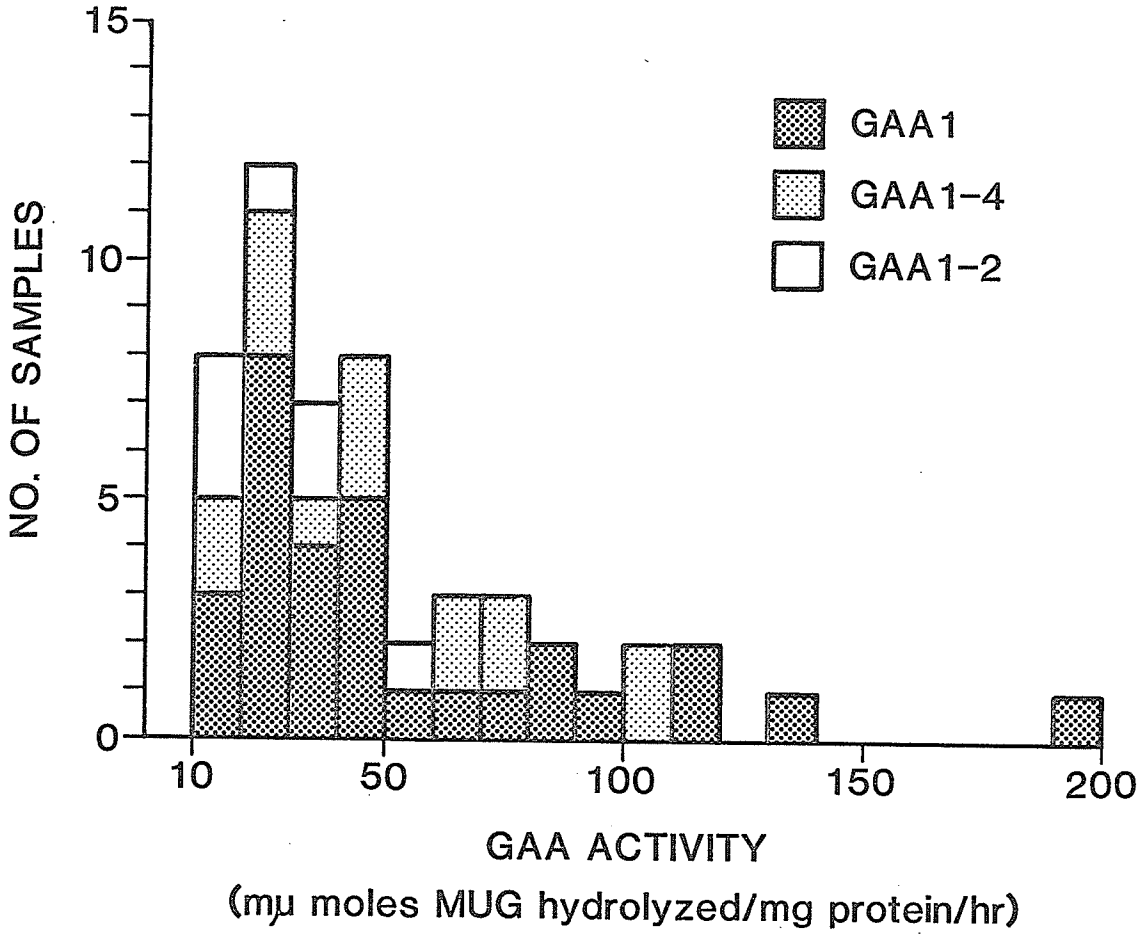

Fig. 5 Bar graph illustrating distribution of GAA activity in GAA1, GAA1-4 and GAA1-2 phenotypes.

from 12-54 units with a mean of 25.71 units and a standard deviation of ± 15.63 units.

Using the GAA activity values obtained above an attempt was made to estimate the GAA activity per allele. GAA*1 appears to be associated with 27.53 units of activity per allele on average, while GAA*4 is associated with 23.88 units of activity per allele on average. Since the mean activity of the GAA1-2 phenotype is less than the average activity calculated for the GAA*1 allele, the GAA*2 allele appears to have an inhibitory effect on GAA activity in GAA1-2 heterozygotes. It was not possible to determine if the activities per allele were truly additive as samples from homozygous GAA2 and GAA4 individuals were not available for assay.

Analysis of variance was used to determine if there were a difference in activity between phenotypic classes. There was no statistical evidence for a difference in activity between phenotypes at a 95% confidence interval ($F=1.835$, $df=2$).

Assay results from samples obtained from seven individuals that were collected using ACD, EDTA, and heparin as anticoagulants are illustrated in Figure 6. The GAA activity of wbc's when ACD was the anticoagulant was, on the average, 72% of that obtained from wbc when heparin was the anticoagulant. When EDTA was employed, the GAA activity of the wbc's was on the average 148% of that of heparinized samples. Statistical testing of these data using analysis of variance revealed that the variance between individuals for each anticoagulant

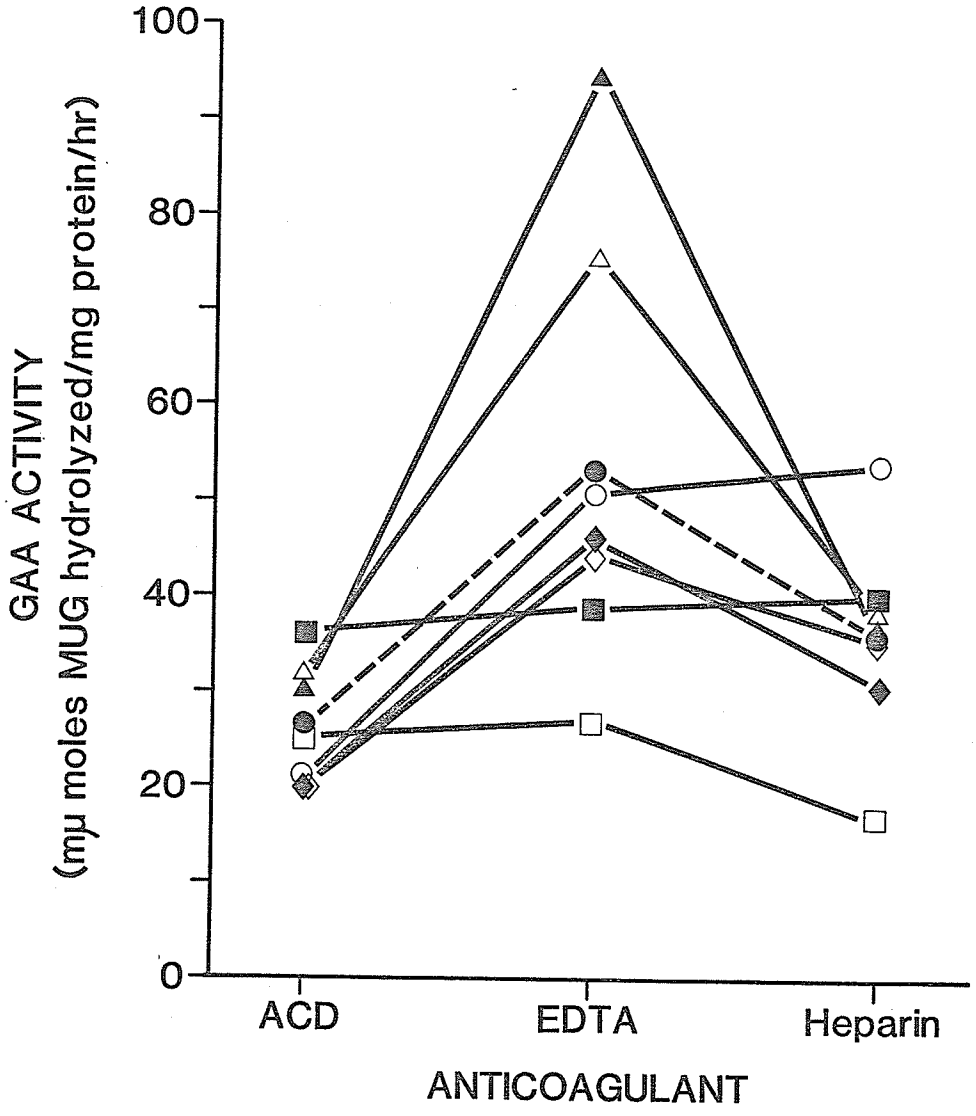

Fig. 6 Graph showing the effect of different anti-coagulants on GAA activity. Solid lines show the relative GAA activity for each individual. The dashed line shows the relative mean GAA activities.

was not statistically significant($F=1.75$, $df=6$), but GAA activity showed statistically significant differences according to the type of anticoagulant used ($F=7.18$, $df=2$, $P<0.01$).

4. Discussion

4.1. Population and family studies

In a survey of 201 placental samples reported here, most that were GAA1 by starch gel electrophoresis showed a major band of enzyme activity with a slightly weaker, more anodal band when subjected to IEF. However, some samples that were GAA1 by starch gel electrophoresis showed two major bands of enzyme activity, implying a heterozygous phenotype involving a previously unrecognized allele, GAA*4, a notion supported by family data. One wbc lysate showed what has tentatively been called GAA1-5, but further studies will be required to determine if the isozyme pattern observed is due to another allele at the GAA locus in heterozygous combination with GAA1 or perhaps due to post-translational modification. From data collected in this study of an ethnically heterogeneous random sample of the Canadian population the allele frequencies of GAA*1, GAA*2, and GAA*4 have been estimated to be 0.91, 0.03, and 0.06, respectively, which are in close agreement with those obtained by Swallow et al. (1975) where the

frequency of GAA*1 was 0.97 (this would include GAA*1 and GAA*4) and the frequency of GAA*2 was 0.03. There was a suggestion that the GAA*4 allele might be more common in people of Oriental descent than in persons of Western European origin.

In the polymorphism of GAA demonstrable by starch gel electrophoresis, separation of GAA isozymes appears to depend on differences in the affinity of the enzyme components for the support medium in which the electrophoresis was conducted rather than differences in molecular size or charge (Swallow et al., 1975). After electrophoresis at pH 6.5, the GAA1 isozyme showed as a single, slightly anodal band of enzyme activity, whereas the GAA2 was more anodal, indicating that the type 2 isozyme did not bind as firmly as the type 1 isozyme to glucose polymers such as dextran or starch.

Following IEF, when the pH gradient was measured using two different techniques, molecules coded for by GAA*1 appeared to be 0.05 pH units more acidic than those relating to GAA*2, and 0.12 pH units more acidic than those relating to GAA*4. These data are consistent with those obtained by column IEF, which despite the actual pI's being different, indicated that the pI of the type 1 isozyme was 0.06 pH units more acidic than that of the type 2 isozyme (Swallow et al., 1975). More recent data obtained from DEAE-cellulose column chromatography confirmed the notion that the type 2 isozyme is more basic than the type 1 isozyme, as the type 2 isozyme was eluted from the column at a lower NaCl concentration than the type 1 isozyme

(Beratis et al., 1980).

The type of qualitative variation of GAA isozymes that was observed in this study could have resulted from single amino acid substitutions, where an acidic amino acid in the type 1 isozyme could have been replaced by a neutral amino acid in the type 2 isozyme and a basic amino acid in the type 4 isozyme. A single amino acid substitution in an area where the "affinity" of the enzyme for the starch would be affected could account for the difference in relative mobilities of the type 1 and type 2 isozymes between starch gel electrophoresis and IEF. For the type 2 isozyme, where a neutral amino acid substitution is proposed, there is an increased anodal mobility in starch gel electrophoresis relative to the type 1 isozyme that has been attributed to increased affinity of this isozyme for the supporting medium. In the type 1 and 4 isozymes, where different charged amino acid substitutions have been postulated, but no mobility difference after starch gel electrophoresis has been observed, it is assumed that the affinity for starch is masking the charge difference between the type 1 and 4 isozymes that is demonstrable by IEF.

IEF which separates proteins according to differences in their pI's has become a useful technique for studying genetic variation because of its ability to resolve proteins which differ by as little as 0.01 pH units (Righetti and Drysdale, 1974). The high resolving power of this technique has been effective in demonstrating qualitative differences between GAA isozymes that were not detectable by previous methods.

Similar demonstrations of an increase in the number of phenotypes detected after IEF have been reported for PGM1, TF and GC (Bark et al., 1976; Kuhnl and Spielman, 1978; Constans et al., 1978). In addition, analysis of samples by flatbed polyacrylamide gel IEF has the advantage that multiple samples can be analyzed in a short period of time and the amount of sample required is less for each test than that required for starch gel electrophoresis.

4.2. Subunit number

The electrophoretic pattern observed in both natural and artificial heterozygotes can often be used to estimate the number of subunits per molecule. Assuming random association and equal levels of activity, the number of subunits present is usually one less than the number of isozymes observed in heterozygotes. There is evidence that bovine liver GAA has four subunits of equal molecular weight (Bruni et al., 1970) and if human GAA is similar in this respect, one might expect to see a five banded pattern of enzyme activity in heterozygotes under ideal conditions. However, after starch gel electrophoresis or IEF of human GAA, only the two primary isozymes are seen; bands with intermediate mobilities or pI's that would represent heteromeric isozymes containing both allelic products are not observed routinely. If samples from heterozygotes are frozen and thawed many times (>10)

one additional isozyme with intermediate pI is observed, but if GAA is a tetramer one would expect to observe a total of five bands in a heterozygote, not three. If the enzyme is a multimer the absence of theoretically expected heteromeric bands in heterozygotes may be explained by dissociation of the protein into subunits, so that during analysis the heteromeric forms are lost, as in the case of the hemoglobins (Benesch et al., 1966). Alternatively, there may be some special structural feature of the alternative polypeptide chains which prevents or limits their association in the same multimeric molecule such as in mitochondrial and soluble forms of certain enzymes (glutamate-oxalate transaminase, isocitrate dehydrogenase, malate dehydrogenase and malic enzyme (Harris, 1977).

If human liver GAA does consist of at least three structurally different subunits with different N-terminal amino acids and different molecular weights as suggested by Belen'ky & Rosenfeld, (1975), one might expect to observe three bands of GAA activity after electrophoresis or IEF. The phenotype of the GAA in their study was unknown, but it is possible a phenotypic variant was studied with two of the bands relating to a heterozygous phenotype such as GAA1-2, GAA1-4 or GAA2-4 and the third band the result of a post translational modification of one of the primary polypeptides or perhaps similar to the band of GAA activity seen after repeated freezing and thawing of the sample. Further analysis is required to resolve this conflict.

4.3. GSD type II fibroblasts

Pompe disease fibroblast strains were examined by IEF to determine if patients with adult onset GSD type II had a qualitative variant of GAA that was responsible for the quantitative variation. One fibroblast strain from a patient with adult onset GSD type II, reported to have 23% activity showed a phenotype that was indistinguishable in pI from GAA1, while in another fibroblast strain from a different patient with GSD type II the GAA activity after IEF was below the level of detection. The two control lines chosen for these experiments both appeared to be GAA1-2 which could have been a problem had quantitative studies using glycogen as the substrate been done because the GAA2 has a reduced catalytic activity towards glycogen (Beratis et al., 1980). These data demonstrate the importance of phenotyping controls before proceeding with further quantitative studies as qualitative variation can make an important contribution to quantitative variation.

4.4. Exclusion mapping

In many cases of autosomal deletions, heterozygosity at a number of genetic marker loci can be demonstrated thereby excluding the location of the structural gene from the deleted segment. The accumulation of

such data for a wide array of deletions should allow the assignment of a locus by a process of exclusion (Ferguson-Smith, 1975). GAA from the wbc's of a patient with the karyotype 46XY, r(17)(p13q25) as well as his karyotypically normal parents were examined by IEF in order to obtain additional information on the chromosomal localization of GAA. The patient and his mother showed the GAA1-2 phenotype while the father showed the GAA1 phenotype. The demonstration of heterozygosity for a locus assigned to chromosome 17 in a patient with a deletion of this chromosome allows the GAA locus to be excluded from that portion of the patient's chromosome 17 that was missing (17q25→qter). Since the GAA locus has previously been localized on chromosome 17q21→qter (Wiel et al., 1979) the demonstration of heterozygosity in this patient allows the assignment for the GAA locus to be reduced to 17q21→q25 (Figure 7).

4.5. Linkage analysis

The extension of the GAA polymorphism by IEF results in an increase in the frequency of detection of heterozygotes to 16%, a more than three-fold increase over that observed after starch gel electrophoresis. This extensive variation makes GAA a useful marker for chromosome 17 in classical linkage analysis of family data as the other genes (thymidine kinase, galactokinase and virus associated

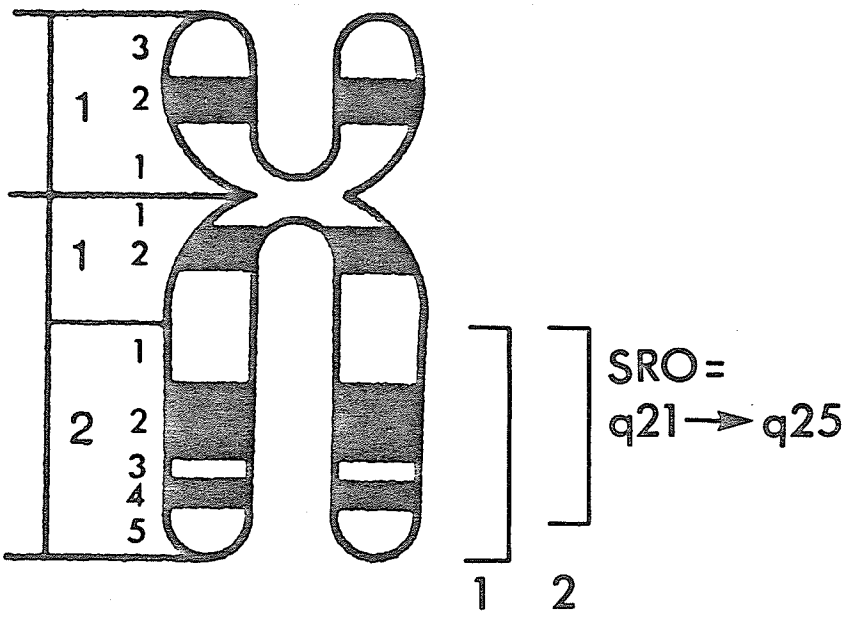

Fig. 7. Regional assignments of GAA on chromosome 17. SRO is the shortest region of overlap of regional assignments.

References: (1) Weil et al., 1979; (2) present data.

markers) assigned to chromosome 17 to date, are unsuitable for family studies.

Paternal lod scores for GAA and the following loci: C6, Di, Do, IGKC, Jk, PI, PLG, Se and TF, all of which were either unassigned or had provisional or tentative assignments at the beginning of this study were ≤ -2.0 at $\theta=0.05$, thereby indicating that close linkage between the GAA locus and any of these marker loci is unlikely. Do and Jk can also be excluded from linkage with the GAA locus at values of $\theta \leq 0.10$. Close linkage of GAA with Co, GPT, GOT2, IGHG, Lu or ORM is unlikely because at least one recombination has occurred between GAA and each of these marker loci. Although paternal lod scores for GAA and C3 and P are slightly positive the data are insufficient to accept or reject linkage.

The lod scores obtained from this study are consistent with data presented at Human Gene Mapping 6 (1981) as most of the loci tested for linkage with GAA were, at least tentatively, assigned to chromosomes other than 17. However as the chromosomal assignments of Co, C6, Di, Do, Jk, GPT and TF still remain uncertain, the possibility of loose linkage between one or more of these marker loci and GAA cannot be excluded at this time.

4.6. Quantitative analysis

GAA activity was assayed using the synthetic substrate MUG in order to determine if different levels of activity are associated with the three common GAA alleles. As 24 of 52 samples assayed were obtained from pregnant women the data were tested to determine if the results from the pregnant women could be pooled with the data from the remainder. No statistically significant difference was found between the two groups of samples so the results were pooled prior to further analysis. The variance in the samples from the pregnant women was notably less than that seen in the other group of samples. This lower variance probably reflects a more uniform manner of handling, as the other samples had been obtained from a number of different sources and handled in a variety of ways. Using the pooled data no statistically significant difference was found in the activity of the three phenotypes tested using MUG as a substrate. Beratis et al., (1980) found that when GAA was assayed using MUG and maltose as substrates no difference was observed between the GAA1 and GAA2 isozymes that were purified from a heterozygous placenta. However, the GAA2 isozyme had reduced catalytic activity for glycogen, as the activity of GAA2 was only 8.6% and 19.2% of GAA1 when assayed with non-saturating and saturating concentrations of glycogen respectively.

It has been proposed that individuals homozygous for GAA*2 would

have a decreased catalytic activity towards glycogen and might develop muscular dystrophy-like disease in late life (Beratis et al., 1980). To test this hypothesis, GAA2 individuals who are expected to occcur in the population with a frequency of approximately 0.001, could be studied to see if they showed any signs of muscular disease as they aged. Alternatively, a sample of individuals with muscular dystrophy-like diseases could be screened for GAA phenotypes to determine if there were an increased frequency of individuals with the GAA2 phenotype. Since both the GAA1 and GAA2 isozymes are effective in cleaving maltose and MUG, an enzyme deficiency of this type would only be detected with the natural substrate, glycogen. Another example of a discrepancy between results with natural and artificial substrates has been described for hexosaminidases where the Tay Sachs "Variant AB" enzymes hydrolyzed synthetic substrates, but not the natural substrate (Sandhoff et al., 1971). Therefore, the importance of using the natural substrate when looking for alterations in catalytic activity of enzymes cannot be underestimated.

Early in the experiments involving estimation of GAA activity in wbc, a variation in activity was noted that appeared to depend on the anticoagulant that had been used when the blood sample was collected. To test if the variation observed was due to the anticoagulant used, blood samples from each of seven persons were collected into tubes containing three different anticoagulants. The variation in GAA activity between individuals for each anticoagulant was not

statistically significant. However, a very significant statistical difference was observed between anticoagulants, with GAA activity being greatest in samples where EDTA had been used, less in samples where heparin had been used and least in samples where ACD had been used. The difference in GAA activity between anticoagulants was sufficiently significant that it would have been observed without having used samples from the same individual.

To explain the differences in GAA activity observed between different anticoagulants one could hypothesize that different populations of wbc's are involved. Leukocytes (mostly granulocytes) contain both GAA and renal maltase, an isozyme of alpha-glucosidase normally present in kidney cells, while lymphocytes contain GAA and little or no renal maltase (Broadhead and Butterworth, 1978; Potter et al., 1980; Dreyfus and Poenaru, 1980). The activity in some samples may be in part due to renal isozyme despite attempts that were made to minimize contamination by this isozyme. Whereas, GAA is almost completely extracted with water, renal maltase remains almost entirely in the sediment after water extraction and requires a more rigorous extraction involving the use of a detergent such as Triton (Dreyfus and Poenaru, 1980). Therefore, aqueous, centrifuged extracts were prepared prior to GAA determinations. More studies will be required to resolve the question of whether different populations of cells are involved and if so, which anticoagulants will give a better estimation of GAA activity without interference from the renal isozyme. Further

experimentation could involve the use of density gradient separation of wbc's to determine if different anticoagulants cause a shift in the wbc population that is used for assays. Also the use of anti-renal maltase antiserum before assaying wbc's from samples collected in different anticoagulants could eliminate the activity due to the renal isozyme and allow for a more reliable estimate of GAA activity.

An alternate hypothesis to explain the difference in GAA activities that was observed with different anticoagulants is that the anticoagulants could be either inhibiting or enhancing GAA activity in some manner. Further experimentation to investigate the effect of anticoagulants on GAA activity might involve the isolation of wbc from defibrinated blood as well as the addition of different anticoagulants prior to the determination of GAA activity.

Reliable determinations of the level of GAA activity are important in studies involved in carrier detection for GSD type II where one is looking for a decrease in activity. There is a remarkable difference in GAA activity observed between samples collected in different anticoagulants and for this reason it is important that all samples, including control samples, be collected using the same anticoagulant. Perhaps further studies will elucidate if one anticoagulant is preferable for these determinations.

The increased detection of heterozygosity at the GAA locus, a more than three-fold increase over that observed after starch gel electrophoresis, may be of help in the investigation in a more detailed

manner the genetic basis of expression of GAA activity in normal and disease phenotypes. Pompe disease already demonstrates allelic diversity, where differences in the precise genetic defects result in the same clinical phenotype as there are both CRM positive patients, in whom a catalytically inactive enzyme protein is synthesized and CRM negative patients, in whom no enzyme protein has been detected (de Barsey et al., 1972; Koster and Slee, 1977; Beratis et al., 1978).

By screening the parents of children with Pompe disease it might be possible to find decreased activity, as well as heterozygosity at the GAA locus. Such an observation could be a starting point for determining if the lack of GAA activity were due to a mutation at the GAA locus or at other loci, possibly involved in post-transcriptional control (Brown, 1981) or production of a protein which activates the primary translation products of the GAA locus.

5. Summary

1. A new method to analyze acid alpha-glucosidase (GAA) activity in human tissues by flatbed polyacrylamide gel isoelectric focusing has been devised.

2. With this method the GAA polymorphism has been extended from three to six commonly occurring phenotypes.

3. After isoelectric focusing the GAA2 isozyme migrated cathodally with respect to the GAA1 isozyme, whereas with the previous starch gel electrophoresis method their relative positions are reversed.

4. The six phenotypes are generated by three common alleles, GAA*1, GAA*2 and GAA*4 with frequencies of 0.91, 0.03 and 0.06, respectively in a sample from a Canadian population.

5. The one fibroblast cell strain from a patient with adult onset glycogen storage disease type II that had sufficient activity to be detectable with the IEF method developed in this study showed a phenotype indistinguishable from GAA1.

6. Paternal lod scores for GAA and the following loci: C6, Di, Do, IGKC, Jk, PI, PLG, Se and TF, all of which were either unassigned or had provisional or tentative assignments at the start of this study were ≤ -2.0 at $\theta=0.05$. These data indicate that close linkage between the GAA locus and any of these marker loci would be unlikely. Although the data did not reach formal levels of significance close linkage of GAA and Co, GOT2, GPT, IGHG, Lu, ORM or P is unlikely because at least one recombination has occurred between GAA and each of these marker loci.

7. The chromosomal localization of the GAA locus has been reduced from 17q21→qter to 17q21→q25 using exclusion mapping in a patient with a deletion of chromosome 17.

8. Using the artificial substrate 4-methyumbelliferyl alpha-D-glucoside, no statistically significant difference was observed in the GAA activity of three different phenotypes, GAA1, GAA1-2 and GAA1-4.

9. The level of GAA activity in white blood cell samples was found to vary with the anticoagulant used when the blood samples were collected. White blood cells from blood samples collected in EDTA had the greatest level of activity, while those from heparinized samples had intermediate GAA levels and those where ACD was the anticoagulant had the lowest levels of activity.

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