DEVELOPMENTAL AND GENETIC STUDIES IN MURINE HISTIDINAEMIA

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ABSTRACT OF THESIS

The development of the histidinaemic (his/his) mouse has been examined as a model of a human aminoacidopathy. The concentrations of histidine and its derivatives have been measured in tissues and body fluids in utero and during the first five weeks after birth. The placental transfer of raised maternal histidine concentrations to developing his/his embryos results in a twenty-fold increase in foetal histidine concentrations. After birth, histidine concentrations are also increased between four and twenty-three fold in the his/his offspring of his/his mothers.

Developmental changes in histidine catabolizing enzymes have been compared in wild-type (+/+) and his/his mice. The histidase defect in the liver does not appear till after birth, although in the skin it is present earlier. Liver histidine amino-transferase activity is two to three times higher shortly after birth than in the adult in both genotypes.

Parallel studies of brain growth and development have been carried out in +/+ and his/his mice. Changes in cell number, cell growth and myelination have been estimated from measurements of DNA, RNA, water, cholesterol and protein content. Although some small differences emerge, the two genotypes show near identical brain growth profiles despite large metabolic differences before and after birth.

The biochemical genetic nature of histidase deficiency has been examined in his/his mice. Structural and kinetic studies show that the residual mutant activity has markedly different properties from the wild-type in crude extracts. Partial purification experiments show that there are two distinguishable components with histidase activity in wild-type liver, only one of which is found in the mutant. The his allele may be a null allele at a histidase structural or regulatory locus, which unmasks a minor histidase isozyme.

CHAPTER I

INTRODUCTION

(i) Inborn errors of amino acid metabolism in man

Since A.E. Garrod (1908) introduced the concept of a genetically determined metabolic block, some 200 - 250 further inborn errors of metabolism have been described in man (Eldjarn et al., 1975). The expression "inborn error of metabolism" refers to those conditions in which a congenital deficiency of a specific protein occurs as a result of an alteration in a single gene. The deficient protein may be involved in catalysis, transport, structure or any other function (Brock, 1978), although Garrod originally envisaged only the first of these. Most known inborn errors show recessive inheritance which is almost the rule with enzyme deficiencies. Deficiencies of non-enzymic proteins may account for many of the dominant conditions whose aetiology is at present unknown. Only 500 out of the 1000 - 2000 single gene disorders known in man show recessive inheritance and of these a specific enzyme deficiency has only been demonstrated in about 200 (McKusick, 1975: Stanbury et al., 1978).

The inborn errors of amino acid metabolism are clinically important despite their individual rarity. This is because while a few are wholly or relatively benign (e.g. α- aminoisobutyric aciduria, albinism) many give rise to severe disability. Sometimes death occurs in infancy (e.g. urea cycle disorders), but permanent disability such as mental handicap (e.g. phenylketonuria), cardio-vascular anomalies (e.g. homocystinuria) or renal impairment (e.g. hyperoxaluria) is a more common outcome.

At the biochemical level, inborn errors of amino acid metabolism generally show typical Garrodian blocks with build up of proximal metabolites. The latter often "overflow" into the urine, so that the term "aminoaciduria" is used as a synonym, although it includes conditions with aminoaciduria secondary to severe renal or hepatic disease. A better term is hereditary "aminoacidopathy" which can be classified into four types according to mechanism (Rosenberg and Scriver, 1974).

(I) Primary defect in catabolism. The tissue concentration of one or more amino acids increases as a result of the block. The amino acid may have a low renal clearance so that it accumulates in the plasma and renal tubules until the tubular reabsorption mechanisms are saturated, leading to "overflow" aminoaciduria (e.g. phenylketonuria, histidinaemia). Alternatively the amino acid may have a high renal clearance which results in relatively low plasma concentrations but a marked ("no threshold") aminoaciduria (e.g. α-aminoisobutyric aciduria).

(II) Primary defect in catabolism with a secondary defect in transport. Accumulation of one amino acid in the plasma and renal tubules saturates the renal transport system shared by other amino acids, so that the latter are reabsorbed at a reduced rate and increase in the urine (e.g. hyperprolinaemia).

(III) Primary defect in membrane transport site. An amino acid carrier protein is defective in the kidney and often other sites, so that there is no plasma increase, but an increased renal loss (e.g. cystinuria, histidinuria).

(IV) Generalized inhibition of transport processes. Tubular reabsorption of amino acids is impaired at the level of ion-dependent or energy-requiring steps, so that a generalized aminoaciduria results, often with excessive loss of other solutes (e.g. oculo-cerebrorenal syndrome).

Inborn errors rarely occur with a frequency greater than 1 in 10,000 live births in outbreeding populations. Those few that show higher frequencies in certain populations (2 - 30 per 10,000 live births) may well be genetically heterogeneous (Brock, 1972). The majority of inborn errors are far less frequent. The incidence of some aminoacidopathies is shown in Table 1:1.

It could be argued that the extensive research into the inborn errors of metabolism has far outweighed their importance; that they are rare causes of clinical disability and that their study is unlikely now to contribute further to our understanding of genetics or metabolism. However, collectively they remain important causes of severe disability. The inborn errors are said to account for almost 10% of mentally retarded individuals in economically advanced

TABLE 1:1

Incidence of certain inborn errors of amino acid metabolism among newborn infants in Massachusetts.
(Data from Levy, 1973; from Harris, 1975).

Disorder	Incidence	Number Screened
Phenylketonuria	1:15,000	1,012,017
Cystinuria	1:15,000	350,176
Hartnup disease	1:16,000	350,176
Histidinaemia	1:17,500	350,176
Argininosuccinic acidaemia	1:70,000	350,176
Cystathioninaemia	1:117,000	350,176
Homocystinuria	1:160,000	480,271
Branched-chain ketoaciduria	1:175,000	872,660
Hyperglycinaemia (non-ketotic)	1:175,000	350,176
Propionic acidaemia	<1:350,000	350,176
Hyperlysinaemia	<1:350,000	350,176

populations (W.H.O. Report, 1969). With the decline in importance of infectious and nutritional disease, genetically determined diseases have become more important. The causes of most of these conditions are still largely obscure. But as Garrod himself realised the inborn errors "are merely extreme examples of variations of chemical behaviour which are probably everywhere present in minor degrees and .... far more subtle than those of form" (Garrod, 1902), so that the principles of biochemical analysis and treatment which are developing from their study are likely to underly further advances in those more subtle or common variations still to be elucidated. Three main areas of research into inborn errors are of outstanding importance in this respect. (1) Increased understanding of the molecular nature of the enzymic lesion and its clinical consequences. This is being facilitated by advances in cell culture and by the use of animal models. (2) Improved means of treatment, which is now shifting in emphasis from dietary means towards enzyme replacement, stabilisation or enhancement. (3) Improved and extended methods of antenatal diagnosis, so that the opportunity to selectively abort those with severe, incurable disease is open to parents. This thesis will be largely concerned with the first of these areas.

(ii) Human histidinaemia

Biochemistry. Histidinaemia was first described in 1961 (Ghadimi et al., 1961a, b) when a phenylketone-like substance in the urine of a speech-retarded child turned out to be a breakdown product of histidine. The condition was shown to be characterised by abnormal accumulations of histidine or its derivatives in blood, urine and cerebrospinal fluid (Auerbach et al., 1962; Ghadimi et al., 1962; Shaw et al., 1963; La Du et al., 1963). The reported concentrations of histidine and its metabolites in histidinaemic and control subjects are shown in Table 1:2. In contrast, the products of histidine degradation such as urocanic acid in skin or sweat (La Du et al., 1962; Whitfield and Shepherd, 1970; Neville et al., 1972) and formiminoglutamic acid (FIGLU) in urine (La Du et al., 1963) were found to be deficient, even after loading the subjects with histidine. These findings suggest a defect in the major pathway

TABLE 1:2 Concentrations of histidine and its metabolites in skin, liver and body fluids from human histidinaemic and control subjects.

Metabolite	Plasma[1] mg./100 ml.		Urine[2] mg./g creat.		CSF[3] mg./100 ml.		Sweat[4] mg./100 ml.		Liver[5] mg./100 g		Skin[6] µmol/g. skin	
	Control	Patient	Control	Patient	Control	Patient	Control	Patient	Control	Patient	Control	Patient
Histidine	1·2	11·0	205·5	1823·8	0·1-0·3	0·9-2·1	-	-	10·3	62·4	-	-
Im pyruvate	-	-	-	64·7	-	-	-	-	-	-	-	-
Im lactate	-	-	9·6	267·6	-	-	-	-	-	-	-	-
Im acetate	-	-	3·4	106·7	-	-	-	-	-	-	-	-
N-ac histidine	-	-	8·4	50·8	-	-	-	-	-	-	-	-
Urocanate	-	-	-	-	-	-	1·9	0·06	-	-	61·2	6·9

1 Control data from Auerbach et al (1962), Stein and Moore (1954) and Baldridge and Greenberg (1963). Histidinaemic data from Appendix (mean of 68 histidinaemics).

2 Mean urinary imidazoles in 7 histidinaemic and 24 controls (Wadman et al 1971).

3 Data of Wadman et al (1967) and Anakura et al (1975) on 3 histidinaemics. Shaw et al (1963) also found a raised CSF histidine in another patient: however, Ghadimi et al (1962) found only a marginal increase and Berlow et al (1965) no definite increase.

4 Data from Auerbach et al (1967) on 7 histidinaemics and 17 controls.

5 Data from Auerbach et al (1967) on liver biopsies from 1 histidinaemic and 2 controls.

6 Data from Whitfield and Shepherd (1970) on 5 histidinaemics and 53 controls.

of histidine degradation, namely deamination to form urocanic acid and ultimately glutamic acid (See Fig. 1:1). The presence of large accumulations of imidazolepyruvic, imidazolelactic and imidazoleacetic acids in the urine of histidinaemics implied an increased flux through the alternative transamination pathway. An oral histidine load in these subjects produced further prolonged accumulations of histidine and its imidazole acid derivatives in urine, but still no demonstrable urocanic acid, imidazolone propionic acid or FIGLU (Auerbach et al., 1962; Ghadimi et al., 1962; La Du et al., 1963, Holton, 1964). When an intravenous urocanic acid load was administered, it was metabolised normally to imidazolone propionic acid and FIGLU, suggesting a normal pathway from urocanic acid to glutamic acid. These findings provided strong but indirect evidence of a specific defect in the first enzyme of the pathway, histidase (histidine ammonia-lyase EC4.3.1.3.). Final confirmation came with the finding of a greatly reduced or absent histidase activity in the epidermal layer of skin in histidinaemics (La Du et al., 1962; Zannoni and La Du, 1963). Histidase activity in liver biopsies taken from two patients with histidinaemia also showed greatly reduced activity compared with controls (Auerbach et al., 1967). The diagnosis of histidinaemia is therefore suggested by a sustained increase in the plasma and urinary histidine and in its urinary transamination products and confirmed by a reduced or absent skin histidase activity. A reduced or absent excretion of urinary FIGLU or urocanic acid after a histidine load and reduced sweat urocanic acid also help to confirm the diagnosis. 136 presumed cases of histidinaemia have been reported in the literature since 1961 (Appendix). Skin histidase activity has been reported in 53 of them. All but three of these show reduced or absent skin histidase activity. The three siblings reported by Woody et al., (1965) had slightly raised plasma and urinary histidine concentrations, increased urinary imidazolepyruvic acid concentrations but normal skin histidase activity. The authors suggested that their subjects had a defective liver histidase and normal skin histidase. This seems unlikely because of the fact that all other histidinaemics apparently lose the activity of both enzymes simultaneously and because,

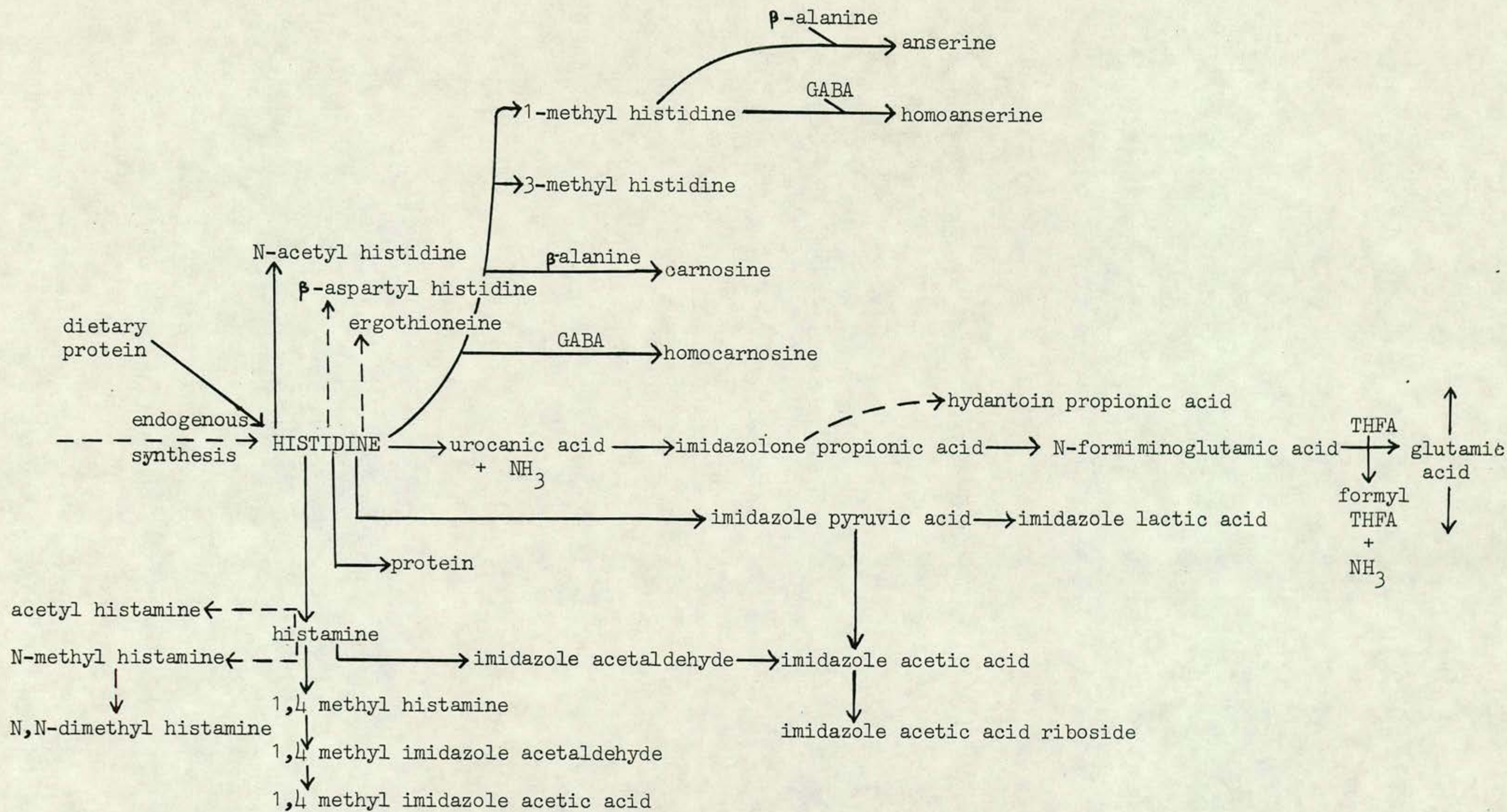


FIG. 1:1 The metabolism of histidine and its derivatives.

at least in the rat, the two enzymes are identical by several criteria [(see Section (iv)]. The family described by Woody et al. is however the best documented example of genetic heterogeneity in histidinaemia. Levy et al. (1974) also report two siblings with slightly elevated plasma and urinary histidine concentrations and skin urocanic acid concentrations only reduced to about one-quarter of normal, which may be another example of "atypical" histidinaemia. Finally, Kothari et al. (1970) have described an atypical histidinaemic with an increase in skin urocanic acid concentration, although the skin histidase activity was not examined.

Other metabolic abnormalities have been reported intermittently. An increased alanine concentration in blood and urine has been reported (Ghadimi et al., 1961; Auerbach et al., 1962, 1967; Ghadimi and Zischka, 1967; Corner et al., 1968; Rosenblatt et al., 1970; Carton et al., 1970; Popkin et al., 1974; Armstrong, 1975). It is not clear whether this represents increased liver transamination of pyruvate with histidine or some other effect of the metabolic error (Ghadimi and Zischka, 1967). Only one of the 26 cases described by Levy et al. (1974) had an increased alanine concentration, so it is a relatively uncommon finding. The concentration of 5-hydroxytryptamine in platelet-rich plasma was found to be reduced by about 40% in one histidinaemic child compared with age matched controls (Auerbach et al., 1962). This was confirmed in some studies (Holton et al., 1964; Corner et al., 1968) but not others (Ghadimi and Zischka, 1967). Holton et al. (1964) reported that platelet 5-hydroxytryptamine returned to normal with a low-histidine diet. A reduction in the glutamic acid concentrations of blood and cerebrospinal fluid (CSF) has been reported (Ghadimi et al., 1962; Auerbach et al., 1967; Carton et al., 1970). The CSF glutamine concentration has also been found to be low (Ghadimi et al., 1962), a finding that was confirmed by Berlow et al., (1965) but not by Holton et al. (1964).

Genetics. Human histidinaemia is inherited in a manner consistent with an autosomal recessive character (La Du, 1972; Ghadimi, 1974). Popkin et al. (1974) examined the frequency of

histidinaemia in 63 siblings of 21 histidinaemic probands and found it to be 0·238 compared with an expected 0·25 with an autosomal recessive character. Levy et al. (1974) also found that, excluding their propositi, 7 out of 26 siblings were affected in 20 families with histidinaemia identified by routine newborn screening. Their parents all had normal blood and urinary histidine concentrations. The ratio of male to female patients identified was 12:14. Heterozygosity in the parents and sibs of histidinaemics has been supported by the demonstration of skin histidase activity at or below the lower limit of normal (La Du et al., 1963; Holton et al., 1964; Cain and Holton, 1968; Gatfield et al., 1969; Neville et al., 1972). La Du (1972) reviewed the reported histidase activities in histidinaemics and their parents and found that the mean parental activity is well below control values. This finding is consistent with the parents being heterozygous carriers of the mutant gene with codominant inheritance of the enzyme defect. Histidine loading has been used to identify heterozygotes (Ghadimi and Partington, 1967) although it is not possible to unequivocally distinguish heterozygotes from normal controls in this way (Hague and Holton, 1971; Neville et al., 1972). La Du (1972) has suggested that a more sensitive method of detecting heterozygotes may be to monitor the urinary excretion of histidine and FIGLU or urocanic acid after a histidine load. Consanguinity in the parents of histidinaemics has been reported in three families with histidinaemia (Woody et al., 1965; Rostenberg et al., 1974; Rao et al., 1974). A family with histidinaemia in two successive generations has been reported by Bruckman et al. (1970). The authors suggested an autosomal dominant mode of inheritance in this instance. However, they assumed that the father of three affected children was not a heterozygote since he showed no increase in serum or urinary histidine and a FIGLU test was normal. However his skin histidase activity was relatively low at 1·4 μmol/hr./g. (lower limit of normal 1·5). Since most authors have not been able to unequivocally distinguish heterozygotes from normals, it seems more likely that this man was heterozygous for histidinaemia, especially since his mother's sister, who was histidinaemic, had seven unaffected children. The hypothesis of dominant inheritance is therefore implausible. All other reported

cases of histidinaemia affect only one generation, with approximately equal numbers of males and females (La Du, 1972).

Incidence. Since many known histidinaemics show no clinical abnormality, an unknown number are likely to remain undetected in the community. Random or total population screening of blood histidine concentrations must therefore be employed to determine the incidence of histidinaemia. Guthrie's bacterial inhibition test is now routinely used in some centres to screen for raised blood concentrations of several amino acids including histidine (Guthrie, 1968; Guthrie and Murphy, 1971). A positive result from screening with the bacterial inhibition or paper chromatographic methods must then be confirmed by a quantitative method, usually ion-exchange chromatography. A diagnosis of histidinaemia is likely if the plasma concentration is consistently raised above 0·3 - 0·4 μmol/ml. (5-6 mg./100ml.). Since transient states of histidinaemia have been described in infants (Thalhammer et al., 1971), the diagnosis is normally confirmed by low or absent histidase activity in skin and a reduction in skin urocanic acid. Newborn populations have been screened for histidinaemia in at least 13 different countries (Table 1:3). In all cases the diagnosis has been confirmed by quantitative amino acid analysis, although in many cases a definitive diagnosis by skin histidase assay was not made. The overall incidence is in the region of 1 in 19,000 live births. Histidinaemia is therefore one of the more common aminoacidopathies (see Table 1:1) with a frequency similar to that of phenylketonuria.

Clinical features. Although many histidinaemics show no overt clinical abnormality, others show varying degrees of mental or neurological impairment (La Du, 1971; Ghadimi, 1974). However the relationship between any clinical abnormality and the metabolic condition is by no means clear. The Appendix contains a summary of the 136 reported cases to date. Intelligence has been assessed with varying degrees of accuracy in 113 of them (excluding 5 atypical cases). About one-quarter (27/113) of this sample of histidinaemics is mentally retarded (IQ < 70) compared with 2% in the general population (Penrose, 1963; Clarke and Clarke, 1974). However this difference can in part be attributed to ascertainment bias. Those histidinaemics with clinical

TABLE 1:3

The incidence of histidinaemia in different populations estimated from newborn screening surveys.

Country	Test	Number Screened	Number Detected	Incidence
Australia	Berry	230,000	3	1:77000[2,5]
Austria	GBIA (His)	141,137	9	1:16000[5]
Denmark	GBIA (His)	30,000	0	0 [5]
Great Britain	GBIA (His)	110,000	10	1:11000[3]
	Scriver	87,982	4	1:22000[5]
	Woolf	6,565	0	0 [5]
New Zealand	GBIA (His)	81,747	7	1:12000[5]
Poland	Berry	72,357	0	0 [5]
United States	Efron	245,379	14	1:18000[5]
	GBIA (His)	70,148	2	1:35000[5]
7 countries		215,503	18	1:12000[4]
	TOTAL	1,290,818	67	1:19000

1 GBIA (His) is the Guthrie Bacterial Inhibition Assay, as applied to histidine screening. The methods of Scriver, Berry and Efron are paper chromatographic methods using blood (Scriver) or dried urinary (Berry, Efron) samples.

2 It is virtually certain that several additional infants with histidinaemia are present in this screened population. The authors were unable to obtain blood specimens from several infants with persistent histidinaemia, to confirm the diagnosis.

3 Neville and Lilly, 1973.

4 Schmid-Ruter, 1972. Survey of newborn in Belgium, France, W. Germany, Ireland, Netherlands, Switzerland and the United Kingdom.

5 Sources quoted in Levy, 1973.

disability are more likely to reach the attention of the medical profession and those with severe disability are more likely to be reported in the literature. Levy et al. (1974) examined the mental status of 59 reported histidinaemics according to the reason for ascertainment. Out of 25 children reported to be mentally retarded (IQ< 85), 19 were ascertained because of an already recognized mental subnormality, 3 because of a speech problem and only 3 for some reason unrelated to their mental function. On the other hand, out of 33 histidinaemic children ascertained in routine newborn surveys or because of a medical problem unrelated to mentality, only 3 were mentally subnormal (2 also had speech defects). 85% of histidinaemics, whose ascertainment was not biased towards the detection of mental subnormality, were therefore found to be normal. Popkin et al. (1974) also tried to exclude the ascertainment bias in a survey of the siblings of 21 late-diagnosed histidinaemic probands. 91% of the probands were retarded compared with only 40% of their histidinaemic siblings whose abnormality only came to light as a result of the index sibling. The 60% normality figure of these workers may be lower than that of Levy et al. (1974) because of stricter clinical criteria which excluded at least one family with normal histidinaemic siblings. Levy et al. (1974) also tested the IQs of 12 histidinaemics identified in newborn surveys and 14 unaffected siblings. The tests were carried out at an average age of 7 and 8 years in the two groups and without a knowledge of the diagnosis or nature of the study. The mean (± S.D.) IQ scores were 107 ± 12 and 108 ± 11 for histidinaemic and non-histidinaemic groups respectively. Further prospective studies of subjects identified in random or total population surveys should help to confirm the above findings which suggest a relatively small (5 - 10 fold) increase in the risk of intellectual impairment in histidinaemia.

Other signs of neurological damage, such as electroencephalogram (E.E.G.) abnormalities, epileptic seizures, poor co-ordination, tremor, ataxia and dysarthria have been reported in some histidinaemics (see Appendix). These signs tend to be worse in the more severely retarded. Again it is not clear if there is a relationship with the metabolic defect. One of the first clinical abnormalities reported in association

with histidinaemia was a speech defect (La Du et al., 1963; Witkop and Hendry, 1963; Gerritsen, 1964, Ghadimi and Partington, 1967; Bakwin, 1968). Witkop and Hendry (1963) suggested that defective speech, particularly impaired articulation and auditory memory, may be a specific consequence of the metabolic defect. Lott et al. (1970) studied the speech of four histidinaemic children and found that all had normal speech, although some had poor auditory memory which was related either to low intelligence, cultural deprivation or a conductive hearing loss. They then examined the relationship between reported IQ and speech in 36 histidinaemics. 92% of those with IQs less than 85 had abnormal speech compared with 38% of those with IQs greater than 85. They concluded that the speech and language abnormalities noted in histidinaemia are not specific to the disorder but are mainly related to concomitant subnormality of intelligence. Other investigators have reached a similar conclusion (Gordon, 1970; Kibel and Levy, 1970; Neville et al., 1972). However some cases with speech defect and apparently normal overall development have been described (Massengill and Smith, 1967). Neville et al. (1972) pointed out the association between seizures, low IQ and speech problems in some histidinaemics. They also noted the occurence of neurological deterioration at times of frequent seizures. Other clinical abnormalities have been intermittently and probably fortuitously associated with histidinaemia (Appendix).

Since at least three-quarters of all histidinaemics are likely to be clinically normal, any causal association between histidinaemia and intellectual impairment is certainly very much weaker than in the biochemically comparable condition of phenylketonuria (PKU). Possible reasons for this difference have been suggested. Firstly, the molar concentration of amino acid is two to three times higher in PKU than in histidinaemia. Possible reasons for this include differences in the flux through alternative pathways (e.g. transamination: see Ghadimi and Partington, 1967), differences in the dietary content of histidine and phenylalanine (Seakins and Holton, 1969) and the higher renal clearance of histidine compared with phenylalanine (Rosenberg and Scriver, 1974). However the occurence of intellectual damage may not simply be a function of the plasma amino acid concentration. Neville et al. (1972) reporting on 4 severely retarded and 3 intellectually normal histidinaemics found no difference in plasma or urinary histidine

or its metabolites between the retarded and normal patients. Anakura et al. (1975) did find such a difference in two siblings, only one of whom was retarded and showed a higher plasma and urinary histidine concentration. Retarded subjects have been reported with plasma histidine concentrations as low as 2 - 4 mg% (Woody et al., 1965; Whitfield and Shepherd, 1970; Rostenberg et al., 1974) and normal subjects with 10 - 13 mg% (Armstrong, 1975; Stevens et al., 1975). Experience in PKU suggests that tissue concentrations correlate more appropriately with intellectual development than plasma concentrations (Andrews et al., 1973). Since the net uptake of phenylalanine from blood into brain is higher than for histidine (Pratt, 1976), it may be that a combination of lower plasma concentrations and restricted brain uptake in histidinaemia is less damaging to the developing brain than in PKU. The importance of the amino acid concentration is supported by the finding of a lower incidence of mental retardation in atypical PKU where the phenylalinine concentration is about half of that in classical PKU (Tourian and Sidbury, 1978) and that partial phenylalanine restriction in PKU, allowing a 5 - 10 fold plasma increase, is not associated with a drop in intelligence (Koch et al., 1973). A second possible reason for the difference between histidinaemia and PKU is in the toxicity of the amino acid metabolites (see Chapter 6).

Intellectual function is influenced by numerous interacting factors, some of which are likely to be particularly important in an aminoacid-opathy. These include the occurence of intercurrent infection, injury or debilitation, diet, fluid intake, renal function, concomitant epilepsy and, perhaps above all, the effect of known illness or handicap on parental attitudes, educational and social opportunities. These and other environmental factors are most likely to be important in the mildly subnormal group who are probably overepresented in histidinaemics compared with the general population. Considerable caution must therefore be used before attributing the occurence of intellectual impairment to a direct effect of altered metabolites.

In 1971 Neville et al. reported a case of maternal histidinaemia, analogous to the condition of maternal phenylketonuria (Stevenson and Huntly, 1967; Allan and Brown, 1968). The case described was that of

a histidinaemic woman with random plasma histidine concentrations in the region 7 - 10 mg./100 ml., giving birth to a boy who was normal in all respects, at least until aged 4½ years. Lyon et al. (1974) found another family of five children all born to a histidinaemic mother. Four of the children were old enough to undergo precise intellectual assessment. The IQs were closely grouped, with mean full scale IQ (86·5 - 96·5) about 20 points less than the mid-parent value (106·5 - 116·5). Since the mean IQ of children is expected to approximate the mid-parent value, the authors suggested a possible causal relation with the maternal histidinaemia. Such a relation has yet to be confirmed by others. Again these findings are in striking contrast with classical phenylketonuria, where mental retardation is almost invariable in the non-PKU offspring. However, Levy and Shih (1974) have suggested that foetal brain damage does not occur in mild (3 - 6 mg%) or moderate (6 - 12 mg%) hyperphenylalaninaemia, occurs to a small extent in atypical PKU (12 - 15 mg%) and to a major extent in classical PKU (20 - 40 mg%). In maternal PKU other abnormalities such as growth retardation, microcephaly and abnormalities of skeleton, heart and eyes have also been reported (Stevenson and Huntly, 1967; Allan and Brown, 1968; Mabry, 1963, 1966; MacCready and Levy, 1972). Dietary treatment of PKU mothers during pregnancy may help to prevent abnormality in the offspring and has led to the birth of a number of normal children (Allan and Brown, 1968). The case for dietary treatment of histidinaemic mothers remains to be demonstrated, especially in view of the hazards of such diets (Cain and Holton, 1968; Neville et al., 1972).

Treatment. The discovery that a reduced dietary intake of phenylalinine, starting in early infancy, reduces the incidence or severity of mental deficiency in PKU (reviewed by Knox, 1972) opened the way to dietary treatment of other aminoacidopathies. Such treatment is mainly applicable to the dietary-essential amino acids. There is some controversy as to the need for dietary histidine in man. Ackroyd and Hopkins (1916) first reported the need for dietary histidine in the young, growing rat. Since then it has been shown that histidine is an essential amino acid during the growing phase of all animal

species investigated including the human infant (Strecker, 1970). Omission of histidine from the diet of human infants results in failure to gain weight, decreased nitrogen retention and the appearance of a rash, all of which disappear on reintroduction of histidine to the diet (Snyderman et al., 1963; Snyderman, 1965). These workers conclude that the dietary requirement for histidine is rather less than 35 mg/Kg/day. In contrast, pre-adolescent children and adults can maintain their weight and nitrogen balance on a histidine-free diet for periods up to four weeks (Rose et al., 1951; Nakagawa et al., 1963; Wixon et al., 1977). Histidine is therefore regarded as semi-essential in man, but in all other animals it is indispensible for growth or maintenance of nitrogen balance (Strecker, 1970; Stifel and Herman, 1972).

There are several reports of the effect of a reduction in the dietary histidine intake in histidinaemia (Table 1:4). These reports show that it is possible to lower the plasma histidine concentration to near normal values with a histidine intake of 20 - 40 mg/Kg/day in infants and young children. Older children (roughly over five years) may require a lower histidine intake and control takes longer and is more difficult to regulate. The need for dietary control in these older children is not established even in PKU (Lancet, 1974). The clinical response to introduction of a low-histidine diet has in the majority of cases been disappointing. Only two patients showed some clear-cut improvement (Thalhammer et al., 1971; Neville et al., 1972). Three patients showed impairment of growth on the diet (Van Sprang and Wadman, 1967; Corner et al., 1968; Cain and Holton, 1968). In view of the dangers and inconvenience of such diets and the evidence that intellectual impairment is much less likely to occur in histidinaemia than in PKU, their routine introduction would seem unjustified. If there are indications for introducing the diet, this should be done as early as possible, preferably within the first four weeks of life, and continued for about two years, provided that growth is adequate (see Griffiths, 1973).

(iii) Murine histidinaemia

Biochemistry. In 1973 Kacser et al. reported the discovery of a mouse mutant with a twenty to thirty-fold increase in the plasma

TABLE 1:4 Summary of the reports on the use of a low-histidine diet in the treatment of histidinaemia.

Authors	Age when diet introduced	Duration of diet	Histidine content of diet	Control of plasma histidine	Clinical change or progress on diet
Ghadimi et al., 1962	3 years 4 years	3 days 3 days	24 mg/Kg/day 374 mg per day	Slight reduction Slight reduction	No change No change
La Du et al., 1963	5 years	4 days	4 mg/Kg/day	No change	No change
Holton, 1964	6 months	Not specified	32-38mg/Kg/day	Good	Not specified
Waisman, 1967	26 months $3\frac{1}{2}$ years $11\frac{1}{2}$ months	1 month 1 month 3 weeks	Not specified Not specified Not specified	Halved Not specified Halved	No change No change No change
Auerbach et al., 1967	$9\frac{1}{2}$ years	5 weeks	Histidine free	Good	No change
Van Sprang and Wadman, 1967	1 year	2 years	15-28 mg/Kg/day	Satisfactory	Favourable progress in speech and motor development. Inadequate growth.
Corner et al., 1968	7 months	$2\frac{3}{4}$ years	30-35 mg/Kg/day	Good for 18 months then poor	Cessation of growth from 20 months till death at 41 months.
Cain and Holton, 1968	$1\frac{1}{2}$ years	11 months	40 mg/Kg/day	Poor	Slowing of growth.
Gatfield et al., 1969	5 years 6 years	4 months 3 months	15-40 mg/Kg/day 20-30 mg/Kg/day	Good Good	No change No change
Thalhammer et al., 1971	$5\frac{1}{2}$ months 5 months $4\frac{1}{2}$ months	6-9 months 9 months 8 months	30-40 mg/Kg/day 30-40 mg/Kg/day 30-40 mg/Kg/day	Good Good Good	Ataxia improved on diet. No change No change

TABLE 1:4 continued

Authors	Age when diet introduced	Duration of diet	Histidine content of diet	Control of plasma histidine	Clinical change or progress on diet
Neville et al., 1972	3 years	Not specified	Not specified	Adequate	Improved
	12 years	Not specified	Not specified	Adequate	No change
	4½ years	Not specified	Not specified	Adequate	No change
Wadman et al., 1973	1 year	10-15 days	20 mg/Kg/day	Good	
	3yrs 5 months	10 days	20 mg/Kg/day	Good	
	10yrs 11 months	12 days	19·5 mg/Kg/day	Moderate reduction	Diet stopped after plasma
		21 days	7 mg/Kg/day	Good; takes longer to achieve than in younger subjects	histidine normalized.
Griffiths, 1973	12 weeks	1¾ years	Not specified	Good	Developing normally
	17 weeks	7 months	Not specified	Good	Developing normally
	8 weeks	1yr 10 months	Not specified	Good	Developing normally
	8 weeks	1yr 10 months	Not specified	Good	Developing normally
Popkin et al., 1974	10 weeks	> 6 months	25 mg/Kg/day	Good	Developing normally
Duffner and Cohen, 1975	1 year	13 months	34 mg/Kg/day	Good	No change
Kotsopoulos and Kutty, 1979	10 years	3 weeks	Histidine free	Moderate reduction	No change

histidine concentration and an enzyme abnormality strikingly similar to human histidinaemia (Table 1:5). The discovery was made in the course of an amino acid screening survey of mutant mice. The mutant was found in a stock of mice derived from animals caught in the wild in Peru in 1962 (Wallace, 1971). Some of the stock showed a "balance defect" consisting of abnormal circling and head tilting behaviour (Wallace, 1970, 1971). Elevated histidine concentrations were found in the animals, some of which were behaviourally normal, others of which were balance defective. The histidine concentration was also found to be increased in the urine, liver, brain and skin.

Increased concentrations of several histidine metabolites were found in the mutant (Kacser et al., 1973; Bulfield and Kacser, 1974, 1975). In the urine, concentrations of imidazolelactic acid (ILA) and imidazoleacetic acid (IAA) were increased seventeen and twentysix-fold respectively. It was not possible to separate N-acetyl histidine from imidazole pyruvic acid (IPA) chromatographically but the combined peak was increased thirty-fold in urine. In the plasma, these compounds were again increased at least ten to twenty-fold, although none were detectable in the wild-type. The concentrations in liver and brain were also increased (Table 1:6). The histamine concentration was measured in whole brain and found to be two-fold higher in histidinaemics, compared with a twelve-fold increase in brain histidine (Bulfield and Kacser, 1975). When histidinaemic mice were put on a 6% histidine supplemented diet, brain histamine was only increased by a further 40%.

Histidine accumulations of this magnitude, coupled with the increased concentration of its transamination products, suggested a block in the major catabolic pathway, namely deamination to urocanic acid. The activity of the first enzyme in the pathway, histidase, was measured in the livers of histidinaemic and non-histidinaemic mice. The former all had drastically reduced histidase activity (less than 5% of normal; Kacser et al., 1973). Skin histidase was also found to be about one-third of normal in 7-day old histidinaemic mice. The mutation therefore appeared to result in defective histidase activity with accumulation of metabolites prior to this step. The

TABLE 1:5

Comparison of mouse and human histidinaemia (Bulfield and Kacser, 1974)

	Mouse	Man
Gene	Autosomal recessive	Autosomal recessive
Enzyme activity in liver	Histidase <5% of normal	Histidase absent
	Histidinaemic/normal ratio	Histidinaemic/normal ratio
Plasma		
Histidine	28·7	5 to 25
Urine		
Histidine	21·5	2 to 20
Im lactate	17·3	15 to 125
Im acetate	25·8	46 to 66
Acetylhistidine	29·0	16 to 22
Skin		
Urocanate	0·45	0·05 to 0·11
Histidase	0·29	0·02 to 0·5
Test for heterozygotes		
Plasma histidine	Not distinguishable	Not distinguishable
Histidase	Distinguishable (sex difference)	Possibly distinguishable
Histidine loading	Distinguishable	Distinguishable

TABLE 1:6

Biochemical characterization of histidinaemic mice (Bulfield and Kacser, 1974, 1975)

Tissue	Histidinaemia	Normal	R = H/N
Liver			
Histidase	0·013	0·280	0·046
Histidine	20·9	0·880	23·7
Ac histidine + Im pyruvate [1]	0·111	0·0082	13·5
Im lactate	0·102	0·0069	14·8
Im acetate	0·017	<0·0044	>3·7
Urine			
Histidine	0·86	0·04	21·5
Ac histidine + Im pyruvate	3·19	0·11	29·0
Im lactate	1·21	0·07	17·3
Im acetate	1·29	0·05	25·8
Plasma			
Histidine	3·30	0·115	28·7
Ac histidine + Im pyruvate	0·016	<0·0008	>20·0
Im lactate	0·012	<0·001	>12·0
Im acetate	0·008	<0·001	>8·0
Brain			
Histidine	2·16	0·169	12·8
Ac histidine + Im pyruvate	0·0044	<0·0006	>7·3
Im lactate	0·0204	<0·0007	>29·1
Im acetate	<0·004	<0·0006	-
Histamine	1·02	0·548	1·9

Note: All values are the means of between 5 - 10 animals. Liver and brain metabolites: µmol/g. wet tissue. Plasma and urine metabolites: µmol/ml. All determinations by column chromatography and ninhydrin or Pauly reaction respectively.

1 In the buffer system used, these two substances were not completely resolved. The values reported are the sum of both.

Im, imidazole.
Ac histidine, N-acetyl histidine.

urocanic acid concentration in skin was measured in 7-day old histidinaemic and control mice (Bulfield and Kacser, 1974). It was found to be reduced from 1·2 μmol/g. to 0·54 μmol/g. This gives a crude estimate of the flux from histidine to urocanic acid as the next enzyme in the pathway, urocanase, is absent in skin. No attempt was made to check the patency of the pathway distal to the histidase step either by urocanic acid or FIGLU loading. Bulfield, Whitehouse and Kacser (in press) measured the flux from histidine to glutamate using tracer methods and found it to be about 40% of normal. The finding of a more than 95% reduction in histidase activity coupled with a 60% reduction in the flux is perhaps reconciled by a substantial mass action effect on the flux as a result of the twenty-fold increase in steady state histidine concentration (Kacser et al., 1979a). There is a sex difference in wild-type hepatic histidase activity with a female/male activity ratio of about 3/2, but this was not apparent in histidinaemic mice (Bulfield and Kacser, 1974).

Genetics. The inheritance of histidinaemia was investigated by crossing a stock of true-breeding "high histidine" mice to biochemically normal mice from an inbred strain (C57BL/6J) (Kacser et al., 1973). The F_1 progeny showed normal histidine concentrations. The intercross (F_2) progeny segregated for high and low histidine concentrations to give a ratio not significantly different from 3:1. These results suggested the presence of an autosomal recessive character with complete penetrance. The mutant allele was designated *his*. The recessive hypothesis was tested further by examining histidase activities in heterozygous matings (*his*/+ x *his*/+). By this means it should be possible to identify heterozygotes since codominant expression is the rule at the level of enzyme activity even if recessive expression occurs at the metabolic or clinical levels (Paigen, 1971). Hepatic histidase activities in the offspring of heterozygous matings showed segregation into three classes. The numbers in each class did not differ significantly from 1:2:1 (Kacser et al., 1973). The mean histidase activity of the intermediate group was 45% of the high group mean. Histidase activity was then examined in animals true breeding for "high histidine" (presumed *his*/

his), wild-type animals (+/+) and the intercross offspring of these two genotypes (presumed his/+). Again three classes of activity were found, corresponding to those seen in the segregating litters. These findings support the hypothesis that the his allele is an autosomal recessive character.

Behaviour. After the discovery of histidinaemia in some of the Peru strain mice, it became clear that the balance defect did not segregate with the metabolic defect. In fact, only the offspring of histidinaemic mothers were balance defective, regardless of their own or their fathers' genotype. This strongly suggested a maternal effect (Kacser et al., 1973). The balance defect showed variable expressivity and consisted of some or all of the following features; circling behaviour, head tilting, deafness, inability to swim, lack of disorientation after spinning and poor maze learning (Kacser et al., 1973). The penetrance of the defect varied in the offspring of histidinaemic mothers. A colony of Peru mice had been established in Cambridge with 80% penetrance of the balance defect. This was maintained by selection, but on relaxation of selection the penetrance dropped to 37% in 1974 and 7% in 1977 (Mya Mya, 1978). Outcrossing to other strains of mice such as AG/Cam or C57BL/6J also resulted in a reduction of the penetrance. The plasma histidine concentrations of the stocks showing different penetrance were not found to be significantly different (Kacser et al., 1977). Penetrance was therefore strongly influenced by the genetic background, but apparently not via differences in plasma histidine concentration.

The lesion underlying this balance defect has been shown to be a series of inner ear abnormalities (Kacser et al., 1973, 1977; Bulfield and Kacser, 1974, Mya Mya, 1978). These abnormalities include distorted semi-circular canals, shortened crus commune, reduced pigmentation, absent or abnormal otoliths, enlarged or distorted cochlea and enlarged ampullae. These changes were present in varying degrees in one or both ears. The inner ears of litters taken from (1) his/his x his/his and (2) his/+ x his/+ crosses, were examined by Dr Mary Lyon without a knowledge of the behavioural or metabolic assessments made on the same animals. It was found that all the balance defective offspring from cross (1) showed severe vestibular damage and two-thirds

of the behaviourally normal offspring of this cross also showed mild damage. All the offspring of cross (2) were behaviourally and histologically normal, whether they were biochemically histidinaemic or normal (Bulfield and Kacser, 1974). A histological examination of balance defective histidinaemic brains and spinal cords was carried out by Dr H. Fraser. There was no evidence of histological abnormality in overall cellular morphology and the nerve fibres and myelin sheaths were intact (Kacser et al., 1973). It therefore appears that the behavioural abnormality segregating within the histidinaemic stock is due to a maternal teratogenic effect on the structural development of the inner ear.

The timing of this maternal effect was investigated further by administering a high-histidine diet to his/+ animals during the second week of pregnancy. Fourteen out of 130 offspring born to heterozygotes on 8% histidine-supplemented diets were balance defective compared with none out of 209 on a normal diet. The diet raised blood histidine concentrations to near histidinaemic values. (Kacser et al., 1977). Administration of the high-histidine diet during the first or third weeks of pregnancy produced no abnormal offspring. When a low-histidine diet was administered to histidinaemics during the second week of pregnancy, the plasma histidine was reduced by a factor of six and the proportion of balance defective offspring declined from 26% to 3% (Kacser et al., 1977). Similarly, changing the diet of histidinaemics during the second week of pregnancy to a 2% histidine-supplemented diet did not change the proportion of balance defective offspring. Loading +/+ mice with 5 or 10% phenylalalanine or lysine-supplemented diets during the middle week of pregnancy did not produce balance defective offspring so that the phenomenon is probably histidine-specific. Mutant mice with prolinaemia were also found to produce non-balance defective litters. Finally, to try and reproduce the balance defect in +/+ mice, +/+ embryos were transplanted into pregnant his/his mice (Kacser et al., 1977). Coat colour markers distinguished the transferred eggs. No balance defective transplant +/+ offspring were found, but eight out of 15 had structural abnormalities in the canals or otoliths of the inner ear, similar to those found in balance defective animals.

Additional maternal effects of the his/his genotype were found on litter size and growth. The mean litter size was reduced in histidinaemic mothers to about 67% of the normal at birth, and a further reduction occurred up to weaning (Bulfield and Kacser, 1974). The three-week and seven-week weights of histidinaemics born to his/his mothers were reduced compared with those born to his/+ mothers.

Preliminary experiments (Bulfield and Kascer, unpublished) showed impaired maze learning in his/his mice. However, a subsequent study was unable to confirm this (MacKenzie and Kacser, unpublished). The occurrence of severe degrees of mental defect in the offspring of human PKU mothers suggested that brain damage may also be occurring in the offspring of histidinaemic mice (probably independent of the balance defect). This seemed to be an interesting possibility since the plasma histidine concentration in his/his mice is about thirty times normal, which is comparable to the difference in phenylalanine concentration in PKU. In human histidinaemia, the difference is only ten-fold. Also, the effect on brain development could be expected to be greater where there is prenatal and postnatal exposure to increased histidine concentrations. For this reason, the experiments on brain development (Chapter 4) were carried out on the his/his offspring of his/his mothers. Any changes found would therefore be associated with a combination of genotypic and maternal effects.

(iv) The metabolism of histidine and its derivatives (see Figure 1:1)

Dietary requirement [see section (ii)]

Protein metabolism. Radioactively labelled histidine has been fed to rats, mice and guinea pigs and was incorporated into body proteins (Tesar and Rittenburg, 1947; Borsook et al., 1950, 1952). A number of amino acids have relatively specific roles in determining the configuration or binding properties of polypeptides. Histidine, with its lone electron pair in the ring nitrogen, may serve as a potential metal ligand as in the iron-binding proteins haemoglobin and cytochrome C. The release of histidine from body protein during periods of increased net catabolism (e.g. fever, trauma) could perhaps exacerbate the metabolic abnormality in histidinaemia. When this was investigated by Holton et al. (1968) in a histidinaemic child during febrile illnesses, no significant increase in urinary or plasma histidine was found. The

experience with PKU has been different, since Woolf et al. (1958) have reported the return of clinical signs and loss of dietary control during infectious illnesses in patients otherwise well controlled on low-phenylalanine diets. However this deterioration was not shown to be specifically related to increased protein catabolism.

Methylation. The 1- and 3-methyl derivatives of histidine were originally thought to be derived only from dietary or bacterial sources. Urinary 1-methylhistidine is derived from the breakdown of the endogenous dipeptides, anserine (β-alanyl 1-methylhistidine) and homoanserine (γ-aminobutyryl 1-methylhistidine) (Meister, 1965). Urinary excretion of 3-methylhistidine, which is largely derived from skeletal muscle, has been used as an index of muscle breakdown (Ward and Buttery, 1978). There is evidence that histidine is methylated in the N (N-3) position of the imidazole ring after its incorporation into muscle proteins and that this compound is not recycled into protein after its release (Munro, 1974). There is also evidence for ring N (N-1) methylation of histidine when it is in the dipeptide carnosine (McManus, 1956). The ring methylated derivatives of histidine are not incorporated directly into body proteins. The 2-methylated derivative of histidine has been reported in a dipeptide with β-alanine (Ophidine), but this has only been found in snake muscle (Meister, 1965).

Dipeptides. There are three histidine-containing dipeptides known. Carnosine is a dipeptide of histidine and β-alanine and is found mainly in the skeletal muscle of vertebrates (Cantarow and Schepartz, 1967). Along with creatinine and anserine, carnosine is one of the three most abundant compounds in the non-protein nitrogen fraction of vertebrate muscle (Martignoni and Winnick, 1954). Margolis (1974) noted that very high concentrations of carnosine are present in the mouse olfactory epithelium and the olfactory bulb which may be acting as a neurotransmitter in the primary olfactory pathway. A rare disorder called carnosinaemia has been described in which there are neurological symptoms, a raised concentration of carnosine in body fluids and carnosinase deficiency (Perry et al., 1967).

Homocarnosine is the dipeptide of γ-aminobutyric acid (GABA) and

histidine. It has been reported in the CNS of mice and several other animal species (Quinn and Fisher, 1977). Both carnosine and homocarnosine can be methylated by an enzyme found in a variety of rat tissues (Pisano et al., 1961; Nakajima et al., 1967) to form anserine and homoanserine respectively.

β-aspartyl histidine is another histidine-containing dipeptide, which has been found in human urine (Kakimoto and Armstrong, 1961), but whose significance remains unknown. Three families with a combination of cerebromacular degeneration and histidine peptiduria (increased carnosine and anserine excretion) have been reported (Levenson et al., 1964; Tocci and Bessman, 1967).

Thiol derivatives. Ergothioneine is the betaine of thiol histidine and was first isolated from the fungus ergot (Tanret, 1909). It is not known to be formed in mammals and is regarded as "strictly exogenous" (Cantarow and Schepartz, 1967).

Acetylation. N-acetyl histidine has been reported in the urine of mammals (Baldridge and Tourtellotte, 1958; Brown et al., 1960). It has been shown to be excreted in increased quantities in both human (Neville et al., 1972) and mouse (Kacser et al., 1973) histidinaemia. The function of N-acetyl histidine is not known, although it has been suggested that it acts as an acetyl donor (Anastasi et al., 1964; Hanson, 1966). It was not found to be synthesised in the mouse brain after intracerebral injection of ^{14}C-histidine (van Balgooy et al., 1972) although this does occur in frog brain (Baslow, 1967).

Transamination. Transamination reactions are important both for the synthesis and breakdown of many amino acids. In mammals histidine has not been found to undergo transamination with the amino-acceptors α-ketoglutarate or oxaloacetate to a significant extent (Lin et al., 1958; Albers et al., 1962; Haavaldsen, 1962; Meister, 1965). Transamination of histidine with α-ketosuccinamic acid has been demonstrated in rat liver preparations to yield imidazolepyruvic acid and asparagine (Meister and Fraser, 1954). An active histidine-pyruvate aminotransferase has been found in the liver of rats, mice and humans (Lin et al., 1958; Spolter and Baldridge, 1963; Auerbach et al., 1967; Budillon et al., 1971; Morris et al., 1973; Noguchi et al., 1976a, b,; Minatogawa et al., 1977; Bulfield, 1978). This is thought to be

the main enzyme catalysing the transamination of histidine. There is disagreement in the literature over the number and intracellular distribution of the histidine aminotransferase isozymes. The existence of cytosolic and mitochondrial forms of the enzyme, differing in Km, heat stability and pH profile, was first reported by Spolter and Baldridge (1963). These two forms of the enzyme were also shown to differ in their response to glucagon (Morris et al., 1973). Recently two isozymes have been purified to homogeneity from rat and mouse liver by Japanese workers (Noguchi et al., 1976a, b,; Minatogawa et al., 1977), who found that both isozymes appear in the cytosolic and the mitochondrial fractions. The isozymes were found to differ in their isoelectric points. Only one of the isozymes was inducible by glucagon. Bulfield (1978) helped to clarify the situation by reporting a variant form of the cytosolic enzyme which occurred independently of the mitochondrial enzyme. This result tends to support the original suggestion of a cytosol isozyme and a mitochondrial one, rather than both isozymes being present in the two locations.

Substrate specificity and affinity studies were carried out by Noguchi et al. (1976a, b) on the purified histidine aminotransferase isozymes. Both isozymes were found to show greater activity with phenylalanine or tyrosine as substrates than with histidine, although the Km for histidine was lower than for phenylalanine in one of the isozymes. Isozyme I was found to occur only in the liver while Isozyme II was present in all rat tissues examined.

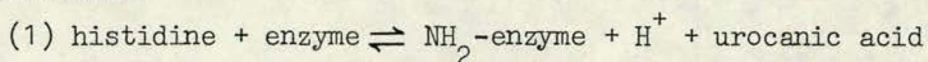
The development of mouse liver histidine aminotransferase activity has not been described. Changes in the developing rat liver phenylalanine-pyruvate transaminase, which may be identical to histidine-pyruvate aminotransferase, have been reported (Auerbach and Waisman, 1959). The activity was low until the day before birth when it increased dramatically until two days after birth, remaining high (about three times adult activity) until about two weeks, then gradually falling to adult levels over the next fortnight. A similar sharp increase in the activity of tyrosine transaminase occurs after birth, reaching levels two to four times those of the adult, in several species (Sereni et al., 1959; Litwack and Nemeth, 1965; Moog, 1971). The premature development of tyrosine transaminase can be reproduced

by glucagon administration (Moog, 1971). The liver isozyme of histidine aminotransferase is also inducible by glucagon (Lee and Harper, 1971; Morris et al., 1972; Noguchi et al., 1976 a). The products of histidine transamination with pyruvate are imidazole pyruvic acid and alanine. The former is either reduced to imidazolelactic acid or decarboxolated to imidazoleacetic acid. Imidazoleacetic acid is also a product of histamine metabolism in some organisms, by the action of histaminase (or diamine oxidase) via imidazole acetaldehyde (Schayer, 1966).

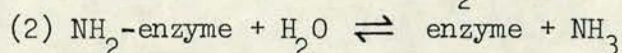
Deamination. Non-oxidative deamination of histidine to urocanic acid is thought to be the major route of histidine catabolism in mammals (Meister, 1965). This route was first described by Edlbacher (1926) who found that the mammalian liver decomposes histidine with with the liberation of ammonia. The degradation of histidine to urocanic acid has since been reported in the livers of rat, mouse, rabbit, dog, cat, guinea pig and man (Hall, 1952; Borek and Waelsch, 1953; Mehler and Tabor, 1953; Coltorti et al., 1966). The enzyme responsible has been called histidase (Edlbacher, 1926) or histidine ammonia-lyase (EC 4.3.1.3.). Although the liver is thought to be the main site of histidine degradation, histidase is also present in the epidermal layer (stratum corneum) of mammalian skin (Schwartz, 1961; Zannoni and La Du, 1963). The histidase activity of the skin appears to be located in the epithelial cells and is not present in fibroblasts (Barnhisel et al., 1970; Melancon et al., 1971). There are few reported systematic studies of histidase distribution in mammalian tissues. Zannoni and La Du (1963) were unable to detect histidase activity in guinea pig kidney or muscle but found very low activity in intestine, testis, spleen and heart. Bulfield and Kacser (1975) were unable to detect histidase activity in mouse brain. Histidase activity has also been studied in fish, where it occurs in liver and muscle (Sakaguchi et al., 1970). Several bacteria are rich in histidase activity and one of the best sources of the enzyme is the bacterium Pseudomonas (Tabor and Mehler, 1955).

Histidase reaction mechanisms. The reaction involves a trans-elimination of the α-amino group and β-proton of L-histidine to form trans-urocanic acid (Tabor, 1954; Kuroguchi et al., 1957; Peterkofsky,

1962; Givot et al., 1969; Retey et al., 1970; Hanson and Havir, 1972). Peterkofsky (1962) suggested that this reaction takes place in two steps. He found that in addition to the irreversible deamination of histidine, histidase catalyses two partial reactions: (1) a β-hydrogen exchange into histidine; (2) a urocanic acid exchange into histidine. Since solvent tritium and ^{14}C-urocanic acid were incorporated into histidine in contrast to $^{15}NH_3$, he suggested that an amino-enzyme complex is formed, which either reversibly reforms histidine or is irreversibly degraded to ammonia and free enzyme. Givot et al. (1969) and Wickner (1969) later suggested that the α-amino group of histidine may bind covalently to a dehydroalanyl residue in the active site of histidase. Removal of the β-proton and rupture of the C-N bond would then result in formation of urocanic acid and the amino-enzyme complex. The two steps envisaged are therefore:-



(1) histidine + enzyme $\rightleftharpoons$ NH_2-enzyme + H^+ + urocanic acid



(2) NH_2-enzyme + H_2O $\rightleftharpoons$ enzyme + NH_3

Reaction (1) is freely reversible and reaction (2), although thought to be irreversible by Peterkofsky, can be reversed under certain conditions, at a very slow rate (Williams and Hiroms, 1967). The equilibrium constant of the overall reaction is about five (Klee et al., 1975). Further support for amino-enzyme formation comes from the non-competitive inhibition of the reaction by urocanate with respect to histidine, suggesting that inhibition occurs by reversal of reaction (1) (Givot et al., 1969). Specificity studies show that only histidine exchanges with tritium and only urocanic acid accepts the amino group in reaction (1) (Givot et al., 1969).

The stimulating effect of metal ions and inhibitory effect of metal chelators on the elimination reaction (Mehler and Tabor, 1953; Tabor, 1954; Peterkofsky and Mehler, 1963; Cornell and Villee, 1968; Rechler, 1969; Givot et al., 1970; Klee, 1972) suggests that metal ions operate at the rate-determining step which may be the rupture of C-H and/or C-N bonds in the α, β-elimination (Klee et al., 1975). Givot et al. (1970) suggest that a divalent metal ion forms part of an enzyme-metal-substrate bridge complex, which would facilitate the removal of the β-proton from histidine in the elimination reaction.

A number of different divalent metal ions (Mn^{+2}, Mg^{+2}, Zn^{+2}, Cd^{+2}, Fe^{+2}) have been shown to stimulate histidase activity although it is unclear which is the cofactor in vivo (Klee, 1972; Okamura et al., 1974). It is likely that the metal is bound to free -SH groups at the active site (Peterkofsky and Mehler, 1963; Klee, 1972; Okamura et al., 1974). Oxidation of active site sulphydryl groups, which occurs during ageing or purification of the enzyme, results in a marked loss of activity (to 10 - 20% of normal), which can be restored by addition of sulphydryl reagents (Tabor and Mehler, 1955; Peterkofsky and Mehler, 1963; Givot et al., 1969; Soutar and Hassall, 1969; Klee, 1970; Frankfater and Fridovich, 1970; Okamura et al., 1974). Oxidation of free sulphydryl groups is associated with a loss of affinity for metal ions (Klee, 1972) and the activating effect of sulphydryl reagents is lost in the presence of the metal chelator EDTA (Okamura et al., 1974). The loss of histidase activity that occurs with ageing, prolonged dialysis or purification (Tabor and Mehler, 1955) is also associated with polymerization of enzyme monomers (Soutar and Hassall, 1969; Klee, 1970; Okamura et al., 1974). This polymerization can be reversed by addition of thiol reagents. There is also evidence from sequencing the SH-containing peptides isolated from the depolymerized enzyme, that disulphide bonds are formed between cysteine residues at the active site of different enzyme monomers (Hassall and Soutar, 1974). Finally, in addition to the divalent cation and sulphydryl groups which are required for optimal activity, an as yet unidentified prosthetic group, which may form part of the active site, is also thought to be present (Smith et al., 1967; Givot et al., 1969; Klee, 1970; Klee et al., 1979).

The enzyme shows a high degree of specificity for L-histidine (Brand and Harper, 1976) although 2-fluorohistidine and 4-nitro-L-histidine also act as substrates for the bacterial enzyme (Klee et al., 1975; Klee et al., 1979). Studies with inhibitors suggest that the enzyme interacts with the substrate specifically through the carboxyl, α-amino and imidazole moieties (Givot et al., 1969; Brand and Harper, 1976). The imidazole ring of the substrate may bind to the divalent metal ion, which in turn is attached to an enzyme-bound - SH group (Klee, 1972). The α-amino group is thought to bind to a highly-

reactive electrophilic dehydroalanine residue on the enzyme (Givot et al., 1969; Givot and Abeles, 1970). The nature of the carboxyl binding site is unknown.

Studies on purified mammalian histidase. Purified mammalian histidase has been less thoroughly studied than the bacterial enzyme. The latter appears to be a tetramer with a molecular weight in the region of 213,000 daltons, which exists in two ("oxidised" and "reduced") forms depending on its state of sulphydryl oxidation (Klee, 1970), as discussed above. Cornell and Villee (1968) purified rat liver histidase about 200-fold, to about 70% purity. Their preparation had an estimated molecular weight of 226,000 daltons by ultracentrifugal analysis. Okamura et al. (1974) purified rat liver histidase 390-fold and showed it to be homogeneous by ultracentrifugal, electrophoretic and immunoelectrophoretic analyses. They estimated the molecular weight to be 190,000 daltons. On electrophoresis there were two minor and one major band with histidase activity, which converted to a single, most anodal band after treatment with thiol reagents. These findings were interpreted as showing a tendency of the enzyme to aggregate by the formation of disulphide bridges, which are dissociated by thiol reagents. No attempt was made to dissociate the 190,000 dalton species into its subunits. Brand and Harper (1976) purified rat liver histidase more than 250-fold to near homogeneity. The molecular weight was estimated to be in the region of 200,000 daltons. Lamartiniere and Feigelson (1977) purified rat liver histidase 319-fold to homogeneity by electrophoretic and ultracentrifugal analyses. The native enzyme had an estimated molecular weight in the region of 230,000 daltons and could be dissociated into 77,000 dalton subunits. This would suggest three subunits, which is uncommon (Harris, 1975). In contrast, Brand and Harper (1975) suggested that the rat liver enzyme is composed of six subunits. Since the bacterial enzyme can be inadvertently hydrolysed to a smaller species during purification, yielding subunits of 35,000 daltons instead of 55,000 daltons, Brand and Harper (1976) later suggested that there may be a similarly labile peptide bond in the mammalian enzyme. A tetrameric structure still remains a strong possibility therefore, although this remains to be established.

Mouse liver histidase has been purified in two inbred strains to 55% and 70% purity respectively (Hanford and Arfin, 1977). Electrophoretic analysis showed a single band with histidase activity.

The rat liver enzyme has a minimum Km for L-histidine of 0·5 mM at pH 9·0, increasing progressively at higher or lower pH (Brand and Harper, 1976). The Km value of 0·5 mM, approximately twice the fasting liver histidine concentration (Herbert et al., 1966), increases to more than 2·0 mM in the physiological pH range of 7·2 - 7·4. The optimal pH for activity is in the region 8·5 - 9·0 (Okamura et al., 1974; Brand and Harper, 1976). The purified enzyme is activated three or more fold by prior incubation with thiol reagents. These reagents have little or no effect at earlier purification stages. The response to thiol reagents and metal chelators is similar in bacterial and mammalian histidase preparations. Rat liver histidase is, like the Pseudomonas enzyme, irreversibly inhibited both in vitro and in vivo by the carbonyl reagent nitromethane (Givot and Abeles, 1970). Hydrolysis of the ^{14}C-nitromethane inactivated enzyme gives identical products with rat liver and bacterial preparations, supporting the existence of a similar electrophilic centre in both enzymes (Givot and Abeles, 1970). Identification of these products suggested the presence of a dehydroalanine residue in the active site of both enzymes.

The properties of partially purified mouse liver histidase (Hanford and Arfin, 1977) have not been studied in detail. The Km for histidine was found to be 0·9 mM at pH 9·3 and pH optimum was 9·0 - 9·5. The enzyme was relatively heat stable with a $t_{\frac{1}{2}}$ of 13 - 14 minutes at 53°C. Baden and Gavioli (1974) reported that at 75°C, partially purified rat liver histidase had a $t_{\frac{1}{2}}$ of 10 minutes. The bacterial enzyme is even more heat stable, retaining its activity after heating at 78 - 83°C for 15 minutes (Tabor and Mehler, 1955).

Developmental changes in mammalian histidase. The development and regulation of rat liver and skin histidase has been extensively investigated (Auerbach and Waisman, 1959; Baden et al., 1968; Makoff and Baldridge, 1969; Sahib and Krishna Murti, 1969; Feigelson, 1968, 1971 a, b, c, 1972, 1973 a, b, c, 1974; Bhargava and Feigelson 1976 a, b,; Lamartiniere and Feigelson 1977; Lamartiniere and Lucier, 1978).

The pattern of developmental changes in histidase activity is quite different in liver and skin. The liver enzyme appears at or shortly after birth, rises during the first week, plateaus and increases further at puberty. In females, the increase at puberty is more rapid and to higher levels than in males (Auerbach and Waisman, 1959; Feigelson, 1968; Makoff and Baldridge, 1969; Feigelson, 1973 b). The skin enzyme is detectable at or a few days before birth, increases in the next week then falls to a low level in the adult, although in the male the activity drops further at puberty (Baden et al., 1968; Sahib and Krishna Murti, 1969; Feigelson, 1972, 1974).

In the liver, several hormones have been shown to modify the expression of histidase, each influencing activity at a particular stage of development and in a characteristic manner. Glucagon (acting via cyclic AMP) and glucocorticoid have been implicated in the induction of the neonatal rise in activity (Feigelson,1973 a, b). Glucocorticoids may also be involved in the adolescent increase in the male (Feigelson, 1973 a, b) and oestrogen in producing the markedly enhanced activity in females (Feigelson, 1968, 1973 b). Glucagon can increase histidase activity at all postnatal stages; glucocorticoids only during the first two postnatal months but not in mature animals; oestrogen only after the first postnatal month (Feigelson, 1973 b). The hormonal regulation of skin histidase has not been extensively studies, although glucocorticoids (Bhargava and Feigelson, 1976 a) and androgens (Feigelson, 1974) may be involved.

Feigelson and coworkers purified histidase to homogeneity and prepared a monospecific antibody to examine the nature of these developmental changes in activity. Bhargava and Feigelson (1976 b) showed that all changes in histidase activity during development are accompanied by parallel changes in the amount of immunoprecipitable enzyme protein, in liver and skin. The rates of in vivo incorporation of (^{3}H) leucine into histidase immunoprecipitates were examined in skin and liver during development. Changes in histidase activity in both tissues were paralleled by corresponding changes in the rates of histidase synthesis (Bhargava and Feigelson, 1976 b). More recently, Lamartiniere and Feigelson (1977) have shown that oestrogen, glucocorticoid, glucagon and cyclic AMP induced changes in rat liver

histidase activity, are each paralleled by equivalent changes in immunoprecipitable histidase protein and in vivo rates of (^{14}C) leucine incorporation into immunoprecipitated histidase. It was therefore inferred that the effect of these hormones on liver histidase development is mediated by a change in histidase synthesis. Hormonal agents capable of supressing hepatic histidase activity (including androgen, ACTH, growth hormone and thyroxine) have been described, but their modes of action remain unclear. The complex interplay of hormones, diet, tissue responsiveness and other factors influencing histidase activity at each stage of development is still being elucidated (Schirmer and Harper, 1970; Lee and Harper, 1971; Morris et al., 1972, 1973; Noda and Nakagawa, 1972; Feigelson, 1973 b, Lamartiniere and Lucier, 1978). Developmental changes in histidase activity have not been investigated in the mouse.

Isozymes. The differing developmental patterns and regulatory properties of skin and liver histidase suggested the possibility of different molecular forms of the enzyme (isozymes) in these tissues. This was investigated by Bhargava and Feigelson (1976 a). They found that purified rat liver and partially (fifty-fold) purified skin histidase were identical by three criteria: isoelectric point, Km and immunological properties. In contrast, crude preparations of rat liver and skin histidase show slight differences in electrophoretic mobility on polyacrylamide gels (Baden and Gavioli, 1974; Bhargava and Feigelson, 1976 a), which are not removed by partial purification. It is not yet clear whether this difference is due to absorption of contaminant protein or to molecular differences, since it was not possible to completely purify the skin enzyme. Baden and Gavioli (1974) also reported heat stability differences between 10 to 20-fold purified skin and liver histidase. The liver enzyme had a $t_{\frac{1}{2}}$ of 10 minutes at 75°C, compared with a $t_{\frac{1}{2}}$ of 20 minutes for the skin enzyme. On the basis of the differing heat stability, electrophoretic mobility and sensitivity to EDTA inhibition, Baden and Gavioli postulated the existence of distinct skin and liver isozymes of histidase. The results of Bhargava and Feigelson (1976 a) using more highly purified enzyme however make this conclusion rather doubtful.

Pathway from urocanate to glutamate. Urocanic acid is thought by

some authors to have a protective action in the skin against ultraviolet light (Zenisek et al., 1955; Hais and Zenisek, 1959; Everett et al., 1961). Urocanate is found in high concentrations in human sweat (Zenisek and Kral, 1953) and is thought to diffuse into the sweat from skin epithelial cells rather than being produced by sweat glands (Brusilow and Ikai, 1968). Histidinaemics have a deficiency of urocanate in the skin, but there is no evidence of unusual skin sensitivity to light (Ghadimi, 1974) although dietary deprivation of histidine in human infants results in a skin rash which disappears on introduction of histidine to the diet (Snyderman, 1965). Urocanate is hydrolysed to 4-imidazolone-5-propionic acid by the action of the enzyme urocanase. This enzyme is found in the soluble cytoplasmic fraction of liver, but is not present in skin (Zannoni and La Du, 1963). Imidazolone propionic acid is converted by imidazolone hydrolase to N-formiminoglutamic acid (FIGLU) (Snyder et al., 1961). Some imidazolone propionic acid is transformed by a liver oxidase to hydantoin-5-propionic acid, which has been found in the urine of the rat, monkey and man (Brown and Kies, 1959; Hassall and Greenberg, 1963). Tetrahydrofolic acid (THFA) combines with FIGLU to form L-glutamic acid and 5-formyl-tetrahydrofolic acid. The latter is converted further to yield "active formaldehyde" (10-formyl tetrahydrofolic acid) and free ammonia. The conversion of FIGLU to L-glutamic acid is catalysed by three separate enzymes (Meister, 1965). Histidine therefore contributes indirectly as a source (along with glycine and tryptophan) of formyl THFA which can be used directly in purine synthesis or converted to hydroxymethyl-THFA for use in transmethylation and pyrimidine synthesis. A genetic defect in the metabolism of FIGLU has been demonstrated in man as a result of a formiminotransferase deficiency (Arakawa et al., 1966; Arakawa, 1970). The syndrome includes mental and physical retardation, neurological abnormalities and increased urinary excretion of FIGLU after an oral histidine load, in the presence of abnormally high serum folate levels.

Decarboxylation. Histidine is converted to the physiologically active metabolite histamine by enzymatic decarboxylation. In adult animals, only an estimated 1% of ingested histidine is metabolised

to histamine (Van Arsdel and Beall, 1960). However, in certain physiological and pathological states (Kahlson and Rosengren, 1968), this percentage is likely to be considerably higher. The role of histamine in physiological processes has recently been reviewed (Schwartz, 1977; Beaven, 1978).

Histidine decarboxylase catalyses the specific formation of histamine from histidine (Schayer, 1966). L-aromatic amino acid decarboxylase can utilise histidine as a substrate together with DOPA or 5-hydroxytryptophan, but is thought to play a minor role in histamine formation in most tissues (Aures et al., 1972; Schayer and Reilly, 1974 a, b). The latter enzyme has a low affinity for histidine and is distinguished from histidine decarboxylase by its pH optimum and response to inhibitors or activators (Weissbach et al., 1961; Ganrot et al., 1961; Lovenberg et al., 1962). Histidine decarboxylase (HDC) requires pyridoxal phosphate as a coenzyme both <u>in vitro</u> and <u>in vivo</u> (Ono and Hagen, 1959; Kahlson et al., 1963). Schayer has shown that the activity of HDC can be induced by a number of non-specific agents. On the basis of these findings, he suggested that histamine is important in the regulation of microcirculatory blood flow. HDC activity can also be regulated by hormones such as oestrogen and catecholamines which enhance activity and glucocorticoids which depress activity (Kahlson and Rosengren, 1968).

HDC is the only enzyme that is known to metabolise histidine to any extent in the mammalian brain. It has been studied in rat brain (Schwartz et al., 1970, 1971, 1972) and mouse brain (Taylor and Snyder, 1972; Schayer and Reilly, 1974 a, 1975). The development of mouse brain HDC has not been studied. Intraperitoneal injection of histidine in the mouse increases whole brain histidine and histamine concentrations about three-fold, with increases evident in all brain regions except the medulla oblongata-pons (Taylor and Snyder, 1972). Bulfield and Kacser (1975) found a two-fold increase in whole brain histamine in histidinaemic mice, which increased to three-fold with a 6% histidine-supplemented diet. These findings suggest that mouse brain HDC is not saturated with histidine under physiological conditions.

Histamine formation by HDC shows some unusual features during

pregnancy in some species. In the pregnant mouse, urinary histamine excretion rises dramatically soon after fetilisation, reaching a peak at about the eighth day of gestation and remaining high until term, when it falls precipitously to near normal values (Rosengren, 1963). Similar increases in urinary histamine excretion are found in the pregnant rat and hamster (Figure 1:2). The increased histamine excretion has been shown to be largely due to high foetal HDC activity, although maternal tissues also show higher than normal HDC activity (Kahlson et al., 1958; Rosengren, 1963; Maudsley and West, 1964). The physiological significance of these changes in histamine formation during gestation are obscure. In the pregnant his/his mouse, exposure of foetal HDC to increased histidine concentrations might lead to even higher rates of histamine formation than occur normally in pregnancy. To speculate further, enhanced gestational histamine formation could be related to the inner ear teratogenic effect of maternal histidinaemia. Schnieder (1975) has suggested that, because of the topography of the perilymphatic vascular network, the inner ear microcirculation is especially sensitive to vasoactive substances such as histamine. Intracochlear administration of histamine is associated with a significant reduction in microcirculatory flow. Increased circulating histamine could perhaps cause prolonged labyrinthine ischaemia, leading to permanent damage to the developing inner ear. A mechanism of this sort is given further credence by the successful use of a histamine analogue (betahistine) to increase the microcirculation in the inner ear in a human condition (Ménière's Disease) characterised by damage to otoliths which may also be the result of labyrinthine ischaemia (Martinez, 1972; Wilmot and Menon, 1976; Frew and Menon, 1976).

There are two major pathways of histamine degredation in mammals (Schayer, 1959). The first is by direct oxidative deamination by histamines (Buffoni, 1966) to imidazoleacetaldehyde, which is rapidly converted to imidazoleacetic acid by the action of aldehyde dehydrogenase (Tabor, 1951). Imidazoleacetic acid is a neuroactive compound which exerts a powerful inhibitory effect on cortical neurons in some species (Green, 1970; Tunnicliff et al., 1972). It is conjugated with 5-phosphoribosyl-1-pyrophosphate to form imidazoleacetic acid ribotide,

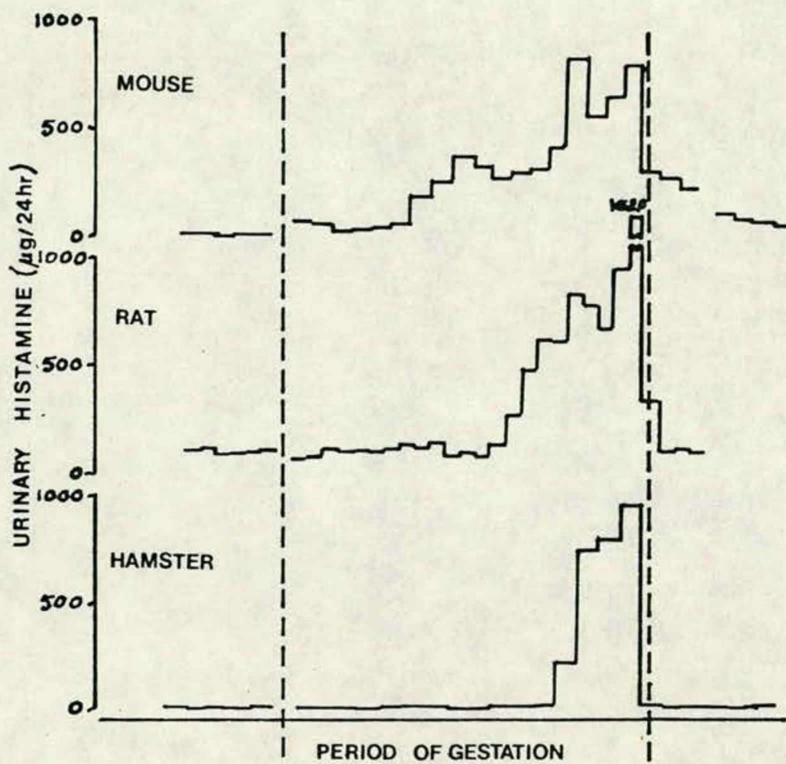


FIG. 1:2 Daily urinary excretion of free histamine in the mouse, rat and hamster. Periods of gestation were 19, 24 and 16 days, respectively (Kahlson and Rosengren, 1968).

which is then dephosphorylated to the riboside (Crowley, 1964). In the mouse, there is an active liver histaminase which oxidises a large proportion of an oral or injected histamine load (Karjala et al., 1956, Schayer, 1956), but in this species, as in man, methylation is the major route for histamine degradation under most circumstances (Schayer 1959).

Methylation of the imidazole nitrogen (N1) of histamine is catalysed by the specific enzyme imidazole-N-methyltransferase (INMT) (Brown et al., 1959). S-adenosyl methionine acts as a methyl donor. The immediate product is N^{τ}-or 1, 4-methyl histamine, which is oxidised to methylimidazoleacetic acid (Schayer and Karjala, 1956). 1, 4-methyl histamine is oxidatively deaminated by monoamine oxidase to form methylimidazoleacetaldehyde (Rothschild and Schayer, 1958; Waldmeier et al., 1977). This is oxidised by aldehyde dehydrogenase to methylimidazoleacetic acid, which is the major urinary degradation product of histamine in the mouse (Karjala et al., 1956; Schayer, 1959). Some other histamine derivatives have been reported, including acetylhistamine (Urbach, 1949) N-methylhistamine, N,N-dimethylhistamine (Kapeller-Adler and Iggo, 1957) N^{π}-or 1, 5-methylhistamine and 1, 5-methylimidazoleacetic acid (Karjala and Turnquest, 1955), γ-glutamylhistamine (Konishi and Kakimoto, 1976). Histamine also forms amides with certain fatty acids (Bachur and Udenfriend, 1966) and peptidoamines in the nervous system (Reichelt et al., 1975). All these compounds are quantitatively minor derivatives of histamine.

(v) Genetic variation in histidase activity between inbred strains of mice.

Arfin and coworkers (Hanford et al., 1974; Hanford and Arfin, 1977) measured hepatic histidase activity in 26 inbred strains of mouse. The inbred strains fell into two groups, showing either a "high" activity (20 - 25 µmol/hour/g. wet weight) or "low" activity (10 - 15 µmol/hour/g. wet weight). A high activity strain (C57BL/6J) was crossed to a low activity strain (C3H/HeJ) and the histidase activity measured in the F_1, F_2 and backcross generations. The results of these crosses and reciprocal backcrosses suggested the presence of a single autosomal genetic difference which could account for the major part of the observed differences in activity. Liver histidase was then

partially purified from the livers of the high and low strains. No difference was found between the two sources of enzyme during purification or on polyacrylamide gel electrophoresis. The final preparations were 55 - 70% pure, but specific staining for histidase activity on the gels revealed a single, identical band in both strains. Comparison of pH optimum, Km for histidine, heat stability, electrophoretic mobility, elution after gel filtration on Sephadex G-200 and inhibition by histidine analogs revealed no difference between the strains. Antibodies to the partially purified enzyme were raised in rabbits and used in immunotitration experiments to determine the quantity of histidase enzyme present in liver supernatants from the two strains. The amount of enzyme antigen was found to be proportional to the level of enzyme activity in both strains, suggesting a difference either in the rates of histidase synthesis or degradation. Rates of enzyme degradation were measured _in vivo_ from the recovery of activity after irreversible inhibition by nitromethane. There was no difference in the rate of histidase degradation. The rate of histidase synthesis, as measured by the incorporation of (^{3}H)-leucine into immunoprecipitated histidase, was about 2·5 times greater for the high activity strain. The authors concluded that the activity difference could be completely accounted for by a higher rate of histidase synthesis in the high strain. These investigations raise the possibility of a regulatory locus influencing histidase synthesis but distinct from the structural locus. The electrophoretic analysis, however, cannot be regarded as definitive and should include comparison at different pH's and gel concentrations or isoelectric focusing. A subtle change in the enzyme structure could have been missed. The presence of intermediate histidase activity in the F_1 generation after crossing high and low activity strains would be equally consistent with a cis-active regulatory gene or codominant expression of structural genes. Definitive evidence for a regulatory mutation requires that the mutant map to a separate site from the structural locus. Identification of the _his_ locus with the structural locus for histidase and its genetic mapping are therefore important steps in the elucidation of this strain difference in histidase activity.

(vi) Outline of investigations undertaken.

The investigations undertaken fall into three parts. Firstly, the biochemical characterisation of the developing his/his mouse. The his/his mice used were all the offspring of his/his mothers, so combining the effects of maternal histidinaemia and histidase deficiency. +/+ Mice on the same genetic background were used for comparison. While the metabolic status of the adult mutant is well documented, the possibility of metabolically induced damage to the developing brain requires that a similar documentation be made during the period of brain development. The relationship between changes in the histidine metabolising enzymes and their precursor and product metabolites has therefore been examined during development.

Secondly, biochemical indices of brain growth and cell proliferation have been examined during postnatal development in the his/his offspring of his/his mothers, in comparison with +/+ mice. Although neuronal multiplication and morphogenesis occur largely before birth (except in the cerebellum), the indices of brain growth were only examined during the first five weeks after birth. This was done because of the greater technical difficulties of ageing and dissecting the animals before birth, while any interference with prenatal brain development is likely to leave a permanent deficit (mainly of cell numbers; Altman et al., 1970), which would therefore still be evident after birth. Also, the effect of histidinaemia on neuronal multiplication has been examined directly in the cerebellum, which is almost wholly formed after birth. The postnatal phase of brain development is characterised by an important "growth spurt". During this growth spurt the brain is particularly vulnerable to metabolic or toxic interference. It is during this phase of development that the brain damage in phenylketonuria occurs.

Thirdly, the nature of the enzyme defect in his/his mice has been investigated. This provides an indirect but important indication of its genetic basis. The residual histidase activity has been scrutinised for evidence of a change in those physico-chemical properties (e.g. heat stability, electrophoretic mobility) suggestive of an alteration in the primary structure. If found, this suggestsa mutation at the structural locus for the enzyme. Alternatively, it could be

the result of a mutant gene concerned with the post-translational processing of the enzyme (e.g. attachment of carbohydrate or other conjugant groups). If there is no change in one of these physicochemical properties, the mutation could be at a regulatory locus controlling only the amount of enzyme protein, provided simple inhibition has been excluded. The properties of the residual mutant activity have been examined in crude and partially purified liver extracts.

CHAPTER 2

MATERIALS AND METHODS

(i) Animals and breeding methods.

The mice used in these experiments were derived from a stock of wild mice trapped in Peru in 1962 (Wallace, 1971) and maintained in the Department of Genetics, Edinburgh University since 1971. After the discovery of histidinaemia segregating in the stock, selection for the *his* allele was carried out. After seven crosses a stock of true breeding *his/his* mice was obtained. Wild type (+/+) and heterozygous (*his*/+) mice on the same genetic background were also maintained. This stock (SEV) was routinely maintained as follows. Three crosses were made:-

(1) *his/his* x *his/his*

(2) *his*/+ ♀♀ x *his/his* ♂♂

(3) +/+ x +/+

The offspring genotypes of the second cross were distinguished on the basis of urinary "Phenistix" (Ames Co.) testing. This is a dip test based on the ferric chloride reaction which gives a grey-green colour in the presence of high urinary imidazole pyruvic acid, which is only found in *his/his* mice. The stock was maintained in this manner until 1976, with avoidance of brother-sister matings which were found to reduce litter size. During the course of experiments in which histidase activity was measured, it was found that some putative +/+ animals had histidase activity about half of that expected in this genotype. Definitive evidence that animals thought to be +/+ were in fact *his*/+ came when the offspring of one "+/+" x "+/+" mating was found to include an unequivocally histidinaemic mouse.

A programme to select unequivocal +/+ and *his/his* mice was carried out by Dr G. Bacon. Six male and six female parent +/+ mice were crossed to known biochemically histidinaemic (*his/his*) mice of the opposite sex. At least ten offspring of each cross were then bled from the tail vein after reaching seven weeks of age. The bloods were analysed for the total imidazole concentration by the Pauly reaction without prior chromatographic separation of individual compounds. This technique was shown to give a clear distinction between histidinaemic and non- histidinaemic bloods. Where female "+/+" mice were

being crossed to male his/his mice, the male was removed as soon as the female became pregnant. Where the test animal was a male being crossed to a his/his female, the male was left in the cage. In all cases, the offspring were removed at weaning (3 weeks) and the sexes separated. An animal was only identified as unequivocally +/+ if no his/his mice were found, out of at least ten test cross offspring. Some apparent +/+ animals tested were shown to be his/+ in this way and the procedure was continued until enough true +/+ males and females were obtained to set up new breeding stocks. Thereafter, only crosses (1) and (3) were routinely maintained, in view of the danger of his/+ being mistaken for +/+. Selection of animals for breeding stocks was routinely accompanied by blood imidazole or histidine analysis, rather than relying on Phenistix urine testing. His/his were only selected for mating if their balance defect was relatively mild or absent (especially in the case of females), since otherwise the survival of litters was reduced, owing to a tendency to trample on the young. Some selection against the balance defect was therefore occurring in the stock (see Kacser et al., 1979 b).

Breeding stocks were checked every morning and late afternoon for the presence of new litters. The day on which a new litter was found, was taken to be "day 0", the following day "day 1" and so on. Weaning was routinely carried out at three weeks after birth, when the sexes were also separated. "Adult" mice were taken to be at least seven weeks old.

(ii) Statistical methods.

The statistical tests used were described by Snedecor and Cochran (1967) and calculated on a Sumlock 325 digital computer (Compucorp).

(iii) Chemicals.

All chemicals were Analar grade obtained either from British Drug Houses, Koch-Light or Sigma Chemical Company unless otherwise specified. (^{3}H)-S-adenosyl-methionine was obtained from the Radiochemical Centre, Amersham.

(iv) Dissection of tissues.

Mice were killed by stunning, followed by cervical dislocation. Guinea pigs used in the extraction of imidazole-N-methyltransferase were killed by carbon dioxide inhalation and decapitation.

The liver was extracted from mice by opening the abdominal wall with sharp scissors, removing the liver and placing it on a square of

aluminium foil on crushed ice. The gall bladder was then removed.

Skin was dissected from the dorsal surface of the mouse with scissors. In older animals the moistened hair was first shaved off with a scalpel.

In the case of brain dissections, mice were first anaesthetised with ether and then decapitated on a chilled metal plate cooled to $4^{o}C$ and lying on an ice-salt mixture. The skull was opened with scissors and the brain carefully removed, blotted with filter paper and placed on the chilled plate. It was dissected by mid-collicular section which separated the forebrain (cerebrum) from the rest of the brain. The cerebellum was then separated from the pons medulla and the latter was discarded. The cerebrum consists mainly of the cerebral hemispheres but includes the corpus striatum, hypothalamus and other parts of the diencephalon and midbrain. The dissection procedure, based on that of Glowinski and Iversen (1968), is shown in Figure 2:1. The cerebral and cerebellar portions were separately wrapped in aluminium foil and immediately dropped into liquid nitrogen, where they were stored until use.

Blood was removed from animals either from the tail vein or at the time of death by decapitation. It was collected into 20 µl heparinised capillary tubes.

Urine was collected first thing in the morning by picking up the mouse and collecting the urine into a watch glass or capillary tube.

(v) <u>Extraction of tissues</u>.

(a) <u>Liver</u>. In preliminary experiments, the liver was extracted for histidase assay using the method of Kacser et al. (1973). This involved homogenisation in 3 x W/V 0·25 M sucrose/1mM EDTA/ 1mM dithiothreitol/ 50 mM Tris HCl pH 7·4, using 5 up and down strokes of a Tri-R glass-Teflon homogeniser (Camlab), at speed setting 10. The homogenate was then centrifuged in an MSE Superspeed 50 centrifuge at 60,000g in an angle rotor for 30 minutes at $4^{o}C$. The clear supernatant was used for histidase assays. In initial experiments it was found that EDTA was inhibiting histidase activity, as reported by others (Tabor and Mehler, 1955; Okamura et al., 1974), so it was omitted from the homogenisation medium. Dithiothreitol was also omitted from the extraction medium since it did not enhance the activity of crude extracts.

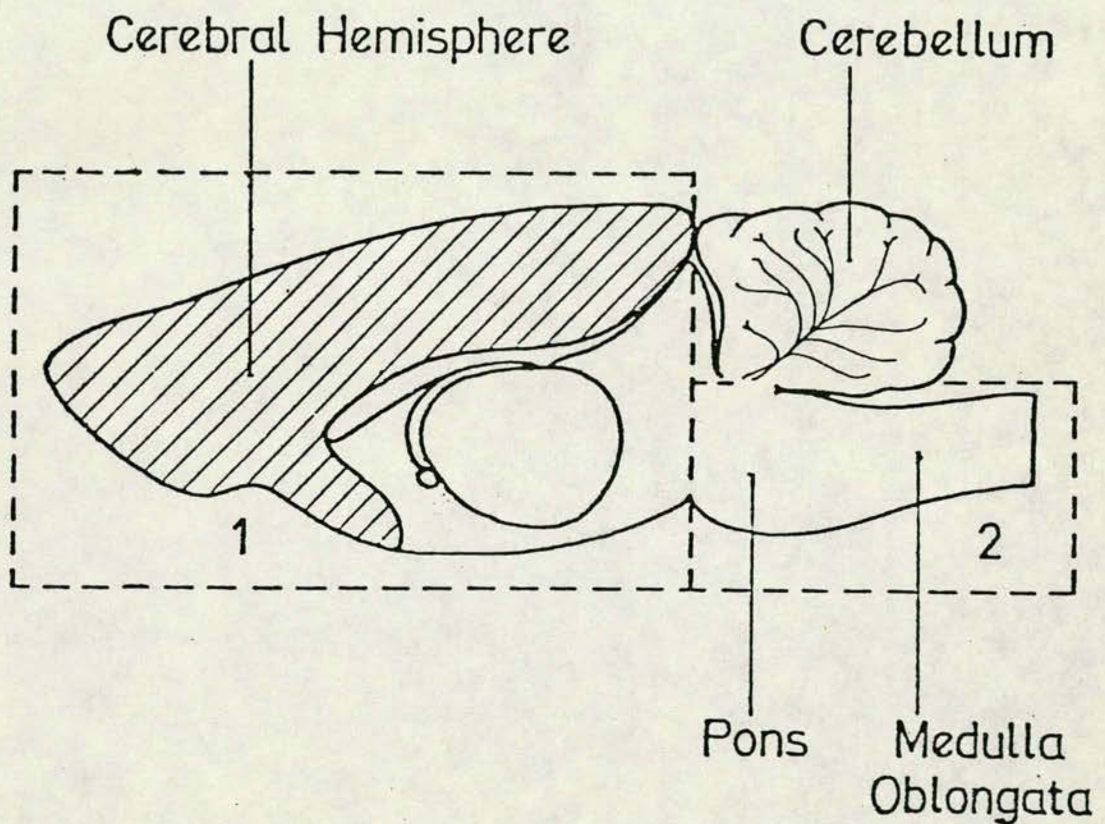


Figure 2:1 Diagram showing dissection procedure for mouse brain.

(1) The portion containing the cerebral hemispheres and diencephalic structures (cerebrum).

(2) The portion containing pons and medulla oblongata, not used in the experiments cited.

The portion containing the cerebellum is labelled.

In experiments on the development of histidase and histidine transaminase activity in mouse liver, the extraction method of Spolter and Baldridge (1963) was adopted. By using this, both enzymes could be assayed in the same sample. The liver was homogenised in 3 x W/V KCl 0·14 M/NaOH 0·005 N, as described above. The homogenate was then centrifuged in an MSE Superspeed 50 centrifuge at 105,000 g for 60 minutes at 4°C, using an angle rotor. The clear supernatant was aspirated and used for enzyme assays. Using this method, however, the mutant histidase activity was diminished or lost altogether. The method was retained for histidine transaminase assays but histidase was assayed separately after extraction in sucrose 0·25 M/Tris HCL 20 mM pH 7·4. Addition of the protease inhibitor phenylmethylsulphonyl-fluoride (PMSF, Sigma) (Lumsden and Coggins, 1977) to the homogenisation medium at a concentration of 1·2 mM did not affect histidiase activity in +/+ or his/his mice.

(b) Skin. Skin histidase activity was extracted as follows. The skin was first minced with scissors, weighed and added to a fixed volume (5 - 10 x W/V) of ice-cold sucrose 0·25 M/Tris HCl 50 mM pH 7·4. Five 10 second applications of a Polytron tissue disrupter were used, followed by 12 up-and-down strokes with a glass-Teflon homogeniser, cooling the tube in melting ice. The homogenate was then centrifuged at 60,000 g for 30 minutes at 4°C in an MSE Superspeed 50 centrifuge. The clear supernatant was used for assays.

(c) Brain. For histamine assays, the mice were killed by immersion in liquid nitrogen for five seconds followed by decapitation on to a plate chilled to 4°C. The skull was opened and the brain rapidly removed and dissected as described above. The samples were weighed and homogenised in 5 x W/V sodium phosphate 0·01 M pH 7·9 / Triton X-100 0·1% with five strokes of a glass-Teflon homogeniser at speed 10. The homogenate was then boiled in a 100°C waterbath for ten minutes in a capped conical glass centrifuge tube, in order to destroy endogenous S-adenosyl methionine. The tubes were cooled and centrifuged in an MSE bench centrifuge (speed 10) for five minutes and the supernatant was frozen at -20°C until use. For histidine decarboxylase assays, brains were extracted as above except that the boiling step was omitted.

Brain samples stored in liquid nitrogen were extracted for assay of nucleic acid, protein and lipid fractions as follows. Samples were removed from the liquid nitrogen store, quickly weighed (frozen) and put into glass homogenisation tubes in melting ice containing 5 - 10 x W/V perchloric acid 0·2 N/ - aminoguanidinopropionic acid (AGPA) 1mM. They were homogenised with five strokes of a glass-Teflon homogeniser at speed 8. Samples were then left for 15 minutes on ice for the protein to precipitate out. They were transferred to 10 ml. conical glass centrifuge tubes and the homogenisation tubes washed with a known volume of perchloric acid 0·2 N/AGPA 1 mM. The tubes were centrifuged in an MSE Highspeed 18 centrifuge at 2,500 g for 15 minutes at $4^{o}C$ (angle rotor). The supernatant was removed and the pellet washed in 1-2 volumes of perchloric acid 0·2 N/AGPA 1 mM and centrifuged at 2,500 g for 10 minutes. The supernatants were combined and used for amino acid analysis after removal of perchlorate. A second wash was then carried out with 15 - 20 volumes perchloric acid / AGPA which was discarded since it only contributed a further 2 - 3% to the amino acid concentration of the combined supernatant and first wash. The pellet was used for DNA, RNA, lipid and protein extraction.

(d) Blood. Blood samples from a heparinised capillary tube were expelled into a plastic test tube and mixed with distilled water and a known volume of internal standard, AGPA 1mM. The sample was vortexed in order to haemolyse the cells. An aliquot of 30% sulphosalicylic acid was added and the tube vortexed to precipitate protein. The sample was then stored at $-20^{o}C$ prior to amino acid analysis. The final mixture was therefore as follows:-

0·02 ml. heparinised whole blood

0·08 ml. distilled water

0·05 ml. AGPA 1mM. Vortex to lyse cells.

0·05 ml. sulphosalicylic acid 30%. Vortex and store at $-20^{o}C$.

(e) Urine. Urine collected from a watch glass or capillary tube was put into a plastic test tube on ice. Protein was precipitated by adding one volume of 30% sulphosalicylic acid to two volumes of urine. The sample was vortexed and stored at $-20^{o}C$ for analysis of urinary imidazoles and creatinine.

(vi) Enzyme assays.

(a) Histidase. The assay method used initially was that of Kacser et al. (1973). This contained sodium phosphate buffer, 0·065M pH 9·4, reduced glutathione 5 mM, L-histidine HCl 0·13 M and 0·05 - 0·10 ml. enzyme extract in a final volume of 1 ml. This was a discontinuous assay at 30°C over 15 minutes, which was stopped by addition of 1 ml. ice-cold trichloroacetic acid 10%. A number of alterations were made. First, the assay buffer was changed to Tris HCl (pK 8·08) 50mM pH 9·0 since the phosphate buffer (pK 7·2) was outwith its effective buffering range. Second, the histidine concentration used by Kacser et al. (1973) (133 mM) was considerably higher than that recommended by Tabor and Mehler (1953) (3mM) or Spolter and Baldridge (1963) (33 mM). The high histidine concentration may have been necessary to overcome histidase inhibition by EDTA which was present in the original homogenisation medium. After omission of EDTA, a histidine concentration of 30 mM was used, which was about 50 times the Km for histidine. Third, the assay was changed to a continuous one, followed on a Beckman DB spectrophotometer at 277 nm. This had the advantage over a discontinuous assay of ensuring that linear initial rates were being measured under all circumstances. Boiled homogenate blanks were found to be associated with a slight, but steady, decrease in absorbance at 277 nm., so blanks containing +/+ liver extract without added histidine were used routinely. In the case of his/his liver extract without added histidine, a small but perceptable increase in absorbance was found, presumably because of the higher endogenous histidine concentration (0·16 - 0·33 mM in the assay mixture) since it disappeared after gel filtration.

The assay method is based on the spectrophotometric assay of Tabor and Mehler (1955) which makes use of the strong ultraviolet absorbance of urocanic acid. A molar extinction coefficient of 18,600 M^{-1} Cm^{-1} was used to calculate the amount of urocanic acid formed. Activity was expressed as µmol urocanic acid/min. per g. wet weight or per mg. protein. The assay is dependent on a low or negligible rate of urocanic acid degradation by the next enzyme in the pathway, urocanase. This is achieved by assaying at a pH (ca.9) where urocanase is inactive.

Initial rates of histidase activity were found to be proportional to enzyme concentration in the range used (50 - 200 µl supernatant from a 25% homogenate). Assays were performed with and without 5 mM reduced glutathione and no difference in activity was found using crude liver or skin extracts. Glutathione was therefore not included routinely in the assay mixture. Addition of a divalent metal ion at 10^{-3} M was also without effect on the activity of crude extracts.

(b) Histidine aminotransferase. The assay method described by Spolter and Baldridge (1963) was used with one modification. It was found to be impossible to use the high concentration (0·57 M) of alkaline borate solution recommended by these authors, because the borate came out of solution at the temperature of the assay. A borate concentration of 0·38 M was therefore used. This solution was first warmed to about 50°C to get it into solution, mixed with the assay constituents and allowed to equilibrate at 30°C before starting the reaction by addition of histidine. The final reaction volume was 3 ml. and contained 50 - 200 µl enzyme extract, in which range the rate was proportional to enzyme concentration. The method depends on the ultraviolet absorbance of enol-borate complexes with aromatic ketoacids such as imidazole pyruvate. The reaction was followed continuously at 293 nm on a Beckman DB spectrophotometer at 30°C. Blanks containing water instead of histidine[1] were used in both genotypes. The reaction rate was linear for about 5 minutes only. A molar extinction coefficient of 12,000 M^{-1} Cm^{-1} for the enol-borate complex of imidazole pyruvate was used.

(c) Histidine decarboxylase. This was assayed in brain samples using the method of Taylor and Snyder (1972). Aliquots of homogenate (12·5 - 50 µl) were added to 0·05 M-sodium phosphate buffer, pH 6·5/ 0·1% Triton X-100 in a 7·5 ml glass test tube on ice. Samples of homogenate containing two concentrations of added authentic histamine (2·5 and 5·0 ng) were also analysed routinely. The tubes were pre-incubated at 37°C for five minutes and the assay started by addition of 12·5 µl 0·1M L-histidine HCl/0·1 mM pyridoxal-5-phosphate. The final reaction volume was 125 µl. The samples were capped and incubated in a shaker bath at 37°C for various times up to 90 minutes. The incubation was stopped by immersion in a boiling water bath for 10

[1] Histidine concentration used was 43 mM.

minutes. The tubes were then cooled and 25 μl 0·1N-NaOH was added to bring the pH to 7·9. The samples were then centrifuged in an MSE bench centrifuge (speed 10) for 15 minutes. An aliquot of the supernatant was frozen at -50°C before being assayed for histamine. All pipetting was carried out with Gilson automatic pipettes (P20 or P200).

(vii) Quantitative separation of acid-soluble, protein, lipid and nucleic acid fractions from brain samples.

The method used was a modification of the Schmidt and Thannhauser (1945) procedure, based on the recommendations of Hutchison and Munro (1961) and Munro and Fleck (1966). Each assay contained equal numbers of +/+ and his/his samples at the same developmental stage and brain region to minimise the effect of methodological variation.

Samples were removed from storage in liquid nitrogen, weighed and extracted in 0·2N-perchloric acid/AGPA 1mM as described above. The acid soluble supernatant and first wash were combined and neutralised by addition of potassium hydroxide, which resulted in precipitation of potassium perchlorate. The samples were left overnight at 4°C to complete the precipitation and then centrifuged on an MSE bench centrifuge for three minutes (speed 10) and quickly returned to ice. The supernatant was removed and acidified with a small volume of HCl before being stored at -20°C for amino acid analysis.

Lipids were extracted from the acid-insoluble pellet first by suspension with ice-cold 90% ethanol containing 2% sodium acetate. Sodium acetate was added to prevent losses of nucleic acid and protein during ethanol extraction following acid precipitation (Steele et al., 1964). The suspension was then centrifuged at 2,500g for 10 minutes at 4°C. The supernatant was removed and the pellet re-extracted with chloroform:methanol (2:1 V/V). This was centrifuged as above and the supernatant was combined with the ethanol extract. A third extraction with ether was carried out as above and the ether, chloroform and ethanol extracts combined. This "lipid fraction" was stored at -20°C prior to cholesterol assay.

Alkaline hydrolysis of the lipid-free pellet was carried out to separate the RNA from DNA and protein fractions. The white powder remaining after ether extraction was suspended in 0·3N-potassium

hydroxide (after first mixing the pellet with 50 - 100 µl 0·3N-perchloric acid to aid solubilization). The sample was then incubated at 37°C for one hour, stirring frequently with a glass rod. A small aliquot (100 µl) was removed at the end of the incubation for protein estimation and the remaining sample was put into an ice bath for 10 minutes. This was then acidified with 1·2N-perchloric acid and left standing in ice for 15 minutes to precipitate DNA and protein, which are resistant to alkaline hydrolysis under these conditions. Samples were centrifuged on an MSE bench centrifuge (speed 6) for five minutes at room temperature and returned to ice. The supernatant was removed and the pellet washed with cold perchloric acid to give a final perchlorate concentration of 0·3N. The wash was combined with the previous supernatant as the "RNA fraction" and the volume of this fraction recorded. RNA analysis was carried out on the same day.

The acid-insoluble residue from the previous step was then extracted in hot perchloric acid to quantitatively hydrolyse the DNA to acid-soluble nucleotides, thus separating DNA from protein. The pellet was suspended in 1N-perchloric acid and heated in a water-bath at 70°C for twenty minutes with frequent stirring. The samples were then removed from the bath, allowed to stand at room temperature for one minute and put into ice for 10 minutes. They were centrifuged on an MSE bench centrifuge (speed 10) at room temperature for five minutes. The supernatant was removed. This procedure was repeated and the supernatants combined to form the "DNA fraction". The volume of this fraction was recorded. DNA assays were set up on the day of extraction and read the following morning.

In preliminary experiments, the RNA concentration of brain samples was estimated after varying periods of alkaline hydrolysis. RNA was estimated both by the orcinol reaction and by measuring the absorption at 260 nM. The RNA concentration was found to reach a plateau within 30 minutes of alkaline hydrolysis, thereafter remaining constant for up to two hours. In another experiment, acid hydrolysis in 1N-perchloric acid was carried out either once for 20 minutes at 70°C, followed by a wash, or twice for 20 minutes at 70°C, as described above. Samples extracted twice at 70°C had DNA concentrations 15% higher than those

extracted once, so this procedure was adopted.

(viii) Determination of creatinine, amino and imidazole acids.

These compounds were separated on a modified Technicon automated amino acid analyser. Amino acids were determined after reaction with ninhydrin on the basis of the amino acid peak area relative to that of the internal standard AGPA, added to samples during extraction. The relative peak sizes of equimolar amounts of amino acid and AGPA at 570 nM were determined to give a "colour value" for each amino acid (Table 2:1). The amino acid concentration was calculated as follows:-

$$\frac{\text{amino acid peak area} \times \mu\text{mol AGPA added to sample} \times \text{colour value}}{\text{AGPA peak area} \times \text{sample weight or volume}}$$

Samples were routinely deproteinized before chromatography using 30% sulphosalicylic acid, in the case of blood and urine samples, or perchloric acid for brain samples. The perchlorate was later precipitated out as described above prior to ion-exchange chromatography. The internal standard method helped to reduce errors due to variation in sample loading and sensitivity.

Imidazole acids (histidine, N-acetyl histidine, imidazole lactate, imidazole pyruvate, imidazole acetate) were determined by the Pauly (Diazo) reaction using diazotized sulphanilic acid (MacPherson, 1942) and the same elution programme as for amino acids. Creatinine was measured simultaneously with imidazole acids by time-switching the column effluent into a line carrying alkaline picrate reagent (Faulkner and King, 1970) for long enough to measure the creatinine peak at 520 nM. The effluent was then switched back to the Pauly reagents. Creatinine chromatographed just after histidine. Standard curves were constructed by loading different concentrations of the authentic compounds. The urinary amino and imidazole acid concentrations were expressed in terms of the creatinine concentration. It was necessary to assay creatinine after ion-exchange chromatography rather than directly on deproteinized samples, because a number of compounds, including histidine, present in tissue or urine extracts were found to react with the creatinine chromogen (see also Polar and Metcoff, 1965). Histidine has been reported by Cook (1975) to interfere with the determination of creatinine in unchromatographed samples. Initially,

TABLE 2:1

Relative peak sizes of equimolar

amounts of amino acids and AGPA

at 570 nm. after reaction with ninhydrin

Amino Acid	Colour Value*
histidine	0·5369
lysine	0·7481
phenylalanine	0·6161
alanine	0·9479
glutamic acid	0·7704
γ-aminobutyric acid	0·9245

* $\frac{\text{AGPA area (i } \mu\text{mol)}}{\text{AA area (i } \mu\text{mol)}}$

an attempt was made to express urine concentrations in terms of the 24-hour urinary creatinine excretion, to correct for variation in urinary dilution. This was abandoned because of the difficulty in obtaining uncontaminated 24 hour urine collections in mice. Two reports have showed that little or no variation in creatinine excretion occurs throughout the day in humans (Gitlow et al., 1965; O'Gorman, 1968). This supports the notion that creatinine is produced at a virtually constant rate from muscle creatinine. Creatinine estimation in random urine samples (as used in the experiments) should therefore be a useful measure of urinary dilution.

(ix) Determination of histamine.

There have been considerable problems in finding a specific and sensitive method for estimating histamine, which has led to the relative neglect of this amine in comparison with other biogenic amines (Green, 1970; Taylor, 1975). The recently introduced radio-enzymatic assay of histamine is reported to be simple, specific and sensitive (Taylor, 1975; Beaven, 1975). Although a double-isotope method was used initially, a single-isotope modification has been widely adopted (Taylor and Snyder, 1972; Kobayashi and Maudsley, 1972; Brownstein et al., 1974; Beaven, 1975; Orr and Quay, 1975; Tuomisto, 1977). The principle of the assay is to methylate histamine with a radioactively-labelled methyl donor (S-adenosyl methionine, SAM) by means of a highly specific enzyme (imidazole-N-methyltransferase, INMT). An organic extraction is then used to separate histamine and SAM from the radiolabelled product, 1,4-methylhistamine.

The method described by Brownstein et al. (1974) was adopted. Glasswear was cleaned with detergent, soaked overnight in acid, rinsed twice in distilled water and dried before use. Stock solutions of histamine dihydrochloride were made up in distilled water at 1 mg/ml. and stored in aliquots for one month at -20°C. S-adenosyl-L- (methyl-^{3}H) methionine (7·5 - 12·2 Ci/mmol) was stored in liquid nitrogen in suitable aliquots which were only thawed once. A 10 µl tissue sample was mixed with 10 µl 0·05N-sodium phosphate buffer pH 7·9 in a 7·5 ml glass screw-cap test tube on melting ice. The reaction was started by adding 10 µl of a mixture containing 0·5 µl 0·5M-sodium phosphate pH 8·0, 2 µl (1 µCi) [^{3}H]-SAM and 7·5 µl INMT which was partially

purified from guinea pig brain as described below. The buffer and enzyme were mixed together before addition of [^{3}H] SAM. The tubes were incubated at 37°C in a shaker bath for varying periods of time. The method of Brownstein et al. (1974) used a 30 minute incubation routinely, but this was changed to 60 minutes since a time course experiment showed that methylation was not quite complete by 30 minutes but was completed by 60 minutes, remaining the same at 90 minutes. The reaction was stopped by addition of 25 µl 0·4N-perchloric acid containing 1,4-methylhistamine 1 mg/ml (Calbiochem) as a carrier. The sample was vortexed and put onto ice. 3N-sodium hydroxide (0·5 ml) was then added to each tube followed by 5 ml chloroform. The original method used 6 ml chloroform but 5 ml gave better mixing and did not alter the recovery of labelled products. Tubes were capped and shaken mechanically for five minutes in a commercial flask shaker. Samples were then centrifuged in an MSE bench centrifuge (speed 10) for five minutes. This gave a clear separation into two phases, with the precipitated protein at the interface. Strong alkali is used in the extraction to increase the partition of methylhistamine into the organic phase. The upper aqueous phase was aspirated and discarded. 3N-sodium hydroxide (1 ml) was then added to each tube, which was shaken mechanically for a further five minutes and centrifuged as above. The aqueous phase was discarded and 4 ml of chloroform was transfered to a glass scintillation counting vial. This was evaporated to dryness at room temperature under a stream of air. A manifold accomodating ten tubes was constructed for this purpose. Scintillant containing ethanol (1 ml) and toluene based PPO 0·4% and POPOP 0·01% (10 ml) was added to each vial after reaching dryness. Evaporation was complete within about 30 minutes. Samples were then counted in a Packard Scintillation Counter.

The number of counts per minute was found to be proportional to the amount of authentic histamine added in the range 0·02 ng to 2 ng. Sensitivity (twice blank) was about 0·1 ng. Buffer blank counts were in the region of 2,000 CPM and 2 ng histamine gave about 60,000 CPM above the blank. Addition of internal histamine standards to brain extracts gave a recovery of 80 - 100% of counts obtained in the

absence of tissue extracts.

A time course experiment, measuring histidine decarboxylase (HDC) activity in brain samples was carried out using the above method for assaying histamine. It became apparent that the HDC reaction product (histamine) was not being formed linearly with time. Instead, it reached a plateau within five minutes of adding the substrate (histidine) and remained relatively constant thereafter. The plateau counts exceeded the amount of endogenous histamine in the sample by up to 60-fold. When the product of the histamine assay was chromatographed with ethyl acetate-butanol-glacial acetic acid-water (1:1:1:1), two radioactive peaks were found, instead of the expected single peak corresponding to 1,4-methylhistamine. One of these products corresponded to authentic 1,4-methylhistamine with an Rf value of 0·77. The other more rapidly migrating product ("X") (Rf = 0·92) had about ten times the number of counts found in the methylhistamine peak. Similarly, when authentic histamine was assayed by the above method in the absence of tissue extract, the organic extract was chromatographed and the same two radioactive peaks were found. Chromatography of the radioactive substrate (^{3}H) SAM in the above solvent system showed that 86% of the total counts corresponded to authentic SAM (compared with 95 - 98% radiochemical purity on dispatch from the suppliers). The remaining counts were in a peak with an Rf value similar to the unidentified radioactive product "X" of the histamine assay (Rf = 0·90). However this radioactive impurity was not simply extracted into the organic phase and carried through the assay. Firstly, buffer blank counts were considerably lower than sample or standard counts (see above). Secondly, histamine standards were assayed in the presence and absence of the INMT enzyme: in the presence of INMT 1 ng histamine gave 61,187 CPM compared with 550 CPM without INMT. The corresponding buffer blanks gave 996 CPM (+ INMT) and 508 CPM (- INMT). The radioactive product "X" was therefore dependent on the presence in the assay mixture of authentic histamine or brain tissue, (^{3}H) SAM and INMT extract. SAM is reported to be rather unstable, particularly at alkaline pH (Beaven, 1975: Product Information, Radiochemical Centre, Amersham). Several (^{3}H)

containing derivatives are possible. SAM is readily cleaved between the sulphur atom and the carbon chain of the amino acid residue to form thiomethyladenosine and homoserine (Baddiley et al., 1953). Thiomethyladenosine (Sigma), homoserine, 1,4-methylhistamine, 1,5-methylhistamine*, methionine and histidine were all chromatographed in ethyl acetate-butanol-glacial acetic acid-water (1:1:1:1) (Table 2:2). The chromatographic methods are described below. Two products were identified on chromatographing thiomethyladenosine, with Rf values of 0·67 and 0·88. Both had spectral maxima at 260 nM, suggesting that they could be adenine and thiomethyladenosine, although this was not verified.

Two possible sources of the unidentified, enzymatically formed product "X" were examined. First, it was possible that the other (^{3}H) labelled reaction product, 1,4-methylhistamine, was being metabolised further by the next enzyme in the pathway, monoamine oxidase, which could also be present in the INMT extract. The assay was therefore carried out in the presence and absence of a monoamine oxidase inhibitor, iproniazid**, at a concentration of 5 mM. When the reaction product was chromatographed, the same two radioactive peaks were found and the relative counts in the two peaks was unaltered by iproniazid. Secondly, the possibility that the decarboxylated derivative of (^{3}H) SAM [S-(5-adenosyl)-3-(^{3}H)methyl-mercaptopropylamine] was acting as a propylamine donor to histamine, methylhistamine or a polyamine (e.g. putrescine, spermidine) present in brain extracts, was investigated. A propylamine transferase enzyme is present in brain tissue and could have been present in the INMT extract. Although polyamines are known to be propylamine acceptors the possibility that methylhistamine can also be an acceptor has not been reported. The expected radioactive products would include (^{3}H) thiomethyladenosine, which appeared to chromatograph in the same region as the unidentified product "X" (See Table 2:2).

* This compound was synthesized and donated by Dr J.C. Emmett of Smith, Kline and French Laboratories Limited, Welwyn Garden City.

** Donated by Dr R.N. Smith of Roche Products Limited, Welwyn Garden City.

TABLE 2:2

R_f values of substances chromatographed in ethyl acetate-butanol-acetic acid-water (1:1:1:1) solvent system.

Compound	R_f Value	Standard Deviation	Number of chromatographic runs
1,4-methylhistamine	0·77	0·04	7
1,5-methylhistamine	0·79	-	1
histamine assay products (1)	0·75	0·05	9
(2) "X"	0·92	0·02	
histamine	0·73	-	1
histidine	0·49	-	1
methionine	0·83	0·02	2
homoserine	0·67	-	
S-adenosyl methionine	0·42	0·01	3
S-adenosyl (methyl ^3H) methionine (1)	0·41	-	1
(2)	0·90	-	
thiomethyladenosine (1)	0·67	-	1
(2)	0·88	-	

Putrescine (2 mM), spermidine (2 mM) and methylhistamine (30 µM) were incubated instead of histamine and carried through the assay procedure. The number of counts in the final assay products were 63 cpm with putrescine, 1383 cpm with spermidine and 1080 cpm with methylhistamine after subtraction of buffer blank counts. None of these counts were high enough to account for the previous findings.

The histamine assay was being developed to measure histidine decarboxylase activity and the histamine concentration in urine during pregnancy and in the developing brain. Because of the above difficulties, the assay was abandoned.

(x) Paper chromatography.

After evaporating the chloroform extract from the histamine assay to dryness, it was taken up in 100-200 µl ethanol. 10 - 20 µl aliquots were spotted on to Whatman No. 1 chromatography paper, dried and equilibrated with the solvent system in a closed tank for thirty minutes. The sample was then chromatographed for 15 - 18 hours at room temperature. The paper was afterwards removed from the tank and dried in a fume cupboard with a hair drier. It was cut into one cm strips, which were put into glass scintill ation vials. One ml ethanol and 10 ml toluene scintillant containing PPO 0·4% and POPOP 0·01% were added and the samples counted in a Packard Scintillation Counter. The solvent system used was ethyl acetate / butanol / glacial acetic acid / water (1:1:1:1). Histidine, homoserine, methionine, SAM, histamine and methylhistamine were all identified by spraying the dry paper with 0·1% Ninhydrin in n-butanol (saturated with water). Thiomethyladenosine was identified by eluting 1 cm strips of the chromatographic paper into three ml distilled water and reading the absorbance at 260 nm. The Rf values of standards and unknowns run in this system are shown in Table 2:2.

(xi) Determination of nucleic acids.

Ribonucleic acid (RNA) was determined by the orcinol reaction and checked by measuring the ultraviolet absorbance in perchloric acid extracts. The orcinol reaction (Ceriotti, 1955) was carried out by diluting the sample with 0·36N-perchloric acid to a volume of 3 ml and adding 3 ml freshly made reagent (orcinol 0·2 g + 10 ml $CuCl_2$ 0·004 M in conc. HCl). The mixture was vortexed and put in a constant

volume boiling water bath for 40 minutes then returned to ice. The green colour was concentrated by adding 3 ml isoamyl alcohol and the tubes were vortexed. They were then centrifuged in an MSE bench centrifuge (speed 6) for five minutes. An aliquot of the upper organic phase was removed and its absorbance read at 675 nM in a Beckman DB spectrophotometer. A standard curve was constructed using yeast RNA (Sigma) which gave a linear relationship in the range 5 - 60 µg/ml. The RNA estimations were checked by measuring the ultraviolet absorbance of perchlorate extracts. Samples or standards were made up in 0·36N-perchloric acid and the absorbance at 260 nM and 232 nM was read. The reading at 232 nM was used to correct for small amounts of hydrolysed protein present in samples, as suggested by Fleck and Begg (1965). The RNA concentration was calculated from the formula:-

$$C_{RNA} = a\,A_{260} - b\,A_{232}$$

- where a and b are constants, A_{260} is the absorbance at 260 nM and A_{232} the absorbance at 232 nM. The constants were estimated from:-

$$a = \frac{1}{r_1 - r_2 \frac{P_1}{P_2}} \qquad \text{and} \qquad b = \frac{1}{r_1 \frac{P_2}{P_1} - r_2}$$

- where r_1 and r_2 are the specific extinction coefficients of RNA at 260 nM and 232 nM respectively. The values for r_1 and r_2 using yeast RNA were found to be 200·7 ($E^{1\%}_{260}$) and 60·7 ($E^{1\%}_{232}$). P1 and P2 are the specific extinction coefficients of the acid-soluble polypeptide contaminants at 260 nM and 232 nM. The ratios P1/P2 were estimated by measuring the absorbance of acid-insoluble proteins (dissolved in 0·3N-KOH) remaining at the end of the acid hydrolysis step, rather than the absorbance of acid-soluble polypeptides contaminating the RNA fraction. The ratio A_{260}/A_{232} was therefore used to estimate P1/P2 and its reciprocal to estimate P2/P1. RNA concentrations were determined by this method in brain samples at the 6, 15 and 24 day stages and gave comparable results to those obtained with the orcinol reaction.

Deoxyribonucleic acid (DNA) was determined by the diphenylamine reaction using the method of Richards (1974). This has been reported

to have advantages over the methods of Burton (1956) or Giles and Myers (1965) in the increased stability of the reagent, increased sensitivity, lowered reagent blanks and shorter overall procedure. A 1 ml aliquot of the perchloric acid extract ("DNA fraction") was added to 2 ml Diphenylamine Reagent (diphenylamine 1·5%, paraldehyde 0·01% in glacial acetic acid). The colour was allowed to develop at room temperature for eighteen hours and read at 600 nM on a Beckman DB spectrophotometer. Stock solutions containing 100 µg/ml calf thymus DNA (Sigma) were hydrolysed by heating at 70°C for 20 minutes in 1N-perchloric acid and stored at 4°C. A standard curve was run with each set of samples in the range 10 - 100 µg/ml.

(xii) Determination of cholesterol.

Cholesterol was determined by the method of Zak (1957) based on the Liebermann-Burchard reaction. Lipid fractions stored at -20°C were evaporated to dryness over a water bath at 40°C in a rotary evaporator and then dissolved in 5 ml glacial acetic acid. Two ml of the colour reagent ($FeCl_3.6H_2O$ 1·1 g in 1 litre glacial acetic acid) was added to 2 ml sample in glacial acetic acid and vortexed. After standing at room temperature for 15 minutes, 2·67 ml concentrated sulphuric acid was added and the tube vortexed. After 30 minutes at room temperature the absorbance at 560 nM was read on a Beckman DB spectrophotometer. A standard curve was run with each batch of samples, using concentrations of authentic cholesterol (Sigma) in the range 0·01 - 0·125 mg/ml which gave an approximately linear relationship. A stock solution containing 1 mg/ml cholesterol in glacial acetic acid was stored at room temperature. The method is not completely specific for cholesterol since it also measures the cholesterol precursor desmosterol, and cholesterol esters, which are, however, only present in small amounts relative to cholesterol in the developing brain (Davison, 1968, 1970).

(xiii) Determination of protein.

Protein was determined by the method of Lowry et al. (1951). The sample was made up to a suitable concentration with water or buffer and to a final volume of 0·4 ml. Two ml of solution "C" [50 ml of solution "A" containing 20 g anhydrous sodium carbonate,

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4 g sodium hydroxide and 0·2 g hydrated sodium potassium tartrate in a 1 litre volume, plus 1 ml of solution "B" containing 2·5 g hydrated copper sulphate in a 0·5 litre volume] was added to the sample and vortexed. This was allowed to stand for 20 minutes at room temperature. Then 0·2 ml of the Folin-Ciocalteau Reagent (freshly diluted 1:1 with water) was added and vortexed immediately. After 30 minutes at room temperature, the absorbance at 750 nM was read on a Beckman DB spectrophotometer. Standard curves were prepared from dilutions (10-400 μg/ml) of a stock dilution of bovine serum albumen (Sigma) containing 0·4 mg/ml. The stock solution was stored in aliquots at -20°C. Standard and blanks containing buffer or distilled water were run with each batch of samples.

(xiv) Determination of water content.

Brain samples were removed from the liquid nitrogen store and allowed to warm to room temperature in a dessicator. Samples were then put on to squares of aluminium foil of known weight and weighed. They were then dried to constant weight in a 60°C oven.

(xv) Desalting.

Liver supernatant was put into Visking dialysis tubing and dialysed overnight at 4°C against sodium phosphate buffer 0·01 M pH 7·4 containing dithiothreitol 1 mM. The wild-type enzyme lost 30 - 40% of its activity and the mutant enzyme 80 - 90% of its previous activity following dialysis. Since commercially obtained dialysis tubing may be contaminated with proteases and sulphydryl compounds, the mutant histidase activity was compared before and after dialysis in tubing previously boiled for 15 minutes in distilled water compared with dialysis in unboiled tubing. This experiment showed a complete loss of activity in the unboiled tubing and 50% loss of activity after dialysis in boiled tubing. The loss of activity was not reversible by preincubation with 2 mM glutathione. Substitution of Tris for phosphate buffer in the dialysis medium was also without effect. Desalting in this way was effective in that the pre-dialysis his/his liver extract had a histidine concentration of 19·1 μmol/g wet weight while the post-dialysis extract contained no detectable histidine. However, the marked loss of mutant histidase activity led to the development of an alternative method.

Desalting by gel filtration was then tried, since this procedure is rapid and suitable for unstable proteins. Sephadex G-25 granules (coarse, 100 - 300 mesh) were swollen and applied to a Pharmacia column sized 1·6 x 28 cm. This was then equilibrated with Tris HCl 0·05 M pH 9 buffer. A sample of crude liver extract in a volume less than 20% of the bed volume was applied to the column in a cold room and eluted at a flow rate of 0·4 ml/min. Thirty drop fractions were collected in a Gilson fraction collecter and the reddish coloured fractions were pooled and concentrated through a Column Eluate Concentrator (CEC-1, Amicon). With this technique, the histidine concentration of his/his liver extracts was again reduced to below the limit of detection but without significant loss of activity.

(xvi) Enzyme purification procedures.

Imidazole-N-methyltransferase (INMT). This enzyme was partially purified for the radioenzymatic assay of histamine by the method of Brown et al. (1959) modified by Snyder (1971). Six adult male guinea pigs were killed by carbon dioxide inhalation and decapitation. The brains were rapidly removed and pooled on ice before being weighed. All procedures were carried out at $4^{o}C$. The brains were homogenised in ten volumes of ice-cold sucrose 0·25M in a blender, then with 5 up-and-down strokes of a glass-Teflon homogeniser (speed 10). The pooled homogenate was centrifuged at 78,000 g in a refrigerated MSE Highspeed 50 centrifuge (angle rotor) for 30 minutes. To 180 ml of the clear supernatant, 50 g ammonium sulphate (grade I, Sigma) was added with stirring (45% saturation). The solution was stirred for 15 minutes on ice and left to stand for 60 minutes. It was then centrifuged at 10,000 g for 20 minutes. The supernatant was removed and 33 g ammonium sulphate was added (70% saturation). This was stirred for 15 minutes and left to stand for 60 minutes on ice before centrifuging at 10,000 g for 20 minutes. The supernatant was decanted and the precipitate dissolved in 25 ml sodium phosphate buffer 0·01M pH 7·4. This was dialysed overnight at $4^{o}C$ in Visking Dialysis tubing (size 18-32, boiled for 15 minutes previously) against two litres of sodium phosphate buffer 0·001M pH 7·4. The final extract was stored in 200 μl aliquots at $-50^{o}C$.

Mouse liver histidase. The reported methods for purifying

mammalian histidase (Mehler and Tabor, 1953; Kato et al., 1955; Cornell and Villie, 1968; Okamura et al., 1974; Lamartiniere and Feigelson, 1975; Brand and Harper, 1975, 1976) are unsuitable for purifying the mutant enzyme, which is likely to differ from the wild-type in its stability and chromatographic properties. Most procedures use a heat step and ammonium sulphate fractionation in the early stages, both of which are likely to result in a marked loss of activity, without achieving more than a three or four fold purification. Since overnight storage of the mutant enzyme at $4^{o}C$ also results in loss of activity, it was felt that a short non-denaturing technique was required.

The first attempt to solve this problem was to use gel filtration, since this offered a relatively rapid means of achieving a limited purification, under conditions in which the enzyme should be stable. Sephadex G-150 (40-120 µ diameter, Pharmacia) is suitable for fractionating proteins in the range 5,000 - 400,000 daltons (cf. histidase molecular weight ca. 200,000 daltons). A Pharmacia column sized 2·7 x 46 cm was packed with swollen Sephadex G-150 gel particles and equilibrated with Tris HCl 0·02M pH 7·4/NaCl 0·1M/dithiothreitol 0·3 mM/$MgCl_2$ 1mM, in a cold room. A 5 ml sample of crude liver supernatant (2% of the bed volume) was applied to the column and eluted with buffer at 0·16 ml/min using a peristaltic pump. The column eluate was run through a Uviscan recorder which measured the absorbance of the eluate at 254 nM. The eluate then passed through a Column Eluate Concentrator (CEC-1, Amicon) set at a concentration ratio of 4:1. One ml fractions were collected in a Gilson fraction collector. Fractions were then assayed for histidase activity. Histidase activity from +/+ extracts was found to elute near the front of the protein peaks (Figure 2:2). The five fractions containing most activity were found to be the first five turbid fractions, which were combined and assayed for histidase and protein content (Warburg-Christian method) in comparison with a sample taken prior to gel filtration. The purification achieved with this method is shown below:-

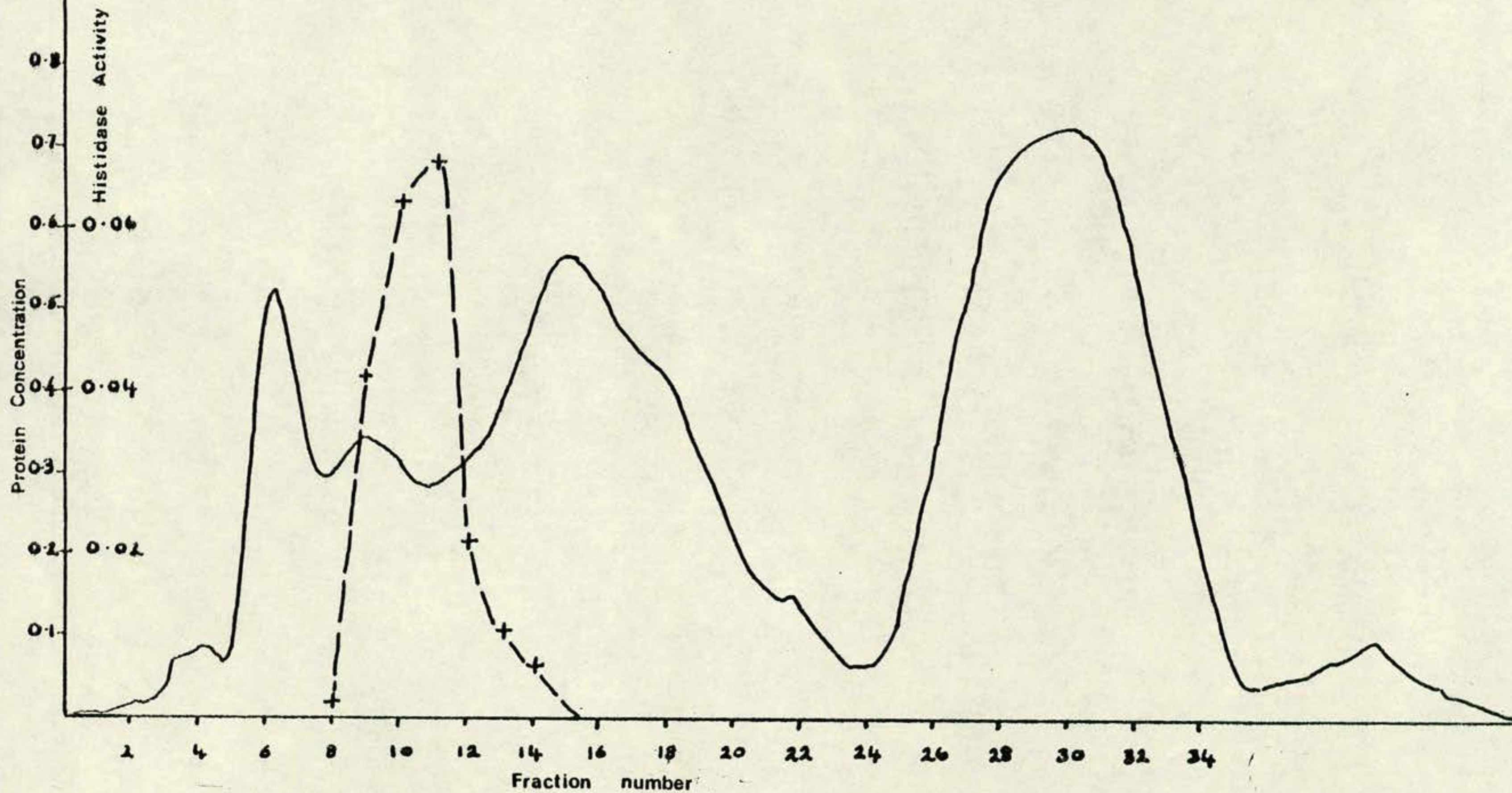


FIG. 2:2 Gel filtration of +/+ liver extract on Sephadex G-150. Histidase activity (change in A_{277} per minute) X- - -X. Protein concentration (A_{254}) ———. The sample was eluted and collected as described in the text.

+/+ liver extract	Specific activity (nmol./min./mg. protein)	Total Units
Before gel filtration	3·22	500·7
After gel filtration	12·12	426·5

This achieved a four-fold purification with 85% recovery in +/+ extracts. The same procedure was repeated with the mutant enzyme. A similar elution profile on the Uviscan was obtained. Since the his/his enzyme was expected to chromatograph in the same way as +/+ extracts on Sephadex (since it depends largely on molecular size), the first five or six fractions containing visible turbidity were pooled, as with the +/+ enzyme. The combined fractions were then assayed for histidase and protein as before, together with a sample taken before gel filtration. The results are shown below:-

his/his liver extract	Specific activity (nmol./min./mg. protein)	Total Units
Before gel filtration	0·052	6·8
After gel filtration	0·151	3·5

This represents a three-fold purification with 51% recovery. It also showed that at least half the mutant histidase chromatographed on Sephadex G-150 in the same region as wild-type histidase.

An alternative method of partial purification was suggested by a communication from W.C. Hanford and S.M. Arfin, who had purified mouse liver histidase 500-fold (later published in Hanford and Arfin, 1977). The procedure started with a protamine sulphate precipitation, which gave a twenty-fold purification and 90 - 100% yield, in a single step. The method was used with some minor modifications which are described below.

Female mice were killed by stunning and cervical dislocation. The liver was rapidly removed and put on to aluminium foil on ice. After weighing, 3 x w/v Tris HCl 0·05M pH 7·4 /$MgCl_2$ 1mM/ sucrose 0·25M was added and the samples homogenised with five strokes of a glass-Teflon

homogeniser. The homogenate was then centrifuged at 100,000g for 30 minutes in an MSE Highspeed 50 centrifuge at 4°C. The clear supernatant (SN) was removed and the volume measured and assayed. One fifteenth volume of 2% protamine sulphate (Sigma, grade II, freshly prepared in distilled water, unneutralised) was added drop by drop to the stirred supernatant, in an ice bath. The sample was then centrifuged at 25,000g for 20 minutes at 4°C. This supernatant (SN-1) was found to contain little active enzyme and so was discarded in subsequent experiments. The pellet was extracted first with one sixth the original extract volume of Pipes 50 mM pH 7·2/$MgCl_2$ 1mM/NaCl 40 mM. The extract was then centrifuged at 25,000g for 20 minutes at 4°C. The supernatant (SN-2) was removed and kept on ice. The pellet was then extracted with one tenth the original extract volume of Pipes 100mM pH 7·2/$MgCl_2$ 1mM/NaCl 200 mM. The extract was centrifuged at 25,000g for 20 minutes at 4°C and the supernatant (SN-3) removed. Aliquots of each fraction were assayed for histidase activity and protein. The results obtained with +/+ liver extract are shown below:-

+/+ liver extract			
Fraction	Total protein	Specific activity (nmol/min./mg. protein)	Total Units
100,000 g SN	372·9	3·3	1230·6
SN-1	-	-	-
SN-2	10·9	3·7	40·4
SN-3	13·6	49·3	669·9

The +/+ histidase activity was found predominantly in the SN-3 fraction, extracted from the protamine sulphate precipitate at high ionic strength, giving an approximately fifteen-fold purification and 54% yield. It was not clear why the yield was lower than that of Hanford and Arfin (1977).

The same procedure was applied to his/his liver extracts. The results of a purification experiment are shown below:-

his/his liver extract			
Fraction	Total Protein	Specific Activity (nmol/min./mg. protein)	Total Units
100,000 g SN	631·6	0·06	37·9
SN-1	580·0	0·01	5·8
SN-2	23·5	2·2	51·7
SN-3	37·5	1·9	71·3

Mutant histidase activity is found almost equally in SN-2 and SN-3 fractions in this experiment. Unmasking of mutant activity occurs since the total units increase by a factor of three after protamine sulphate precipitation. This could be the result of the removal of urocanase activity, although this was not verified. In general, the effect of urocanic acid removal by another enzyme will be greater with low histidase activity than with high activity. In the wild-type, recoverable enzyme activity did not increase on purification. The specific activity of mutant histidase increased thirtyfold with this procedure. In SN-3 extracts, the specific activity was variable, ranging from 0 to 4·6 nmol/min./mg. protein in four experiments. This variation may have been due to differences in the duration of exposure to the high ionic strength extraction buffer.

Since the protamine sulphate method achieved a better increase in histidase specific activity it was used in preference to the gel filtration method to contrast the mutant and wild-type enzymes.

(xvii) Disc Electrophoresis.

Analytical polyacrylamide gel electrophoresis was carried out by the procedure of Davis (1964). The electrophoresis reservoirs, gel tubes and electrodes were made by Shandon Southern. The gels were prepared from the Davis stock solutions "A" to "F". A 7% small-pore gel was prepared by mixing 2 ml solution "A" with 4 ml solution "C" and 2 ml distilled water. The solution was mixed and degassed under vacuum. 1·3 ml of this solution was rapidly pipetted into a series of 6 mm (i.d.) x 65 mm plastic gel tubes, sealed at the lower end with Parafilm. The gels were then carefully overlayed with distilled

water (or Tris HCl 0·38M pH 8·9 if prepared the night before) using a 1 ml syringe (23G needle). The tubes were placed vertically in the reservoir grommets and allowed to polymerise for 30 - 60 minutes at room temperature. The 3% large-pore gel was prepared by mixing 0·5 ml solution "B", 1 ml solution "D", 0·5 ml solution "E" and 2 ml solution "F". The solution was degassed as before. The gel tubes were given a preliminary rinse with a small volume of this solution, which was then discarded and 0·15 ml was pipetted into each tube and carefully layered with water. The gel tubes were positioned vertically in the reservoir grommets and allowed to polymerise under an ultraviolet light for about 60 minutes. The Parafilm was removed and the gel tubes and reservoirs placed in a cold room at 4°C. The reservoirs were partially filled with two litres of ice-cold Tris glycine pH 8·3 reservoir buffer (diluted 1:10 prior to use). After cooling the gels for one hour the samples were applied to each gel. Samples were prepared in two volumes of 40% sucrose containing 5 µl of 0·05% bromophenol blue marker dye (0·1% in initial experiments) and applied to the gels using a plastic-tipped Gilson pipette. The samples were carefully overlayed with Tris glycine buffer and the upper reservoir filled with the remaining buffer. Bubbles of air were removed from the bottom of the gels with a syringe. The electrodes were placed into the reservoir and connected to a Vokam stabilised DC power pack (Shandon Southern) set to deliver a constant current of 1·5 - 2·0 mA per tube. The current was switched off when the bromophenol blue marker was close to the bottom of the gel tube (3 - 4 hours). The gels were rimmed with a blunt needle and propelled out of each tube with an air-filled syringe. The position of the bromophenol blue tracking dye was marked by cutting the gel with a scalpel. The gels were kept on ice until assayed for histidase activity. In a preliminary experiment the gels were removed and frozen on dry ice. The frozen gels were then cut into 1 mm slices with an automatic gel slicer. Gel slices were then immersed overnight in ice-cold buffer and crushed to elute the enzyme, which was later assayed for histidase activity.

The position of the histidase band was routinely detected by a method based on that of Hassall et al. (1970). These workers detected

histidase on polyacrylamide gels by photographing the gel in a medium containing histidine with transmitted ultraviolet (UV) light. The position of histidase is indicated by the appearance of a UV-absorbing band because of the high extinction of urocanate at 277 nm. In these experiments, the gel was placed in a quartz cuvette and overlayed with 7 ml Tris HCl 0·02M pH 9 (diethanolamine buffer was used initially) containing 10% sucrose. The gel was equilibrated with this mixture for five minutes before addition of 1·5 ml histidine HCl 0·18M pH 9, which was mixed with the buffer using a pipette. A clock was started on addition of histidine and the cuvette containing the gel was then scanned with a Joyce-Loebl chromoscan containing a UV light source and 254 nm filter. The gel was scanned at regular, timed intervals. Each scan was recorded on a chart recorder. The position of the histidase band became apparent within a few minutes in gels containing +/+ extract by a sharp increase in the UV absorbance. After about fifteen minutes, the band broadened markedly, due to diffusion, which decreased resolution. Gels were stained for protein by immersion in 1% naphthalene black (BDH) in 7% acetic acid for 30 - 60 minutes and destained in 7% acetic acid. Stained gels were scanned on a Kipp and Zonen (KS3 densiscan) microdensitometer.

CHAPTER 3

DEVELOPMENT OF THE HISTIDINAEMIC MOUSE I ENZYME AND METABOLITE PROFILES

The status of the histidine catabolic enzymes and their major products have been well characterised in the adult his/his mouse (Kacser et al., 1973; Bulfield and Kacser, 1974, 1975). The aim of this part of the work is to extend that characterisation to the developing his/his mouse in parallel with a study of brain growth and differentiation (Chapter 4). Any disorder of brain growth in his/his mice can therefore be compared directly with concurrent metabolic differences between +/+ and his/his mice. His/his offspring of his/his x his/his crosses have been examined throughout these experiments, since the combination of maternal histidinaemia and histidase deficiency could be expected to have a more drastic effect on brain development than histidase deficiency alone.

The activity of the various enzymes utilizing histidine are likely to develop at different rates; substrate and cofactor concentrations may also change as development proceeds, so that the relative fluxes through these pathways may differ markedly from those in the adult. Other factors can accentuate these differences, including changes in the compartmentation of enzymes or metabolites and developmental changes in isozyme pattern. The activity of skin and liver histidase and histidine aminotransferase have been investigated during postnatal development. Experiments investigating the development of histidine decarboxylase were abandoned because of the histamine assay difficulties discussed in Chapter 2. Amino and imidazole acid concentrations have also been estimated in foetal and maternal samples and in blood and brain during postnatal development.

(i) The development of liver histidase activity.

Liver histidase activity was examined in +/+ and his/his mice during the first five weeks after birth. Males and females were examined separately after the first two weeks because of the reported sex difference in liver histidase activity. The results are shown in Table 3:1 and Figure 3:1. On the day after birth, +/+ mice have 1-2% of the five week activity, which then increases steadily during the third and fourth weeks. The sex difference starts to emerge in the

TABLE 3:1

The development of liver histidase activity in +/+ and his/his mice.

Genotype	Age (postnatal days)	Sex	Specific Activity ± S.E.M. (µmol./min./g. wet weight)	No.
+/+	1	pooled	0·0052	8
	2	pooled	0·0087	4
	3	pooled	0·0181	3
	5	pooled	0·0258	4
	8	pooled	0·0645	2
	11	pooled	0·0581	2
	15	female	0·105 ± 0·025	3
	15	male	0·118 ± 0·063	3
	24	female	0·347 ± 0·027	4
	24	male	0·308 ± 0·010	4
	35	female	0·439 ± 0·024	4
	35	male	0·284 ± 0·059	2
his/his	0 (birth)	pooled	0·0067 ± 0·0018	8
	1	pooled	0·0041 ± 0·0007	8
	3	pooled	0·0012 ± 0·0008	4
	5	pooled	0·0046 ± 0·0011	3
	7	pooled	0·0071 ± 0·0015	4
	10	pooled	0·0094 ± 0·0013	8
	15	pooled	0·0176 ± 0·0020	4
	24	female	0·0056 ± 0·0011	3
	24	male	0·0045 ± 0·0014	3
	35	female	0·0044 ± 0·0005	4
	35	male	0·0050 ± 0·0008	4

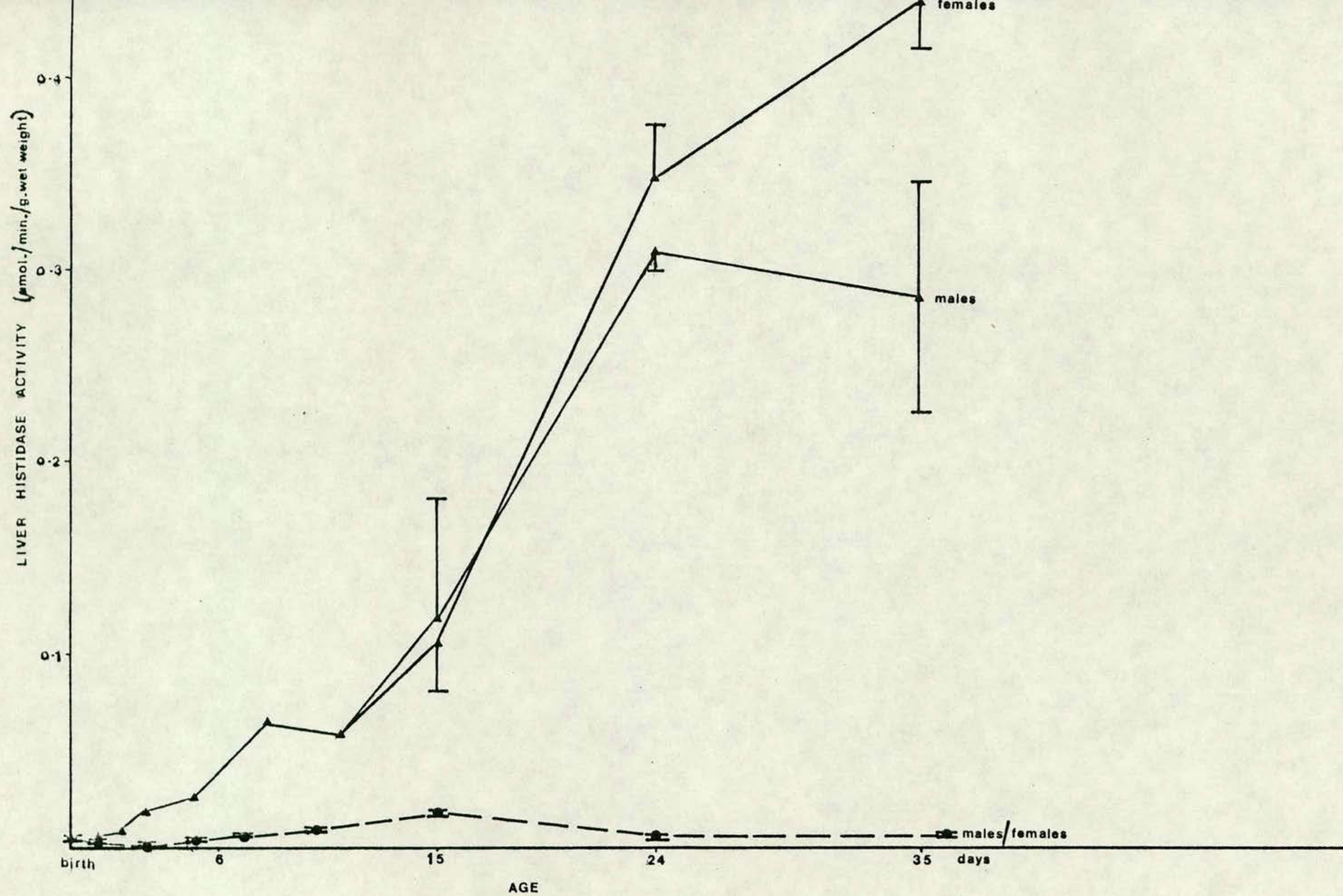


FIG. 3:1 Liver histidase activity in +/+ (▲——▲) and his/his (●— —●) mice during postnatal development.

the fourth week and by five weeks the female has about 50% higher specific activity than the male.

In his/his mice, the activity at birth and day 1 is about the same as in the +/+ genotype, showing that the genetic difference at the his locus only becomes manifest in the liver after birth. In contrast to the +/+ enzyme, mutant histidase then declines briefly before rising to a maximum at 15 days after birth. At this stage, the specific activity is about 15% of the wild-type. Mutant histidase activity then declines steadily to reach one-quarter of the 15-day activity by five weeks. The mutant histidase at five weeks has only 1-2% of wild-type activity. No sex difference is present in the mutant.

There are differences in the pattern of liver histidase development between the two genotypes. Firstly, the mutant reaches a peak at two weeks after birth compared with at least five weeks in the wild-type. Secondly, after reaching a peak, mutant histidase declines by a factor of four over the next 3 weeks. Thirdly, there is loss of the sex difference in histidase activity in his/his mice which suggests that the mutation also causes a loss of responsiveness to pubertal oestrogen (Feigelson, 1973b). This cannot wholly explain the genetic difference in histidase activity since the loss of mutant activity is apparent before the sex difference is manifest. These differences in the development of histidase activity in +/+ and his/his mice could also be explained if the residual activity in the mutant is due to a separate enzyme. This will be examined further in Chapter 5.

(ii) The development of skin histidase activity.

Skin histidase activity was assayed during the first two weeks after birth in +/+ and his/his mice. Although the development of skin histidase has not been reported in the mouse, the most striking activity changes in the rat occur during the first two weeks after birth (Lamartiniere and Feigelson, 1976a). Confining the study to the first two weeks also avoids the difficulty of extracting the enzyme from older animals in which the skin is tougher and the hair denser.

The results are shown in Table 3:2 and Figure 3:2. At birth, +/+ histidase activity is already about 60% of its maximum while mutant activity is undetectable. Both genotypes then rise to a maximum

TABLE 3:2

The development of skin histidase activity in +/+ and his/his mice.

Genotype	Age (postnatal days)	Specific activity ± S.E.M. (μmol./min./g. wet weight)	% maximum activity	No.
+/+	0 (birth)	0·0757 ± 0·0035	59	4
	1	0·1290 ± 0·0118	100	4
	3	0·0953 ± 0·00037	74	4
	5	0·1040 ± 0·0243	81	2
	7	0·0775 ± 0·0041	60	4
	10	0·0151 ± 0·0016	12	4
	15	0·0034 ± 0·0012	3	4
his/his	0 (birth)	zero	0	4
	1	0·0005 ± 0·0005	5	3
	3	0·0053 ± 0·0016	58	4
	5	0·0091 ± 0·0025	100	4
	7	0·0053 ± 0·0005	58	7
	10	0·0038 ± 0·0005	42	4
	15	0·0038 ± 0·0004	42	4

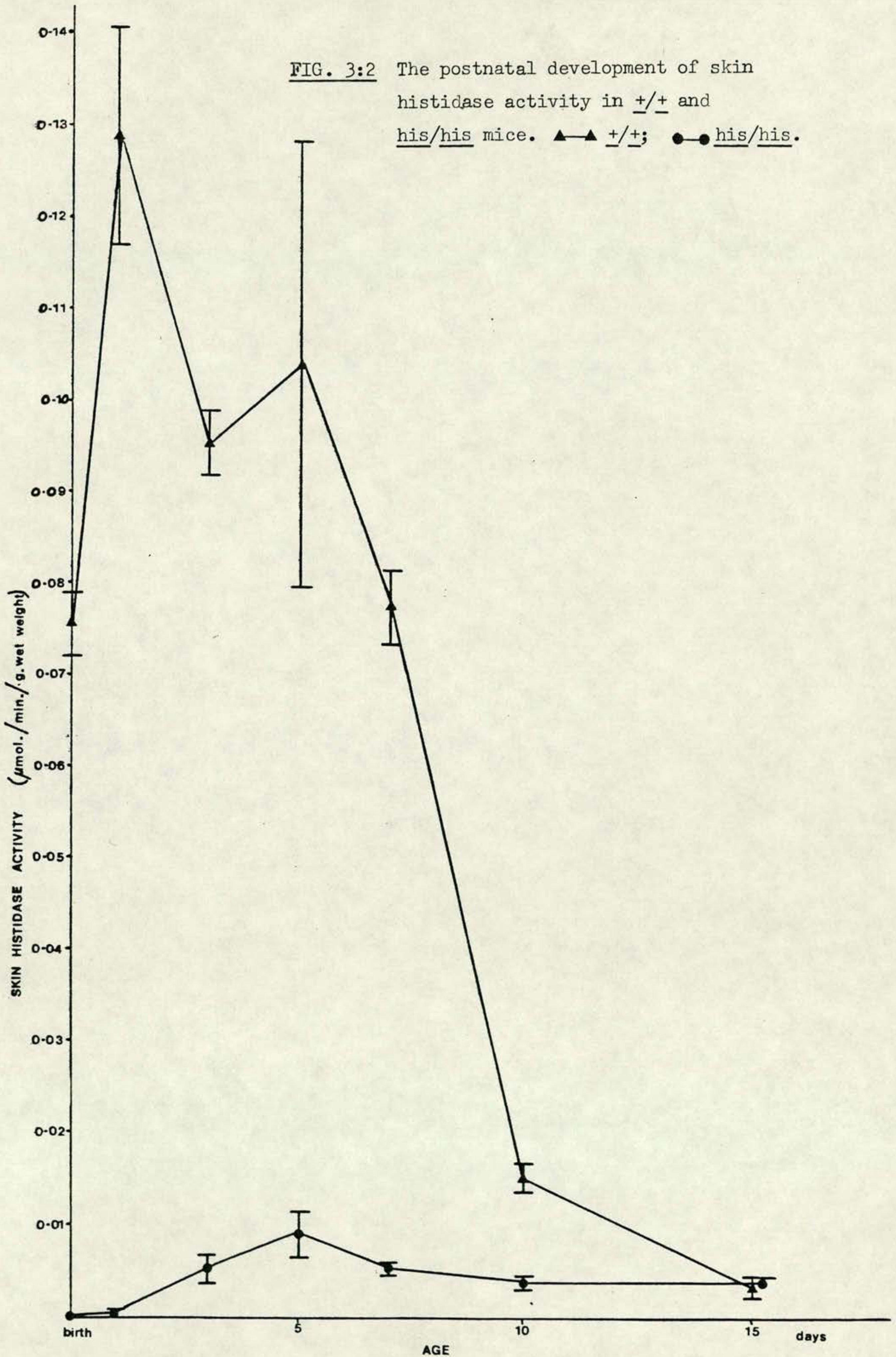


FIG. 3:2 The postnatal development of skin histidase activity in +/+ and his/his mice. ▲—▲ +/+; ●—● his/his.

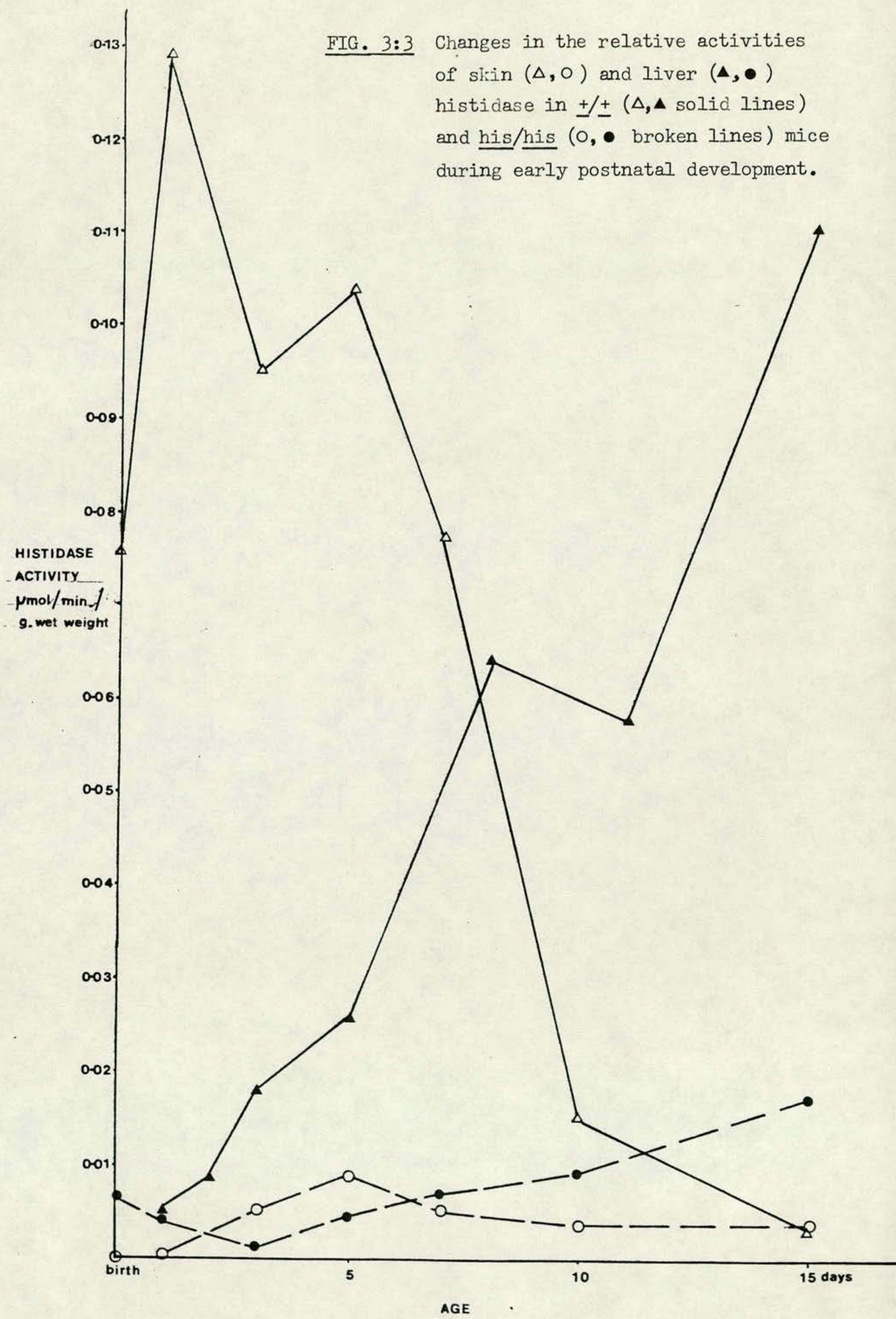


FIG. 3:3 Changes in the relative activities of skin (△, ○) and liver (▲, ●) histidase in +/+ (△, ▲ solid lines) and his/his (○, ● broken lines) mice during early postnatal development.

within the first week, and fall off sharply in the second. By fifteen days, the activity in +/+ extracts has fallen to only 2-3% of its activity during the first five days after birth, reaching a specific activity almost identical to the mutant.

There is more similarity between the development of histidase activity in +/+ and his/his skin than in the corresponding liver extracts. Both reach a maximum within the first week and decline in the second. However their activities at birth are very different and at fifteen days, his/his skin activity is 42% of five day activity compared with 3% in +/+ skin. Two further points are of interest. First, the genetic difference at the his locus would seem to be manifest before birth in the skin, unlike the liver. Second, there is no difference in skin histidase activity between +/+ and his/his mice at fifteen days. If this were simply a consequence of incomplete extraction of histidase activity in the maturing skin, the genetic difference should still remain, unless activity was undetectable in both genotypes. In fact, a low but measurable activity was discerned in each extract.

The relative activities of skin and liver histidase during the first two weeks after birth are shown in Figure 3:3. In the wild-type, during the first week the liver histidase specific activity is considerably lower than in skin, but as the skin activity declines during the second week, liver activity increases sharply, so that by fifteen days it already has some 30 times higher specific activity. In the mutant, the skin activity is also higher than the liver for part of the first week, then it declines as the liver increases. By fifteen days, the liver specific activity is 4-5 times higher than skin. Since the total wet weight of skin in the mouse is considerably greater than that of liver during the first ten days, the skin would appear to be the more important site of histidine degradation at this stage. However the blood supply to the liver and its relationship to the gut may provide a richer supply of substrate.

(iii) The development of liver histidine aminotransferase activity.

The development of liver histidine-pyruvate aminotransferase (combined mitochondrial and cytosolic) activity was investigated in +/+ and his/his mice during postnatal development. The results are

TABLE 3:3

The development of liver histidase aminotransferase activity in +/+ and his/his mice.

Genotype	Age (postnatal days)	Specific Activity* (µmol./min./g. wet weight)	No.
+/+	1	0·720	8
	3	2·190	3
	4	1·580	2
	5	1·530	4
	8	3·070	2
	11	3·040	2
	15	3·490 ± 0·283	6
	24	1·534 ± 0·111	8
	35	1·427 ± 0·174	11
	>50	1·383 ± 0·135	6
his/his	1	0·430	7
	3	1·750	4
	6	2·450	6
	9	1·685	6
	15	3·167 ± 0·193	10
	24	2·311 ± 0·199	5
	35	1·617 ± 0·172	6
Litter segregating for +/his and his/his.	20	3·355 ± 0·298	6

*Livers were pooled up to the 15 day stage. Sexes were analysed separately from the 15 day stage but since no sex difference in activity was found, the results from males and females are combined in this table. Means ± SEM are given from the 15 day stage onwards.

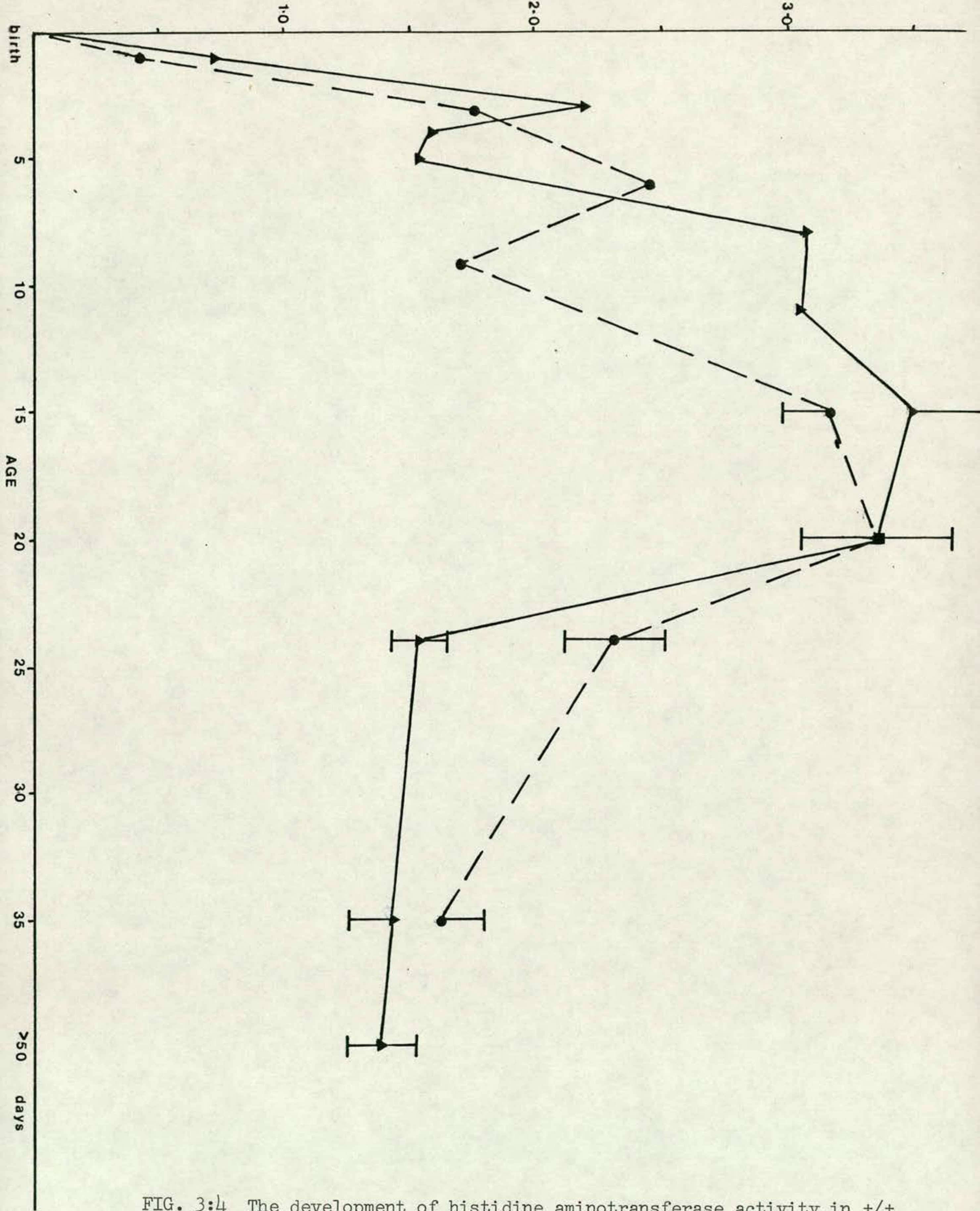
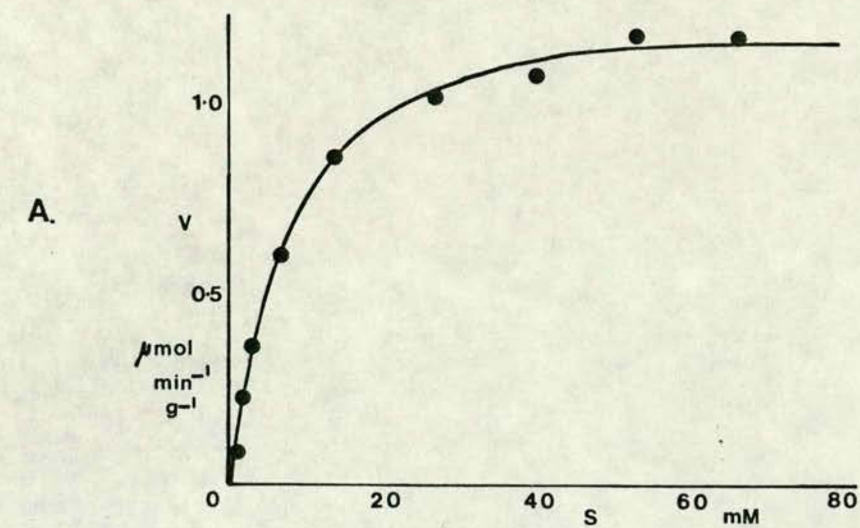


FIG. 3:4 The development of histidine aminotransferase activity in +/+ (▲), his/his (●) and segregating +/his and his/his (■) litters during postnatal development.

TABLE 3:4

The effect of histidine concentration on histidine aminotransferase activity in adult mouse liver.

Histidine mM	Histidine aminotransferase µmol/min /g wet weight
0·7	0·09
1·3	0·23
2·7	0·36
6·7	0·60
13·3	0·86
26·7	1·02
40·0	1·08
53·3	1·18
66·7	1·18



B.

1/V

µmol min⁻¹ g⁻¹

10

5

0

0·5

1·0

1·5

1/S mM

FIG. 3:5 A. The effect of histidine concentration on histidine aminotransferase activity in mouse liver extracts.

B. Lineweaver-Burke plot of histidine aminotransferase activity.

S = histidine concentration, mM.

V = reaction rate, μmol min^{-1} g^{-1}.

Data from Table 3:4

shown in Table 3:3 and Figure 3:4. The activity rises steeply after birth to reach a specific activity 2-2·5 times that of the adult by about ten days. It then plateaus for a further ten days before falling sharply to reach adult activity levels by four to five weeks. No consistent difference between the +/+ and his/his aminotransferase activities is present, despite the marked difference in flux through this pathway caused by the high mutant histidine concentration [see (iv)].

The effect of histidine concentration on histidine aminotransferase activity was determined in adult wild-type liver extracts dialysed overnight against 0·02M Tris HCl pH 7·4 at 4°C. The results are shown in Table 3:4 and the Lineweaver-Burke plot in Figure 3:5. The apparent Km for histidine is 14·7 mM which is in the same region as the mutant 15-day liver histidine concentration (8·8 µmol/g). The Vmax is 1·80 µmol/min/g liver. Since the enzyme does not appear to be saturated, a difference in histidine concentration between wild-type and mutant could result in a corresponding difference in flux, provided the enzyme is not compartmented separately from the substrate or limited by pyruvate [see (vi)].

(iv) Blood amino acid concentrations.

The concentrations of histidine, alanine, phenylalanine, lysine and glutamic acid were determined in whole blood during postnatal development. Alanine and glutamate were examined since there are reports of a raised plasma alanine and a lowered glutamate concentration in human histidinaemia (Ghadimi, 1974). Alanine is one of the products of histidine transamination with pyruvate, while glutamate is one of the major products of histidine catabolism via urocanic acid. The concentrations of phenylalanine and lysine were determined since the former is thought to share a transport system for neutral amino acids with histidine, while the latter uses a separate system for dibasic amino acids (Neame, 1968). A raised histidine concentration would therefore be more likely to influence the transport of phenylalanine than of lysine.

The results are shown in Table 3:5. Marked increases in blood histidine concentration are apparent in his/his mice from day 1 onwards (Figure 3:6) and by five weeks there is a 16-fold difference between the two genotypes. In contrast, there are no statistically

TABLE 3:5

Whole blood amino acid concentrations during postnatal development in +/+ and his/his mice.

Values are means ± standard errors in µmol g^{-1}.

Genotype	Age[2]	Histidine	Alanine	Phenylalanine	Lysine	Glutamic acid	No.
+/+	1	0·092 ± 0·007	0·298 ± 0·028	0·061 ± 0·005	0·346 ± 0·028	0·110 ± 0·008	3
	6	0·161 ± 0·019	0·443 ± 0·029	0·077 ± 0·008	0·625 ± 0·039	0·149 ± 0·010	6
	15	0·123 ± 0·016	0·428 ± 0·085	0·102 ± 0·014	0·607 ± 0·036	0·118 ± 0·008	4
	24	0·065 ± 0·008	0·483 ± 0·061	0·055 ± 0·006	0·423 ± 0·037	0·180 ± 0·044	4
	35	0·090 ± 0·016	0·396 ± 0·069	0·091 ± 0·017	0·442 ± 0·038	0·211 ± 0·035	4
his/his[1]	1	0·548 ± 0·051**	0·307 ± 0·027	0·057 ± 0·006	0·389 ± 0·041	0·089 ± 0·008	3
	6	0·713 ± 0·071**	0·464 ± 0·059	0·076 ± 0·009	0·566 ± 0·038	0·166 ± 0·006	8
	15	1·243 ± 0·073**	0·294 ± 0·041	0·062 ± 0·006*	0·600 ± 0·049	0·111 ± 0·014	4
	24	1·019 ± 0·063**	0·696 ± 0·112	0·059 ± 0·007	0·358 ± 0·011	0·152 ± 0·024	4
	35	1·439 ± 0·108**	0·530 ± 0·116	0·057 ± 0·011	0·486 ± 0·028	0·163 ± 0·010	4

[1] Offspring of his/his x his/his crosses.   * $p<0·05$: ** $p<0·01$

[2] Age in days after birth.

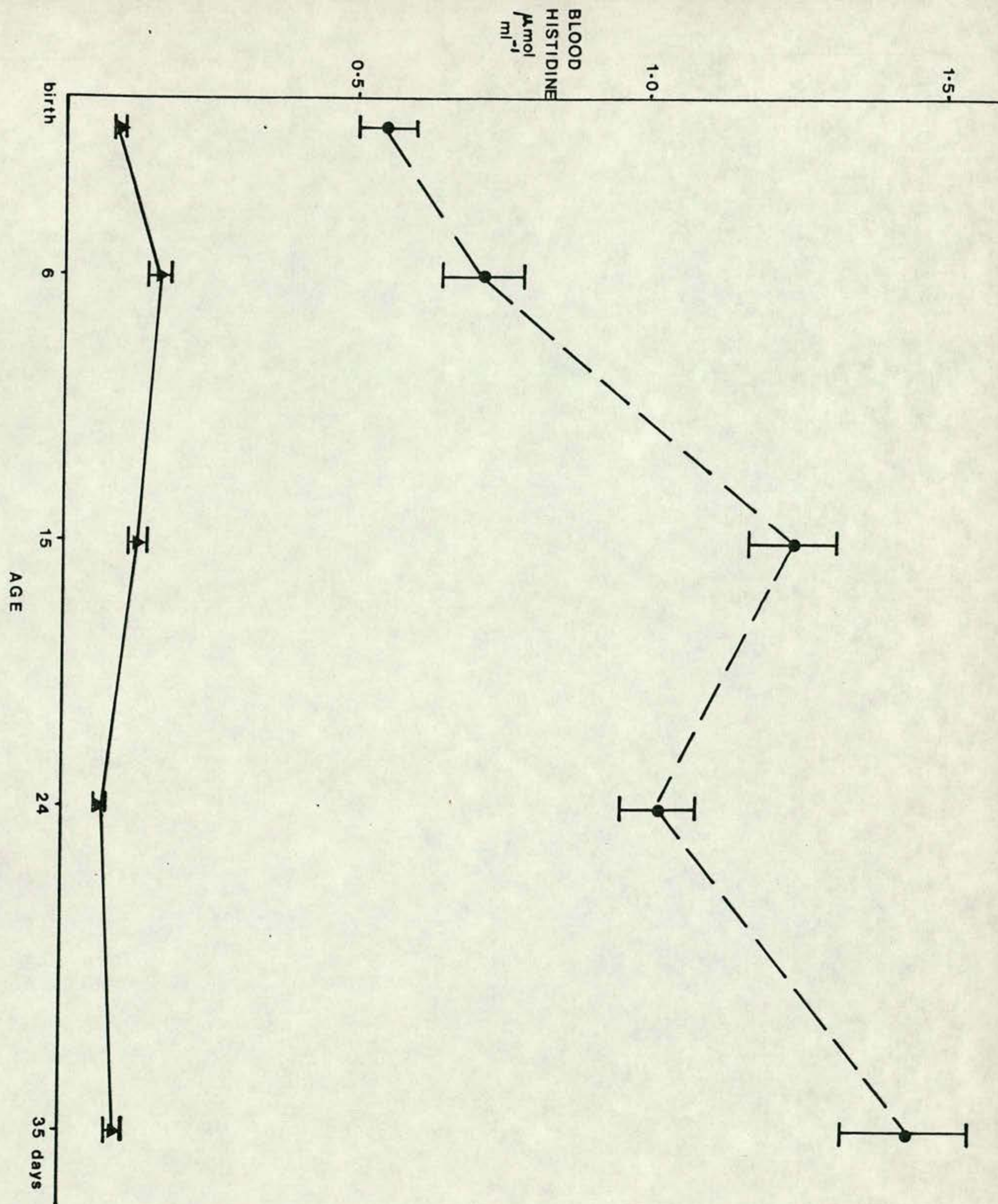


FIG. 3:6 Blood histidine concentration in +/+ (▲——▲) and his/his (●— —●) mice during postnatal development.

significant differences in the blood alanine, lysine or glutamate concentrations at any stage. The blood phenylalanine concentration is significantly reduced ($P < 0{\cdot}05$) in the mutant at the 15-day stage only, perhaps suggesting competition for amino acid entry in the gastro-intestinal tract. The concentration of histidine in gastro-intestinal fluid was not determined, but may be raised in the mutant sufficient to inhibit entry of amino acids using a common carrier. However the blood concentration is the net result of uptake, removal by the tissues and excretion, any or all of which could be altered by raised histidine concentrations.

The increase in blood histidine concentration in histidinaemic mice is apparent as early as day 1, presumably because of the transfer of a raised maternal histidine concentrations via the milk. A prenatal skin histidase deficit may also contribute (see above). As the histidase defect becomes increasingly manifest after weaning (21 days), the histidine concentration difference increases further.

(v) Amino acid concentrations in foetal and maternal samples.

The concentrations of histidine, lysine, alanine and glutamic acid were determined in foetal and maternal blood or amniotic fluid samples on the 16th, 17th and 18th days of gestation. It has been reported that in man (Butterfield and O'Brien, 1963), rat (Boggs and Waisman, 1964), monkey (Kerr and Waisman, 1966) and the mouse (Lajtha and Toth, 1973), foetal amino acid concentrations are higher than maternal ones. An active transport system is therefore presumably present in the placenta, but the significance of the higher foetal concentrations is not known. The foetal/maternal ratio of amino acid concentrations is reported to be between 1 and 3 for most amino acids. It has also been shown that this ratio is maintained even in the presence of elevated amino acid concentrations, achieved by dietary loading in rats (Kerr and Waisman, 1966), or in human PKU (Thomas et al., 1971). These findings suggest that in histidinaemia, the offspring of *his*/*his* mothers could be exposed *in utero* to even higher histidine concentrations than the mother.

The results are shown in Table 3:6. At the 16th gestational day (G) stage, the histidine concentration in the blood of *his*/*his* embryos

Cross	Sample	Genotype	Age	Histidine	Lysine	Alanine	Glutamic acid	No.
I	Maternal blood	+/+	Adult	0·071	0·814	0·601	0·157	1
	Foetal blood	+/his	16G	0·136±0·006	1·433±0·122	0·971±0·069	0·717 ± 0·080	4
	Amniotic fluid	+/his	16G	0·158±0·022	0·907±0·102	0·579±0·062	0·203 ± 0·033	4
	Maternal blood	his/his	Adult	1·338	0·871	0·992	0·389	1
	Foetal blood	his/his	16G	2·568±0·036***	1·095±0·022**	1·361±0·051**	0·973 ± 0·111	6
	Amniotic fluid	his/his	16G	1·357±0·954	0·535±0·379	0·683±0·471	0·524 ± 0·389	2
II	Maternal blood	+/+	Adult	0·102	0·980	1·026	0·505	1
	Foetal blood	+/+	17G	0·138	2·219	1·077	0·596	1
	Amniotic fluid	+/+	17G	0·430	2·818	1·824	0·784	1
	Maternal blood	his/his	Adult	0·829±0·122	0·542±0·021	0·821±0·037	0·256 ± 0·085	2
	Foetal blood	his/his	17G	2·687±0·049	1·394±0·066	1·200±0·246	0·421 ± 0·043	8
	Amniotic fluid	his/his	17G	3·729±0·294	1·337±0·026	1·565±0·129	0·283 ± 0·060	6
III	Maternal blood	his/his	Adult	0·930	0·506	0·544	0·288	1
	Foetal blood	his/his	18G	2·479±0·306	1·700±0·191	1·066±0·085	0·747 ± 0·049	3
	Amniotic fluid	his/his	18G	5·874	2·571	2·940	0·655	1

*$P < 0·05$ **$P < 0·01$ ***$P < 0·001$

TABLE 3:6 Amino acid concentrations in blood and amniotic fluid of foetal samples at 16, 17 and 18 days of gestation.

I Maternal and foetal concentrations from a cross of +/+ female x his/his male, in comparison with his/his x his/his (16 days gestation).

II Maternal and foetal concentrations from a cross of +/+ x +/+, in comparison with his/his x his/his (17 days gestation).

III Maternal and foetal concentrations from a cross of his/his x his/his (18 days gestation). Values are means ± S.E.M. in μmol/ml.

TABLE 3:7

Foetal/Maternal ratios of amino acid concentrations in whole blood in _+/+_ and _his/his_ mice.

Genotype	Age	Foetal/Maternal ratios[1] Histidine	Lysine	Alanine	Glutamic acid
_+/+_ and _+/his_	16G[2]	1·9	1·8	1·6	4·6
his/his	16G	1·9	1·3	1·4	2·5
_+/+_	17G	1·4	2·3	1·05	1·2
his/his	17G	3·2	2·6	1·5	1·6
his/his	18G	2·7	3·4	2·0	2·6

[1] Amino acid concentration in foetal blood/amino acid concentration in maternal blood, in $\mu mol\ ml^{-1}$.

[2] The suffix G refers to gestational days.

is 19-fold higher than in +/his embryos, which is about the same as the genotypic difference in maternal bloods. The molar histidine concentration in his/his foetal blood is almost double that in his/his adults however. Despite the 19-fold difference in maternal histidine concentrations, the foetal/maternal ratio (F/M ratio) of histidine concentrations is 1·9 in both genotypes. The F/M ratios for all the amino acids measured are shown in Table 3:7. Each amino acid has a F/M ratio greater than 1 at all stages, regardless of the maternal concentration. At the 17G stage, foetal blood histidine is again 19-fold higher in his/his than +/+ embryos and the F/M ratio is even higher at 3·2 compared with 1·4 in +/+ animals.

At the 16G stage, foetal blood lysine and alanine concentrations also appear to be different in his/his and +/his embryos (Table 3:6). The lysine concentration is 24% lower in his/his embryos while the maternal blood lysine concentrations are about the same. At the 17G stage, the lysine concentration is again reduced in mutant embryos but this may only reflect a lower maternal concentration in the mutant. The blood alanine concentration in 16G his/his embryos is higher than in +/his embryos although this may again reflect a higher maternal concentration, since there is no marked difference at the 17 day stage.

Amniotic fluid histidine concentrations are also higher (by a factor of 9) in his/his compared with +/+ embryos at the 16G and 17G stages. The lysine concentration is lower in his/his samples at both the 16G and 17G stages, as found in foetal blood.

(vi) Imidazole acid concentrations.

The concentrations of imidazolepyruvic acid (IPA), imidazolelactic acid (ILA), imidazoleacetic acid (IAA) and N-acetyl histidine were determined in foetal and maternal samples on the 17th day of gestation in +/+ x +/+ and his/his x his/his crosses (Table 3:8). These compounds were undetectable in amniotic fluid, foetal and maternal blood samples in the wild-type. In the mutant, the largest chromatographic peak was IPA combined with N-acetyl histidine (not separable chromatographically at this stage), which had a concentration of 0·024 μmol/ml in maternal blood and 0·096 μmol/ml in amniotic fluid. ILA and IAA were undetectable in his/his maternal blood and present at concentrations of 0·057 μmol/ml and 0·017 μmol/ml in pooled his/his amniotic fluid samples.

TABLE 3:8

Imidazole acid concentrations (A) in foetal blood and amniotic fluid, in comparison with maternal blood ($\mu mol\ ml^{-1}$): (B) in the brain (cerebrum) of mice from his/his x his/his crosses during postnatal development ($\mu mol\ g^{-1}$).

Cross	Tissue	Age[2]	Im lactate	Im acetate	Im pyruvate + Ac histidine[1]	No.
(A) his/his x his/his	Foetal Blood	17G	< 0·009	<0·008	<0·025	6
	Amniotic Fluid	17G	0·057	0·017	0·096	3
	Maternal Blood	Adult	<0·007	< 0·006	0·024	1
+/+ x +/+	Foetal Blood	17G	< 0·009	<0·008	<0·025	1
	Amniotic Fluid	17G	< 0·003	<0·002	<0·004	1
	Maternal Blood	Adult	< 0·007	<0·006	<0·01	1
(B) his/his x his/his	Cerebrum	6P	<0·018	<0·015	<0·025	5
		15P	<0·018	<0·015	0·144	1
		24P	<0·018	<0·015	<0·025	1
		35P	<0·018	<0·015	<0·025	1

[1] These compounds were not resolved chromatographically. Values represent the sum of both peaks.

[2] The suffix G refers to gestational days and P to postnatal days.

Im, imidazole · Ac, N-acetyl

None of these imidazoles were detectable in foetal his/his blood samples. As in adult mice, the imidazole acids are barely detectable in tissue or body fluids other than urine. In amniotic fluid, concentrations are considerably higher which supports the suggestion that this fluid serves as an excretory reservoir for the embryo.

These same imidazole compounds were then measured in his/his brain (cerebrum) during postnatal development (Table 3:8). The combined IPA/N-acetyl histidine peak was then the only measurable peak, and this only on the 15th day after birth, when a concentration of 0·144 μmol/g was found. The higher brain concentration at the 15 day stage is paralleled by higher imidazole acid concentrations in the urine at 10 - 20 days (see below).

Urinary histidine, IPA, ILA, IAA, and N-acetyl histidine concentrations were determined in +/+ and his/his mice during postnatal development. The results are shown in Table 3:9 and Figures 3:7 - 3:11. Changes in urinary histidine are shown in Figure 3:7. On day 1, the +/+ urinary histidine concentration is thirty times that on day 30, despite the similarity in blood histidine concentrations at these stages (Figure 3:6). The renal reabsorption capacity for amino acids may be poorly developed at this stage, contributing to the almost four-fold drop in +/+ urinary histidine concentration in the first five days after birth as transport systems mature. While the wild-type histidine concentration falls steadily through postnatal development, in the mutant it maintains a high concentration. The highest absolute concentration is found at ten days, but owing to the decline of wild-type levels, the ratio of mutant/wild-type increases as development proceeds.

The concentration of IPA in urine is about the same in the two genotypes on day 1, then increases in the mutant to plateau between 10 and 21 days before falling slightly at 30 days (Figure 3:8B). Wild-type IPA concentrations gradually fall throughout development so that the ratio of mutant/wild-type increases between birth and 30 days, when it reaches 19. ILA concentrations show a similar trend (Figure 3:8A). IAA concentrations in urine (Figure 3:9) are highest in both genotypes on day 1, falling off by a factor of 2 - 2·5 within the first five days. Between 3 and 30 days, IAA maintains a high concentration

TABLE 3:9

Urinary imidazole concentrations in +/+ and his/his mice during postnatal development.

Values are means ± standard errors in µmol mg^{-1} creatinine.

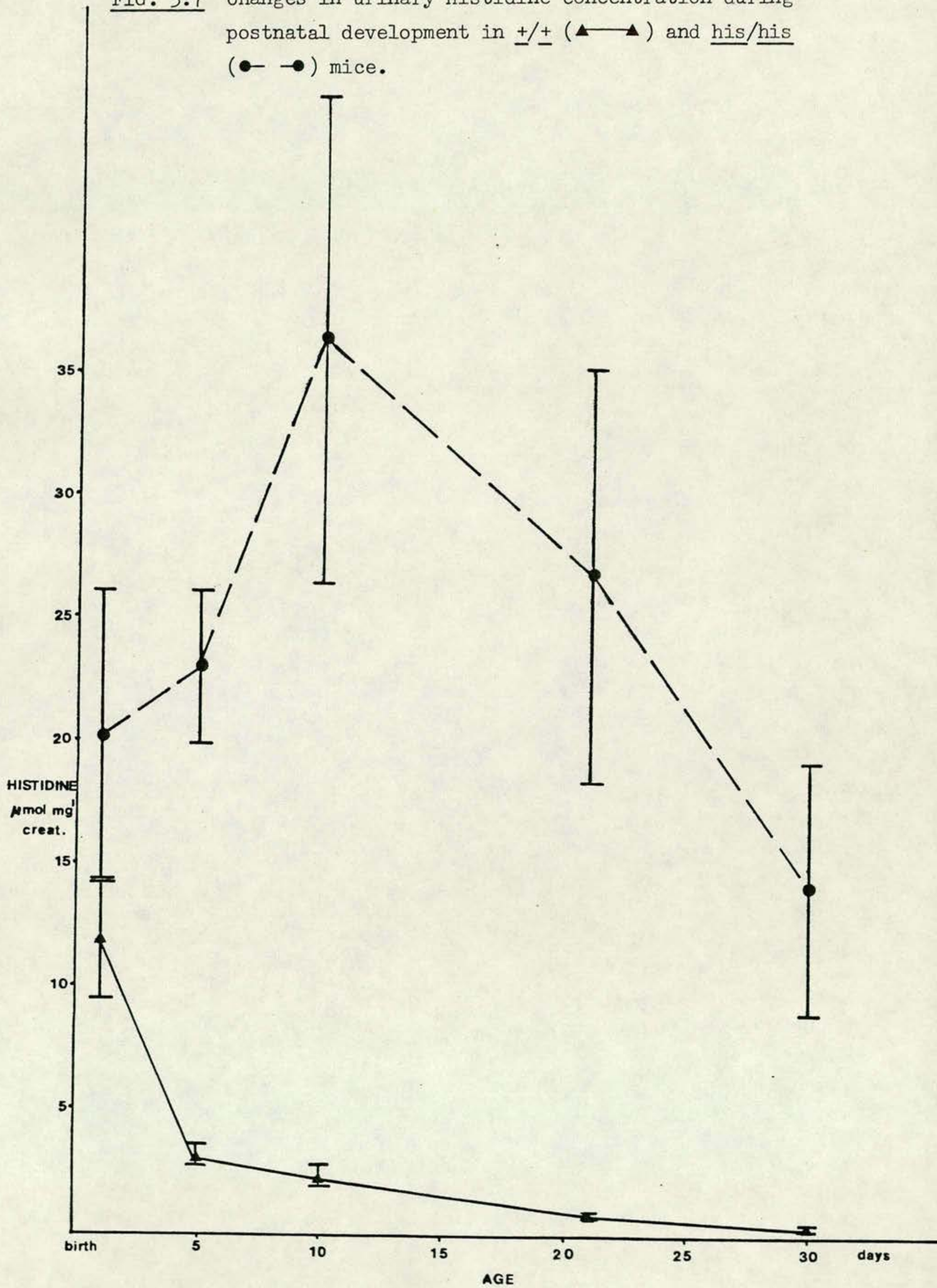
Genotype	Age[2]	Histidine	Im pyruvate	Im lactate	Im acetate	Ac histidine	No.
+/+	1	11·93 ± 2·42	2·80 ± 0·44	0·57 ± 0·08	2·85 ± 0·12	0·51 ± 0·04	2
	5	3·10 ± 0·39	2·26 ± 0·62	0·36 ± 0·10	0·62 ± 0·08	0·23 ± 0·03	7
	10	2·34 ± 0·46	1·56 ± 0·62	0·73 ± 0·11	0·60 ± 0·04	0·41 ± 0·03	6
	21	0·82 ± 0·13	1·78 ± 1·41	0·12 ± 0·02	0·55 ± 0·16	0·37 ± 0·13	3
	30	0·37 ± 0·08	0·37 ± 0·13	0·04 ± 0·001	0·13 ± 0·02	0·19 ± 0·02	3
his/his[1]	1	20·21 ± 5·92	3·48 ± 1·31	0·56 ± 0·14	2·62 ± 0·63	0·80 ± 0·16	5
	5	23·03 ± 3·12***	6·15 ± 3·16	0·64 ± 0·17	1·35 ± 0·25**	1·20 ± 0·15***	4
	10	36·43 ± 9·89**	8·77 ± 1·87**	2·05 ± 0·41**	1·89 ± 0·19***	2·76 ± 0·70**	5
	21	26·94 ± 8·46*	9·27 ± 3·82	1·74 ± 0·30**	2·27 ± 0·34**	2·69 ± 0·58*	4
	30	14·18 ± 5·14	6·91 ± 2·18*	0·82 ± 0·18*	1·13 ± 0·19**	3·21 ± 0·60**	3

*p<0·05 : **p<0·01 : ***p<0·001

[1] Offspring of his/his x his/his crosses.

[2] Age in days after birth.

FIG. 3:7 Changes in urinary histidine concentration during postnatal development in +/+ (▲—▲) and his/his (●– –●) mice.



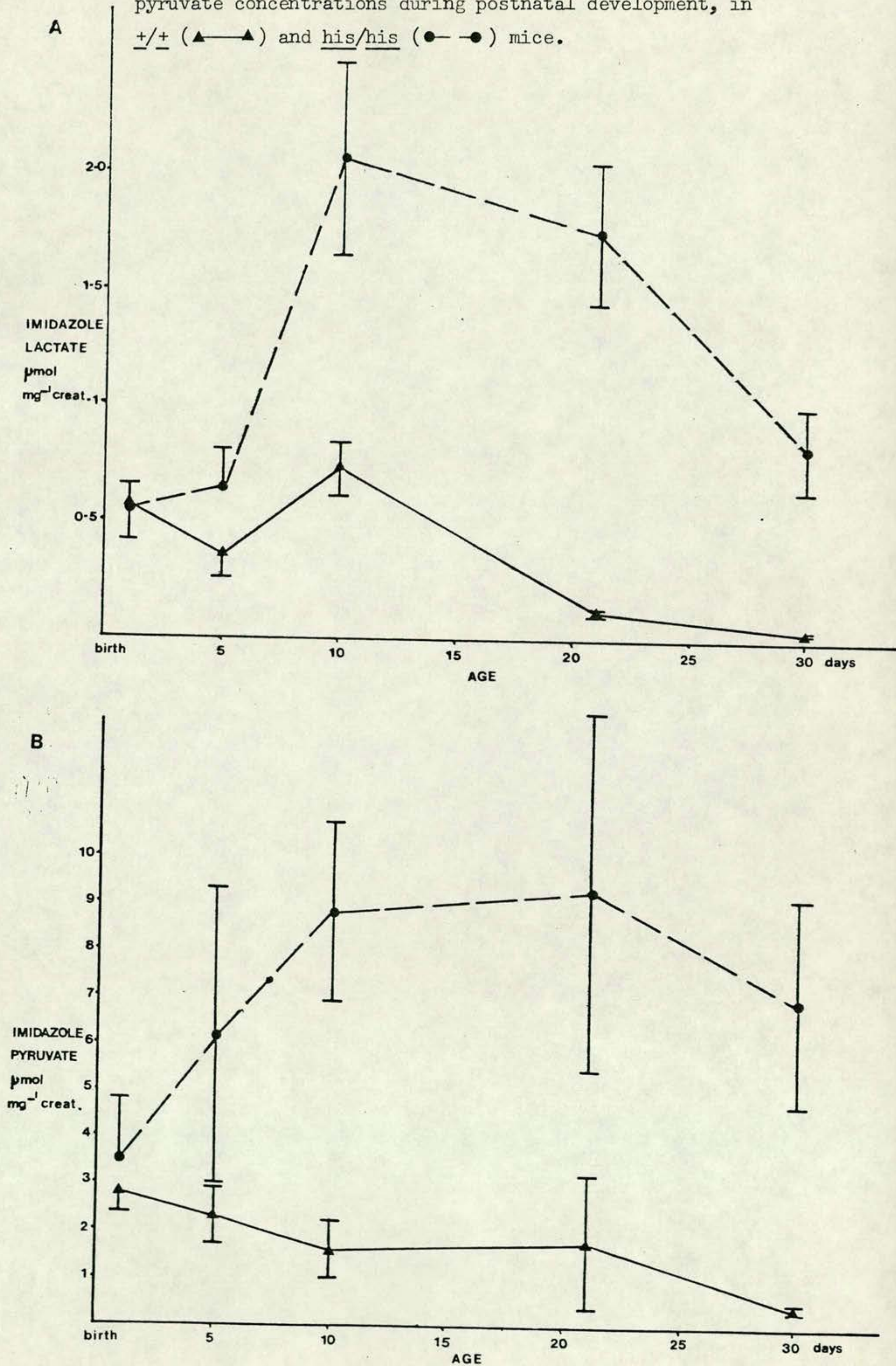


FIG. 3:8 Changes in urinary (A) imidazole lactate and (B) imidazole pyruvate concentrations during postnatal development, in +/+ (▲——▲) and his/his (●– –●) mice.

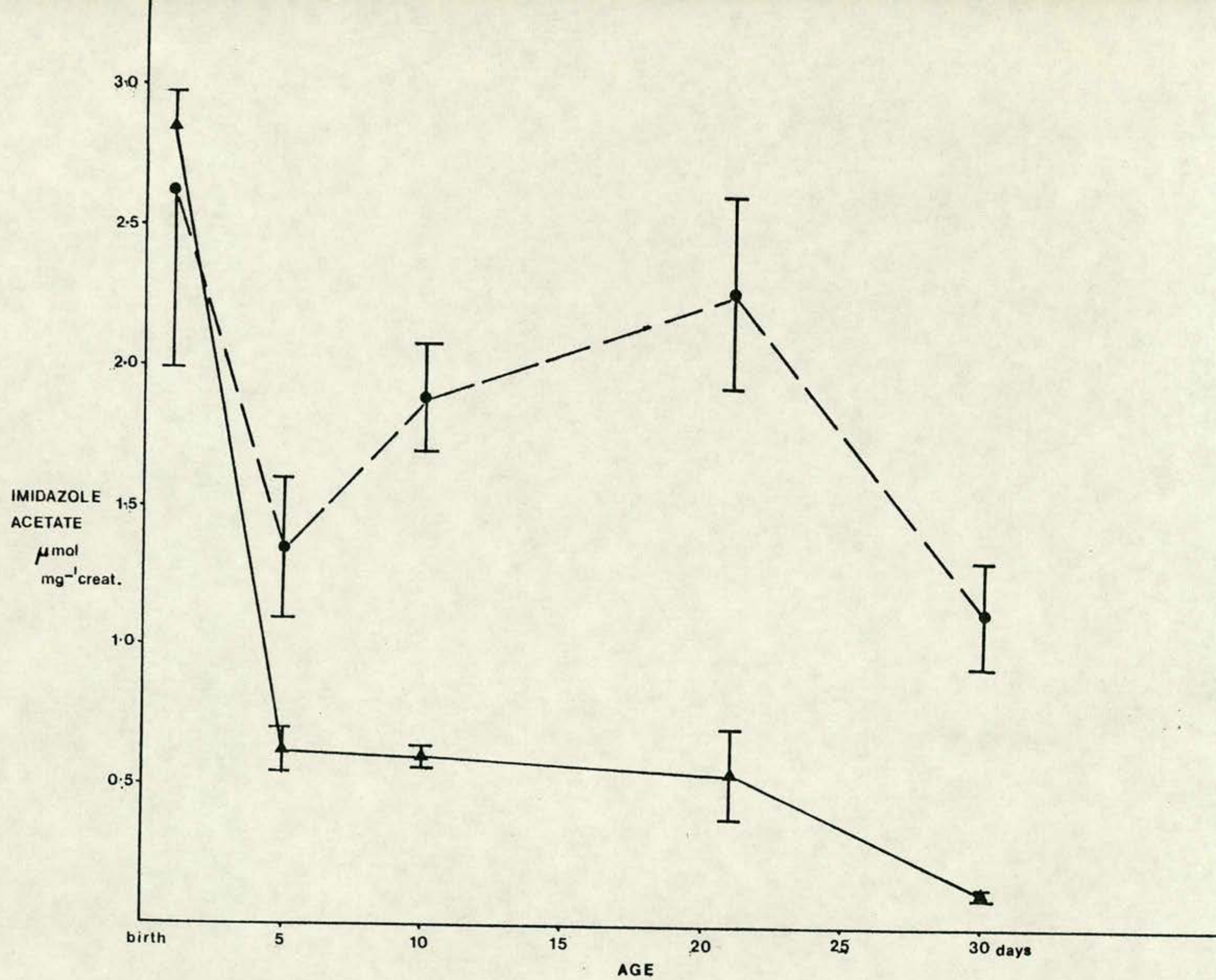


FIG. 3:9 Changes in urinary imidazole acetate concentration during postnatal development in +/+ (▲——▲) and his/his (●— —●) mice.

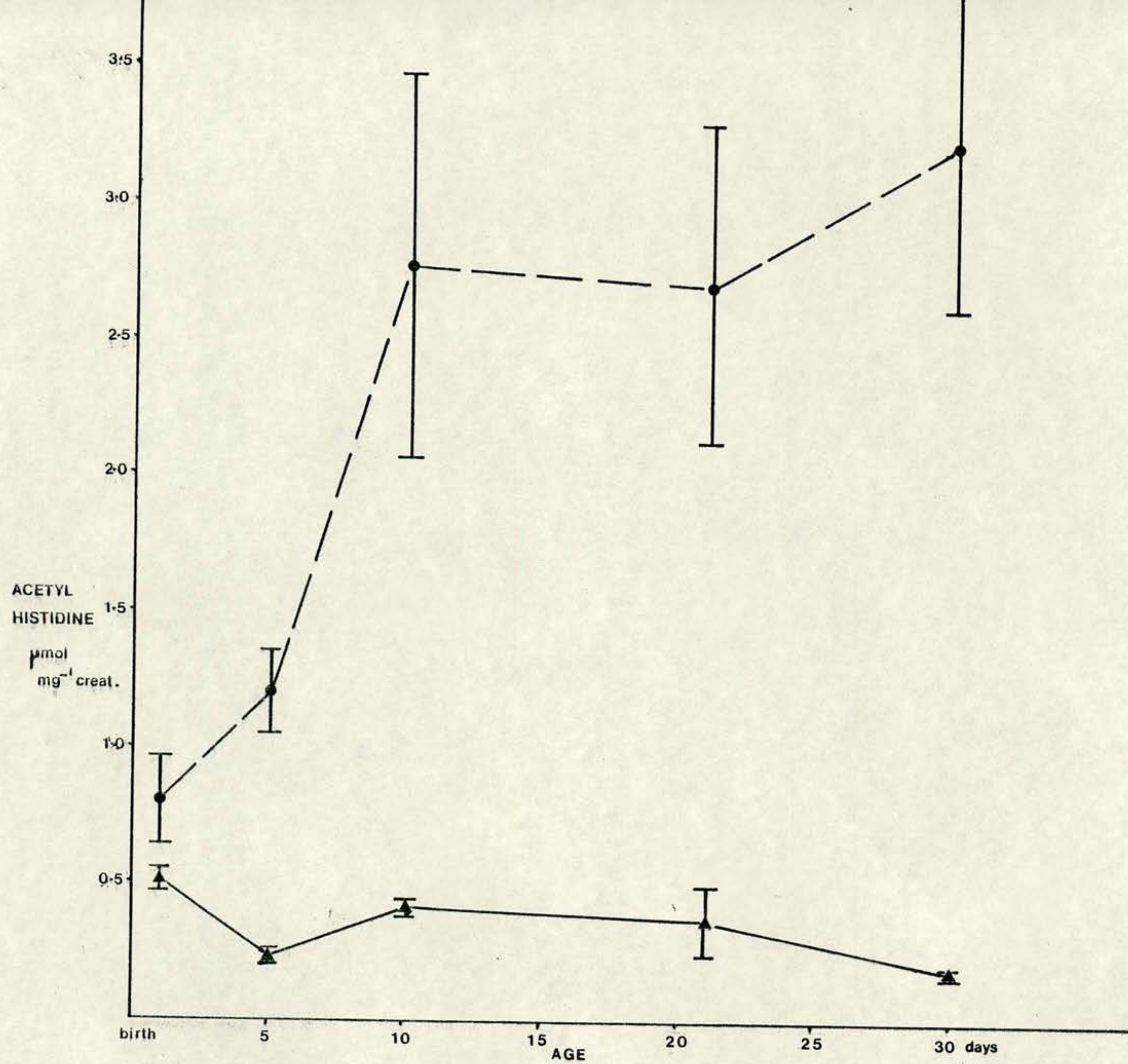


FIG. 3:10 Changes in urinary acetyl histidine concentration during postnatal development in +/+ (▲——▲) and his/his (●– –●) mice.

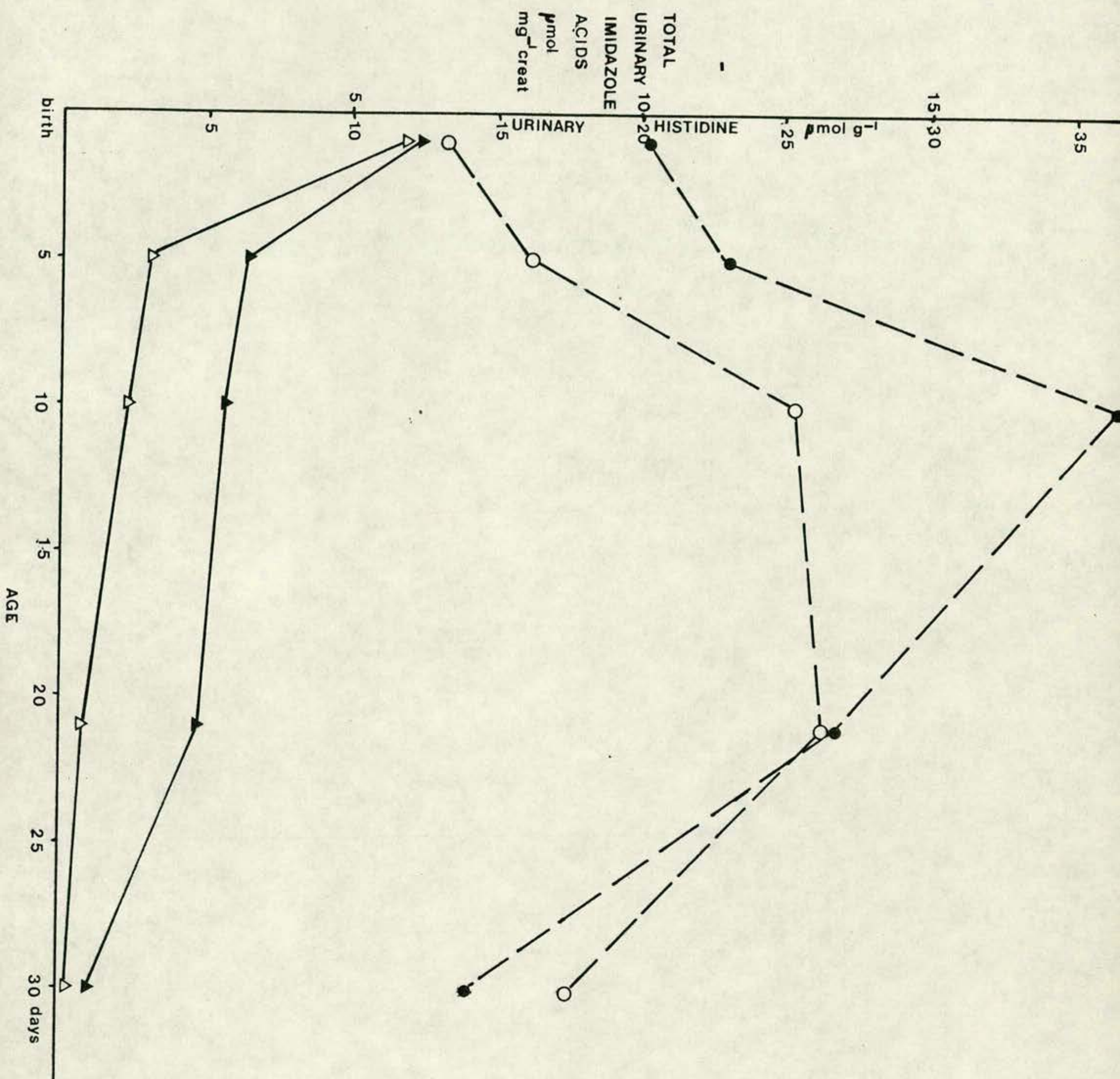


FIG. 3:11 Total urinary imidazole acid (IPA + ILA + IAA) concentrations (▲, ●) in comparison with urinary histidine concentrations (△, ○) during postnatal development, in +/+ (▲, △) and his/his (●, ○) mice.

in the mutant while steadily falling in wild-type. A comparison of total urinary imidazole acids (IPA + ILA + IAA) with histidine concentrations during development (Figure 3:11) shows that in both genotypes, the two run parallel. The blood histidine concentration (Figure 3:6) shows a similar trend. Comparison of the histidine aminotransferase activity (Figure 3:4) with the urinary histidine and imidazole acid concentrations (Figure 3:6) shows that the high imidazole acid excretion between 10 and 20 days is probably not a consequence of the high liver aminotransferase activity at this time. It is explicable in terms of changes in the histidine concentration.

Urinary N-acetyl histidine concentrations (Figure 3:10) rise sharply in the mutant during the first ten days after birth, then level out. In +/+ animals, N-acetyl histidine concentrations show only a slight decline during the same period. By 30 days the concentration in his/his urine is 17-fold higher than in +/+ urine.

One other unidentified Pauly-positive chromatographic peak was found to be increased in the mutant compared with wild-type urine. It chromatographed close to, but distinct from, IAA. It could be methyl-imidazoleacetic acid but this was not verified.

(vii) Discussion.

Changes in the histidine catabolic enzymes have been examined during the first five weeks after birth. Wild-type skin histidase activity is high at birth and remains so for the first five days, then declines sharply to about 2-3% of the peak activity (Figure 3:2). Wild-type hepatic histidase activity is low at birth, increases steadily during the first and third weeks in both sexes, then rises some 50% higher in females than males during the fourth and fifth weeks (Figure 3:1). These findings are very similar to those obtained in the liver (Auerbach and Waisman, 1959; Feigelson, 1968; Makoff and Baldridge, 1969) and skin (Bhargava and Feigelson, 1976a) of the developing rat. Mutant histidase activity shows a rather different development. In the liver, activity increases steadily during the first 2 weeks after birth, then declines slowly to about a quarter of the peak activity (Figure 3:1). No sex difference is present. It is therefore unlikely that the mutant and wild-type enzymes differ only in catalytic efficiency (turnover number). If this was the case, their developmental courses

should run parallel. The fact that they do not suggests (1) different rates of enzyme synthesis, perhaps due to differing responses to hormonal induction; (2) different rates of enzyme degradation; (3) different susceptibility to activators or inhibitors; (4) methodological difficulties with the histidase assay due to interference from the developing urocanase activity; (5) that the residual histidase activity is due to a distinct enzyme with a low activity that is normally masked by the more active enzyme species. This last possibility is also suggested by the development of skin histidase activity in the two genotypes. Wild-type activity is considerably higher than the mutant during the first week, but then declines sharply to the same level as the mutant (Figure 3:2). This could be explained by the occurrence of two skin isozymes of histidase with high and low activities. The former is present in +/+ skin before birth and during the first week of life then disappears. The low activity isozyme is present in both genotypes but is masked by the high activity enzyme until this disappears in the second week. The mutant may either have a variant form of the high activity enzyme with greatly reduced activity accounting for the slight developmental rise or a complete absence of this enzyme. Against this interpretation is the fact that the enzyme in adult rat skin is reported to be identical to the major liver enzyme by three criteria (immunological properties, Km for histidine, isoelectric point) which suggests that there is only a single histidase species in the rat (Bhargava and Feigelson, 1976a). However these studies were carried out on purified enzyme and the purification procedure could have led to loss of a minor histidase species, especially if it is less stable. Another explanation for the identical skin histidase activity in the two genotypes at 15 days, is that the *his* locus mutation does not alter the catalytic efficiency of the enzyme, only its responsiveness to hormonal induction. This is also compatible with the identical, low levels of liver activity in +/+ and *his*/*his* mice on the day after birth, after which only the wild-type shows the expected increase. However the rise in histidase activity after birth can be attributed to a number of distinct processes (Feigelson, 1968; 1973a, b). The neonatal rise has been attributed to the effects of glucagon and gluco-

corticoids. The adolescent increase in the male may also be due to glucocorticoid action and the pubertal rise in females to oestrogen. It would therefore be necessary to invoke a loss of responsiveness to two or three distinct hormonal influences to explain the findings. The neonatal increase in mutant activity is greatly reduced and the adolescent and pubertal increases absent. Mutation at a regulatory locus controlling the induction of histidase by several hormones is however a possibility. An alteration in the rate of histidase synthesis could also arise from mutation at the histidase structural locus (see Chapter 5).

An increased rate of histidase degradation could account for the observed differences in histidase development. This could arise either from a structural locus mutation leading to altered stability (often coupled with impaired catalytic function) or from mutation at another, processing locus which specifically increases the rate of histidase degradation (see Rechcigl and Heston, 1967).

An alteration in the response of mutant histidase to activating or inhibiting influences could lead to the different developmental courses observed. A structural locus mutation could for example lead to increased inhibition either by substrate or product. Reduced activation or increased inhibition by one or more effector molecules could arise from the same cause or from mutation at another locus.

Finally, the differing profiles of histidase development in the liver in the two genotypes might be due, in part at least, to interference with the histidase assay by the next enzyme in the pathway, urocanase. This is suggested by the progressive decrease in mutant histidase activity from 15 days onwards, which could be related to a developmental increase in urocanase activity, and by the unmasking of mutant activity after protamine sulphate precipitation (see Chapter 2). Urocanase activity is greatly reduced, but not eliminated, by the high pH of the histidase assay, but any interference is expected to be greater with a low activity than a high activity histidase.

One of the main alternative pathways through which the rising liver histidine concentration can be catabolized is by transamination with pyruvic acid, by means of histidine aminotransferase. The combined mitochondrial and cytosolic activity increases from a low level at birth to reach a high activity during the second and third weeks. It

then declines sharply to 40 - 50% of the peak activity (Figure 3:4). This finding has not been reported previously but is similar to the high neonatal phenylalanine-pyruvate aminotransferase activity in the rat liver, which then falls to a lower level (Auerbach and Waisman, 1959). There is no consistent difference in liver histidine aminotransferase activity between +/+ and his/his mice. The high in vitro activity found early in development, could lead to a corresponding increase in flux in both genotypes. Unless the enzyme is already saturated with histidine or limited by pyruvate, the tenfold higher blood histidine concentration in the 15 day old mutant will also increase this flux. In the adult mutant, an increased concentration of urinary IPA is a cardinal feature showing that neither of these limitations apply. The apparent Km of the adult liver enzyme for histidine was determined and found to be 14·7mM, so that the liver histidine concentration (20·9 µmol/g wet weight or roughly 20 mM) would not be expected to saturate the adult enzyme. However, regardless of substrate concentrations, the high neonatal aminotransferase activity should lead to a corresponding increase in the flux to IPA, compared with older animals, unless it is compartmented such that the increased activity occurs in a quantitatively minor histidine (or pyruvate) pool, so contributing little to the overall flux. The urinary concentrations of IPA in the wild-type are not in fact higher in the second and third weeks after birth than in older animals (Figure 3:8B). In the mutant, IPA is higher at this stage but so is the concentration of histidine, which is therefore a sufficient explanation. In wild-type urine, IPA is higher on days 1 and 5 than on day 10 despite a considerably lower aminotransferase activity. It therefore seems unlikely that the high neonatal aminotransferase activity is reflected in a corresponding increase in flux to IPA or its derivatives (see Figures 3:8-9). Since only the mitochondrial isozyme of histidine aminotransferase is inducible by glucagon in the rat (Morris et al., 1973; Noguchi et al., 1976a), the high total activity observed between 10 and 20 days may be due to an increase in the activity of this component only. This would suggest that the mitochondrial isozyme contributes little to the overall flux to IPA.

The concentration of histidine in the blood of histidinaemic mice varies considerably during development. In 16-18 day old embryos the concentration is 2·5-2·7 μmol/ml compared with 1·0-1·5 μmol/ml in the adult. Although most amino acid concentrations are higher in foetal than maternal blood, the results confirm that histidine is transported into the foetus against a concentration gradient, even when the maternal concentration is raised almost 20-fold (Table 3:7). The blood histidine concentration of his/his embryos is 19 to 20-fold higher than in +/+ or +/his embryos. After birth, the enzyme defect is not immediately apparent in his/his mice, so that the neonatal increase in blood histidine concentration (Figure 3:6) may be due to the transfer of a raised histidine concentration via the his/his mother's milk. This is supported by the fact that after weaning at 21 days, the blood histidine falls by almost 20% before rising again at five weeks, by which time the enzyme defect is almost fully developed (Figure 3:6).

The effect of a raised histidine concentration on other amino acids was investigated during embryonic and postnatal development (Tables 3:5-6). Alanine, which is one of the products of histidine transamination, is increased by 20 - 30% in the blood of his/his mice at some stages before and after birth, but this did not reach statistical significance. The concentration of lysine in foetal blood and amniotic fluid samples from his/his mice is lower than from +/+ mice, although the reason for this is obscure.

In some aminoacidopathies, the metabolites may be more toxic than the affected amino acid itself (e.g. branched chain ketoaciduria). The concentrations of the histidine metabolites IPA, ILA, IAA and N-acetyl histidine were therefore measured in the brain and body fluids during development (Tables 3:8-9). In +/+ mice, none of these metabolites are detectable in 17-day foetal blood or amniotic fluid or in maternal blood. Bulfield and Kacser (1974) were also unable to measure these compounds in the plasma or brain of +/+ adult mice. In the developing histidinaemic brain, the only measurable chromatographic peak was a combined IPA and N-acetyl histidine peak, found only at 15 days of age at a concentration of 0·144 μmol/g wet weight. The

ratio of these two compounds can be estimated from their relative urinary concentrations at the same stage of development (when they were separable chromatographically). This gives a ratio of approximately 2·5:1 for IPA to N-acetyl histidine, so that the peak brain concentration of IPA would be in the region of 0·1 μmol/g (roughly 0·1mM). The concentrations of ILA and IAA in the developing brain are less than 0·018 μmol/g and 0·015 μmol/g respectively. These concentrations are too low to act as inhibitors of any enzymatic reactions known to be influenced by these compounds. Small et al., (1970) have reported that IPA and IAA can inhibit rat brain glutamate decarboxylase in vitro by about 10 - 30%, but the concentrations required (7·5 mM) are two orders of magnitude higher than those found here. However the leucine metabolite, α-ketoisocaproic acid, has been reported to inhibit rat brain pyruvate dehydrogenase with a Ki as low as 0·1mM (Bowden et al., 1970), so that IPA cannot be ruled out as a metabolic inhibitor.

These same histidine metabolites were measured in the his/his embryos from histidinaemic mothers (Table 3:8). Although undetectable in foetal blood, amniotic fluid samples showed the concentrations of IPA/N-acetyl histidine, ILA and IAA to be 0·096, 0·057 and 0·017 μmol/ml respectively. In the mouse the amniotic fluid composition is largely foetal rather than maternal in origin (Renfree and McLaren 1974; Renfree et al., 1975). These compounds would therefore appear to be readily excreted by the foetal kidney so that blood and tissue concentrations are kept low. IPA is unlikely to be formed to any extent by transamination of histidine in the foetal liver, since the aminotransferase activity is very low until after birth (Figure 3:4). It would seem therefore that these metabolites of histidine are passively transported into the foetus via the placenta and rapidly cleared into the amniotic fluid. It was not possible to determine foetal:maternal concentration ratios.

The urinary concentrations of histidine and its metabolites are measurable in both wild-type and histidinaemic mice, so that the two genotypes can be compared directly (Table 3:9). In +/+ urine, the histidine concentration falls by a factor of 30 between day 1 and

day 30 (Figure 3:7). This may be due to the increasing reabsorption capacity of the renal tubular transport system. In the mutant, urinary histidine concentrations rise from about twice the wild-type level on day 1 to a peak at 10 days which is sixteenfold higher than the wild-type then fall to about 40% of this value at 30 days, although the mutant/wild-type ratio increases to almost 40. The changes in urinary histidine concentration in the mutant parallel those in the blood (Figures 3:6-7).

The concentrations of IPA, ILA and IAA in his/his urine tend to change in parallel (Figures 3:8-9). Also the combined IPA, ILA and IAA concentrations show a close relationship to changes in the urinary histidine concentration (Figure 3:11). This suggests that the availability of histidine is the major determinant of the transamination flux.

One unexpected finding is the high urinary IAA concentrations found on day 1 in both genotypes (Figure 3:9). In the wild-type, this falls to about 20% of this level over the next four days and in the mutant to about half. One possible reason for this finding is the high histamine formation during the pre- and perinatal periods (Rosengren, 1963), which can be metabolized in the mouse to IAA (Schayer, 1959; Kapeller-Adler, 1970). It is also possible that the IAA chromatographic peak is a composite of IAA and methylimidazoleacaetic acid, the latter (or its riboside) being the major end-product of histamine metabolism. It was not possible to verify this, since the compound is not available commercially.

Urinary N-acetyl histidine shows a slightly different developmental course from the other histidine metabolites (Figure 3:10). It increases sharply in the mutant during the 10 days after birth, plateaus then increases further at 30 days. In +/+ urine, its concentration remains fairly constant although there is a slight decline at 30 days.

The relative molar concentrations of the various urinary metabolites of histidine show relatively little change during development. The major histidine metabolite at all stages is IPA, comprising

about 30% of the total urinary imidazoles. The IAA concentration is about one-quarter of this and ILA about one-tenth. The relatively high IAA concentration on day 1 is one exception. Another is the four to five-fold increase in the proportion of the total imidazoles found in N-acetyl histidine between days 1 and 30. The relative concentrations of these urinary histidine metabolites is different from those in human histidinaemia, where ILA is the major urinary metabolite (Wadman et al., 1971, 1973).

These results show that in the his/his offspring of his/his mice, the histidine concentration is raised about twenty-fold towards the end of gestation and an average ten-fold during the first five weeks after birth. The enzyme defect in the liver does not appear till the first week after birth although in the skin it is probably present before birth. Whether the latter gives rise to a difference in histidine concentration before birth could be answered by examining the his/his offspring of heterozygous mothers. This last cross might also help to distinguish between the relative contributions of maternal histidinaemia and histidase deficiency in producing a raised neonatal histidine concentration. However, since it would be difficult to distinguish young his/+ from his/his offspring in the segregating litter, a reciprocal cross of +/+ x his/his mice would be a better way of examining the maternal effect per se. Differences in the transport or metabolism of histidine in foetal and postnatal mice are shown up by the considerably higher molar histidine concentration in the former. A previously unreported increase in liver histidine aminotransferase activity has been found, although this is not accompanied by a corresponding increase in urinary transamination products. Finally, an examination of +/+ and his/his skin histidase activity during development shows that, while the activities are widely different during the first week, they gradually converge until they are identical by 15 days after birth. The implications of this finding on the nature of the his mutation will be discussed further in Chapter 5.

CHAPTER 4

DEVELOPMENT OF THE HISTIDINAEMIC MOUSE II BIOCHEMICAL INDICES OF BRAIN DEVELOPMENT

A dramatic series of morphological and biochemical changes occur in the developing brain about the time of birth (Davison and Dobbing, 1968). These changes coincide with the maximal increase in brain wet weight and have come to be known as the brain "growth spurt". In the mouse, this occurs during the first three weeks after birth (Uzman and Rumley, 1958; Himwich, 1962; Kobayashi, 1963; Agrawal et al., 1968). In man, the growth spurt starts about six months before birth, reaches a peak at birth, and extends for about eighteen months after it (Dobbing, 1970). Multiplication of neuroblasts to form the adult number of neurons has largely occurred before this stage, although some short-axoned interneurons (microneurons) develop later (Davison and Dobbing, 1968). The growth spurt is thought to be due primarily to (1) neuronal expansion, in which axonal and dendritic terminals grow out and establish their synaptic connections, (2) the multiplication of glial cells and (3) the deposition of myelin sheaths around the neuronal axons. These events occur approximately in the above sequence, although at different times in different parts of the brain. Major biochemical changes occur during this period leading to the achievement of adult patterns of metabolic and electrical activity (Davison and Dobbing, 1968; Berl, 1973). Further growth is restricted to lengthening of axons and keeping pace with the increasing body size.

In the mouse, the morphological and biochemical changes associated with the growth spurt occur particularly during the second and third weeks after birth. The ground substance (meshwork of nerve connections) of mouse brain appears on days 5 - 6 (Hess, 1955). Electrical activity changes rapidly from the patterns of the immature brain to those of the adult between the 6th - 9th days and 16th - 17th days (Kobayashi, 1963). Dendritic growth is evident by day 8 and markedly increased or completed by day 15 (Stefanowska, 1898; Raman y Cajal, 1960). The cortex reaches its full thickness around the 17th day after birth (Sugita, 1918). The packing density of neurones decreases rapidly between the

3rd and 7th days and does not change after the 17th day (Haddara, 1956). By the 15th - 17th days after birth, the neurohistological development of cerebral cortex is indistinguishable from the adult and the growth rate has subsided dramatically (Kobayashi, 1963). Fox (1965) divided the behavioural and neurological development of the mouse into five characteristic periods. The third period, between the 9th and 15th days after birth, is associated with a rapid transition to the adult pattern of behavioural and neurological responses. Fox regarded this as a "critical period" of behavioural and neurological ontogeny. Other workers have considered the brain growth spurt as a whole to be a "critical" or "vulnerable" period of development (Flexner, 1955; Dobbing, 1968, 1974; Agrawal and Davison, 1973). Although the concept has been criticised (Dobbing, 1968) there is no doubt that a wide range of nutritional, metabolic and hormonal factors acting on the brain at this time can permanently affect its structure or function. In man, the extended period of myelination is particularly susceptible to interference, although it is perhaps the formation of synaptic connections and proliferation of glial cells that are most crucial to laying the foundations of intelligence (Davison, 1977).

Biochemical measures of mouse brain maturation also change most dramatically during the three weeks after birth. There is a loss of water and increases in total protein, lipid and nucleic acid. Changes in DNA content reflect changes in cell number (Mandel and Bieth, 1952; Gayet and Bonichon, 1961; Hunt and Jacobson, 1971; Dickerson and Dobbing, 1967; Howard, 1968; Altman, 1969), since the great majority of cells in the brain are diploid (Swift, 1950; Heller and Elliot, 1954; Lapham, 1968) and the amount of DNA per diploid cell is constant (Boivin et al., 1948; Vendrely and Vendrely, 1949; Mirsky and Ris, 1951). The water content of the brain probably falls as a result of the accumulation of solid matter (Davis and Himwich, 1973). Because of these changes in the solid constituents of the developing brain, it is useful to express the concentrations of chemical constituents in terms of the DNA content. The concentrations of RNA and protein per unit of DNA give some indication of changes in cell size or cytoplasmic/nuclear ratio (Hess and Thalheimer, 1965; Balazs et al., 1968; Davison

and Dobbing, 1968; Winick and Rosso, 1973; Yates et al., 1974). Such parallels between structural and biochemical entities should be drawn with caution however, especially in a heterogeneous tissue such as the brain. Brain cholesterol has been suggested as a useful "marker" for the deposition of myelin lipids (Davison and Dobbing, 1968). The major myelin lipids are cholesterol, phospholipids and galactolipids (cerebrosides and sulphatides), which are present in approximate molar ratios of 4:3:2 (Autilio et al., 1964). Cholesterol estimations in the developing brain have been found to give much the same information as more complex lipid analyses (Davison and Dobbing, 1968).

Changes in constituents such as DNA, RNA, protein and cholesterol provide a relatively crude insight into brain differentiation compared with the dramatic functional changes occurring for example in amino acid compartmentation (Berl, 1973) or energy metabolism (Gaitonde and Richter, 1966). However these static measures of brain maturation and growth have shown up important pathological changes in conditions such as thyroid deficiency (Balazs et al., 1968), undernutrition (Winick and Rosso, 1973), X-irradiation (Patel and Balazs, 1975), and experimental amino acid imbalance (Chase and O'Brien, 1970).

Brain development has been investigated in +/+ and his/his mice using the biochemical indices of growth discussed above. His/his mice were obtained from his/his x his/his crosses, so combining the effects of maternal histidinaemia and histidase deficiency. His/his offspring of heterozygous (his/+) mothers would only be exposed to increased histidine concentrations during the latter part of the brain growth spurt. Intrauterine exposure to high histidine concentrations might be expected to interfere predominantly with morphogenesis and neuronal multiplication. Postnatal exposure to high histidine concentrations, particularly during the "critical period" of brain growth, might be expected to impair neuronal expansion, glial multiplication and myelination.

Brains were therefore removed from mice on 1, 6, 15, 24 and 35 days after birth. To allow for variation in the extraction and assay procedures between experiments, equal numbers of same-aged animals from

both genotypes (+/+ and his/his) were examined in each experiment. The brain had previously been split into forebrain (cerebrum) and cerebellum, while the remainder of the brain stem was discared. The cerebrum was of particular interest because of the reported effects on higher mental function in human aminoacidopathies. The cerebellum was investigated separately, since with the exception of a small number of macroneurons which are laid down before birth, its morphogenesis and maturation are essentially postnatal phenomena (Altman, 1969). The cerebellum is therefore a relatively discreet brain region, easily isolated from other parts of the brain, whose development can be examined almost in toto after birth.

(i) Brain and body weight.

Body weight was measured in +/+ and his/his mice from birth to 35 days. The results are shown in Table 4:1 and Figure 4:1. At birth, +/+ mice are slightly (7%) heavier than his/his mice ($P < 0{\cdot}05$). There is no significant difference in litter size. In +/+ mice, the mean (± S.E.M.) litter size is 7·68 ± 0·53 (n = 25) compared with 7·00 ± 0·53 (n = 29) for his/his mice ($P > 0{\cdot}1$). On the 3rd and 6th day after birth, his/his body weights are again lower by some 9 - 12%. This reached statistical significance on the 6th day ($P < 0{\cdot}01$). Thereafter the two genotypes converge and grow at the same rate, with the exception of animals at the 18 day stage. Here the his/his mice are lighter than their +/+ counterparts ($P < 0{\cdot}001$). There are two possible reasons for this difference. Firstly the numbers of his/his mice at this stage are much lower than for +/+ and all come from a single litter, which may have been neglected or ill-nourished by the mother. Secondly, it was noticed that the mother often stops feeding the litter before weaning and that some balance defective offspring have more difficulty in getting food from the cages at this stage than behaviourally normal or older mice. This could lead to a temporary reduction in growth in his/his litters, but the fact that the body weights are again identical in the two genotypes at 21 days and subsequently makes this unlikely. These results show that there is a small reduction in the weights of his/his compared with +/+ mice at birth and during the first week but thereafter the genetic difference at the his locus does not affect overall growth.

Brain weight was examined in +/+ and his/his mice between birth and 35 days. The results are shown in Table 4:2 and Figure 4:2. On

TABLE 4:1

Body weight in +/+ and his/his mice during postnatal development.

Age[2]	+/+		his/his[1]	
	Weight, g ± S.E.	No.	Weight, g ± S.E.	No.
0 (birth)	1·297 ± 0·031	45	1·211 ± 0.025*	42
3	2·34 ± 0·07	21	2·13 ± 0·10	26
6	3·49 ± 0·10	49	3·08 ± 0·09**	44
9	4·40 ± 0·24	14	4·07 ± 0·20	13
12	6·01 ± 0·23	40	5·93 ± 0·39	29
15	7·83 ± 0·24	28	7·51 ± 0·19	55
18	8·53 ± 0·21	34	7·07 ± 0·18***	9
21	10·96 ± 1·09	4	10·88 ± 0·47	13
24	11·90 ± 0·32	31	11·80 ± 0·34	25
27	12·96 ± 0·67	10	14·11 ± 0·39	16
30	13·85 ± 1·08	6	-	0
35	17·43 ± 0·41	18	15·98 ± 0·72	25

*$p<0·05$: **$p<0·01$: ***$p<0·001$

[1] Offspring of his/his x his/his crosses.

[2] Age is given in days after birth.

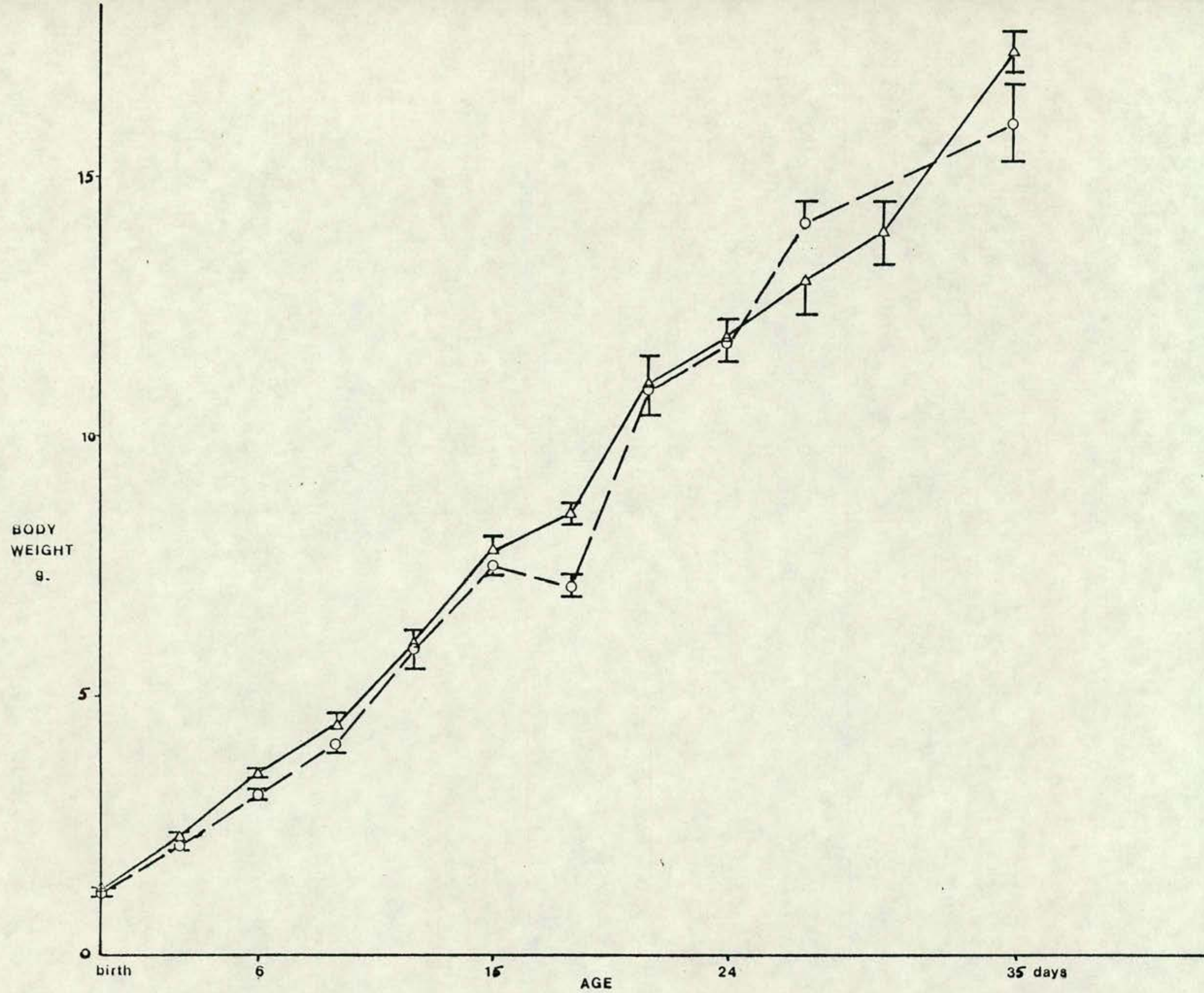


FIG. 4:1 Body weight in +/+ (△——△) and his/his (○– –○) mice during postnatal development.

TABLE 4:2

Brain weight in +/+ and his/his mice during postnatal development.

(1) Cerebrum

Age[2]	+/+		his/his[1]	
	Weight, g. ± S.E.	No.	Weight, g. ± S.E.	No.
1	0·068 ± 0·005	4	0·071 ± 0·002	4
6	0·147 ± 0·007	9	0·169 ± 0·005*	17
15	0·282 ± 0·007	7	0·256 ± 0·010*	6
24	0·316 ± 0·006	4	0·281 ± 0·006**	4
35	0·298 ± 0·005	4	0·293 ± 0·014	4

(2) Cerebellum

Age[2]	+/+		his/his[1]	
	Weight in g.	No.	Weight in g.	No.
1	-		-	
6	0·024 ± 0·002	6	0·023 ± 0·002	13
15	0·051 ± 0·000	7	0·053 ± 0·002	6
24	0·060 ± 0·002	4	0·062 ± 0·002	4
35	0·068 ± 0·002	4	0·065 ± 0·001	4

*$p<0·05$: **$p<0·01$: ***$p<0·001$

[1]Offspring of his/his x his/his crosses.

[2]Age in days after birth.

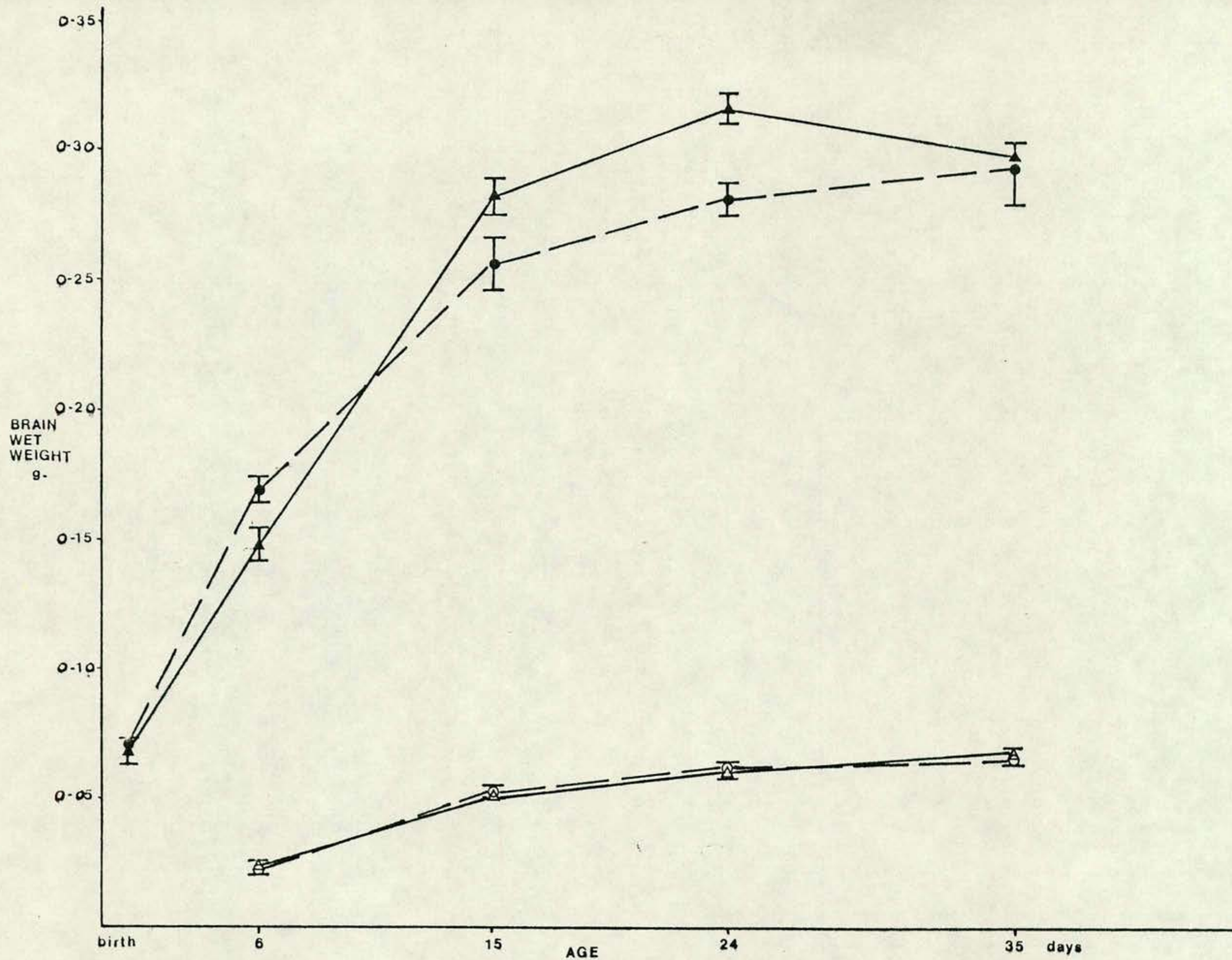


FIG. 4:2 Cerebral (▲, ●) and cerebellar (△, ○) wet weight in +/+ (△, ▲ solid lines) and his/his (○, ● broken lines) mice during postnatal development.

TABLE 4:3

Ratios of brain/body weight in +/+ and his/his

mice during postnatal development.

Values are means ± S.E.M.

Genotype	Age[1]	Brain/Body weight ratio	No.
+/+	6	0·043 ± 0·001	8
	15	0·041 ± 0·002	6
	24	0·027 ± 0·001	4
	35	0·017 ± 0·0003	4
his/his[2]	6	0·046 ± 0·002	9
	15	0·039 ± 0·004	6
	24	0·026 ± 0·002	4
	35	0·020 ± 0·001**	4

** $P < 0·01$ [1]Age in days after birth

[2]Offspring of his/his x his/his crosses

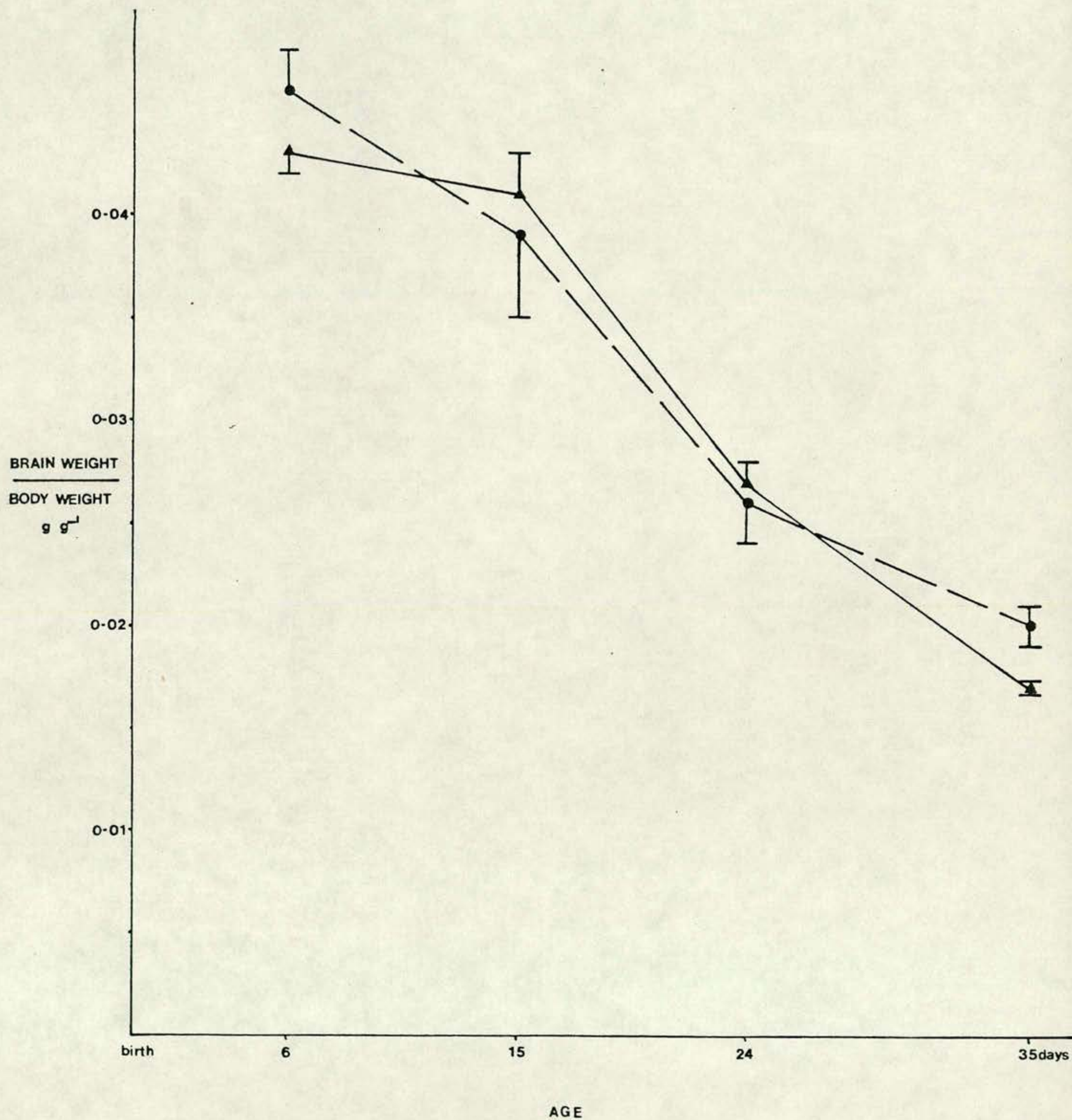


FIG. 4:3 Brain/body weight ratios in +/+ (▲—▲) and his/his (●- -●) mice during postnatal development.

day 1 there is no difference in cerebral weight between the two genotypes. Between the 6th and 24th days, the pattern of cerebral growth appears slightly different in his/his mice. At six days, cerebral weight is 13% lower in +/+ mice ($P < 0{\cdot}05$) but by the 15 and 24 day stages, cerebral weight is about 10% lower in his/his mice. By five weeks, cerebral weight is again the same in both genotypes. In the wild-type there is a 6% fall in weight between 24 and 35 days, as observed by others (Uzman and Rumley, 1958), but this is not seen in his/his brains.

In contrast to the pattern of cerebral growth, there is no difference in the cerebellar weights of +/+ and his/his mice at any stage. It was not possible to dissect out the small cerebellar primordium on the first day after birth.

Brain weight is very closely related to body weight during the development of all species examined and the ratio of brain weight / body weight is high in early life and falls as the animal matures (Dobbing, 1968). An analysis of brain weight / body weight ratios during postnatal development in several species shows an abrupt change in this ratio coinciding with the period of brain maturation (Kobayashi, 1963). In the mouse this was found to occur around the 15th day. This ratio has been plotted in +/+ and his/his mice between 6 and 35 days after birth (Figure 4:3 and Table 4:3). The timing of the fall in the ratio almost coincides in the two genotypes, although the ratio is slightly higher in his/his mice at 35 days ($P < 0{\cdot}01$) because of the lower body weights of his/his mice used for analysis (Table 4:12).

(ii) Amino acids.

The concentrations of cerebral histidine, alanine, phenylalanine, lysine, glutamic acid and GABA were determined in +/+ and his/his mice during postnatal development. The concentrations of histidine, glutamate and GABA were also examined in a small number of cerebellar samples for comparison. The results are shown in Tables 4:4, 4:5 and Figures 4:4, 4:5.

The cerebral histidine concentration is significantly raised in the mutant at all stages of development (Figure 4:4). Unlike the non-essential amino acids, the net entry of histidine from blood into the

TABLE 4:4

Cerebral amino acid concentrations during postnatal development in +/+ and *his*/*his* mice. Values are means ± standard errors of 4-8 samples, in µmol g^{-1}.

Genotype	Age	Histidine	Alanine	Phenylalanine	Lysine	Glutamic acid	GABA
+/+	1	0·087 ± 0·012	0·620 ± 0·124	0·106 ± 0·004	0·265 ± 0·018	4·575 ± 0·180	1·013 ± 0·030
	6	0·309 ± 0·020	1·348 ± 0·163	0·137 ± 0·014	0·427 ± 0·033	8·454 ± 1·057	2·509 ± 0·218
	15	0·245 ± 0·025	1·738 ± 0·227	0·120 ± 0·010	0·638 ± 0·052	12.692 ± 0·445	3·364 ± 0·262
	24	0·182 ± 0·040	0·906 ± 0·108	0·114 ± 0·020	0·918 ± 0·242	15.662 ± 0·164	4·717 ± 0·519
	35	0·140 ± 0·020	1·092 ± 0·099	0·142 ± 0·028	0·510 ± 0·064	18.374 ± 2·309	4·440 ± 0·496
his/*his*	1	0·624 ± 0·052***	0·862 ± 0·148	0·090 ± 0·008	0·381 ± 0·041*	4·293 ± 0·549	1·308 ± 0·189
	6	1·290 ± 0·072***	1·452 ± 0·095	0·119 ± 0·015	0·571 ± 0·040*	8·663 ± 0·675	2·444 ± 0·151
	15	3·501 ± 0·485***	1·248 ± 0·207	0·139 ± 0·021	0·878 ± 0·034**	12.137 ± 1·998	3·430 ± 0·533
	24	2·831 ± 0·272***	1·271 ± 0·023	0·120 ± 0·013	0·486 ± 0·066	16.269 ± 0·438	4·706 ± 0·527
	35	3·230 ± 0·323***	0·997 ± 0·110	0·147 ± 0·028	0·455 ± 0·058	17.953 ± 1·469	4·165 ± 0·312

* $p < 0{\cdot}005$: ** $p < 0{\cdot}01$: *** $p < 0{\cdot}001$

TABLE 4:5

Cerebellar amino acid concentrations in +/+ and his/his mice during postnatal development. Values (means ± standard errors in 6 and 15 day samples) are given in $\mu mol\ g^{-1}$.

Genotype	Age[2]	Histidine	Glutamic acid	GABA	No.
+/+	6	0·206 ± 0·039	0·997 ± 0·078	3·378 ± 0·455	4
	15	0·188 ± 0·043	2·579 ± 0·309	11·947 ± 1·257	4
	24	0·234	3·634	14·153	1
	35	0·150	3·020	15·349	1
his/his[1]	6	1·422 ± 0·509	1·165 ± 0·382	3·407 ± 0·589	4
	15	2·619 ± 0·263 ***	1·989 ± 0·285	9·592 ± 0·818	4
	24	3·088	4·958	20·600	1
	35	2·598	2·422	11·845	1

[1] Offspring of his/his x his/his crosses.

[2] Age in days after birth.

*** $p < 0{\cdot}001$

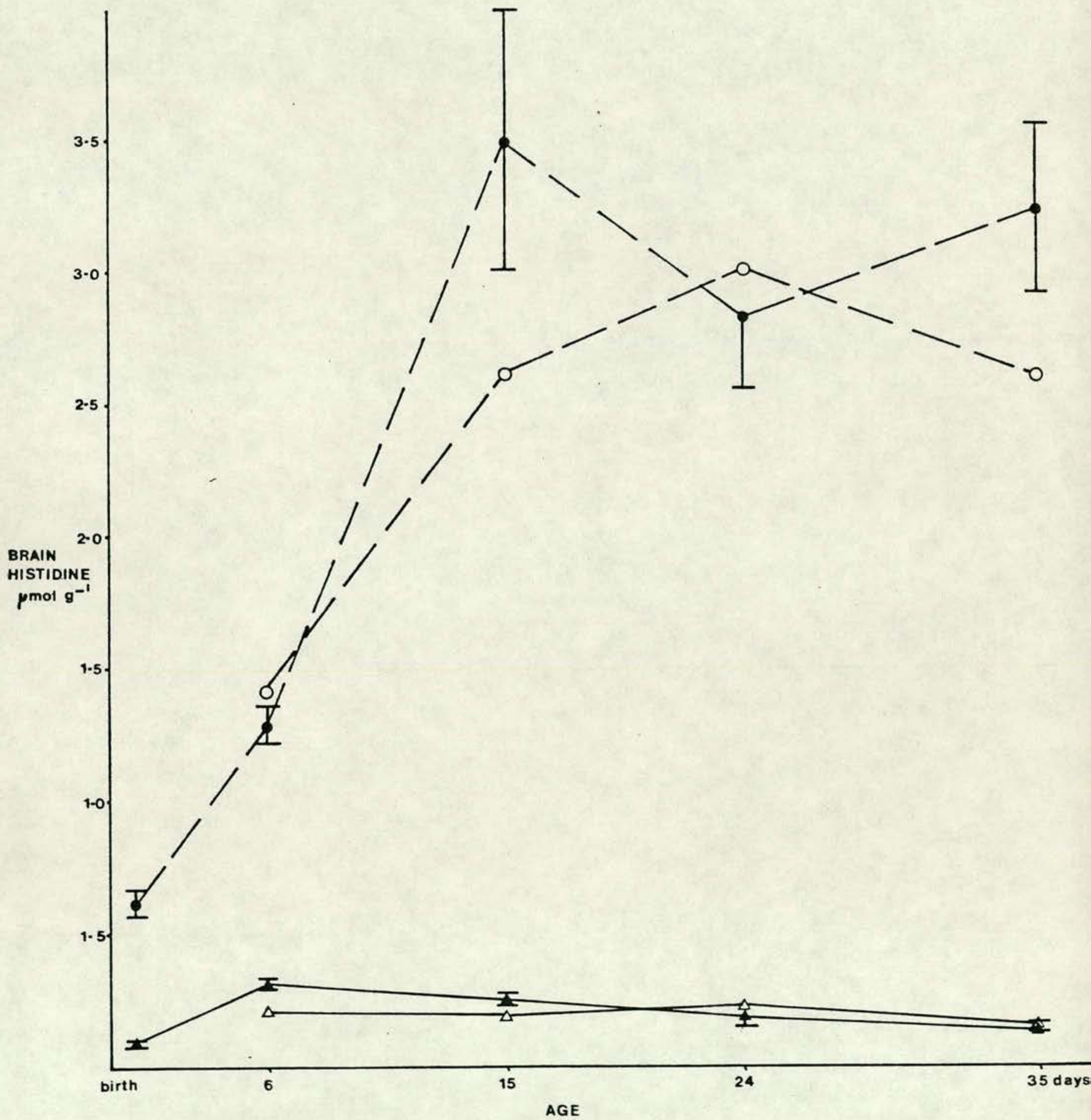


FIG. 4:4 Brain histidine concentrations in +/+ (cerebrum ▲, cerebellum △, solid lines) and his/his (cerebrum ●, cerebellum ○, broken lines) mice during postnatal development.

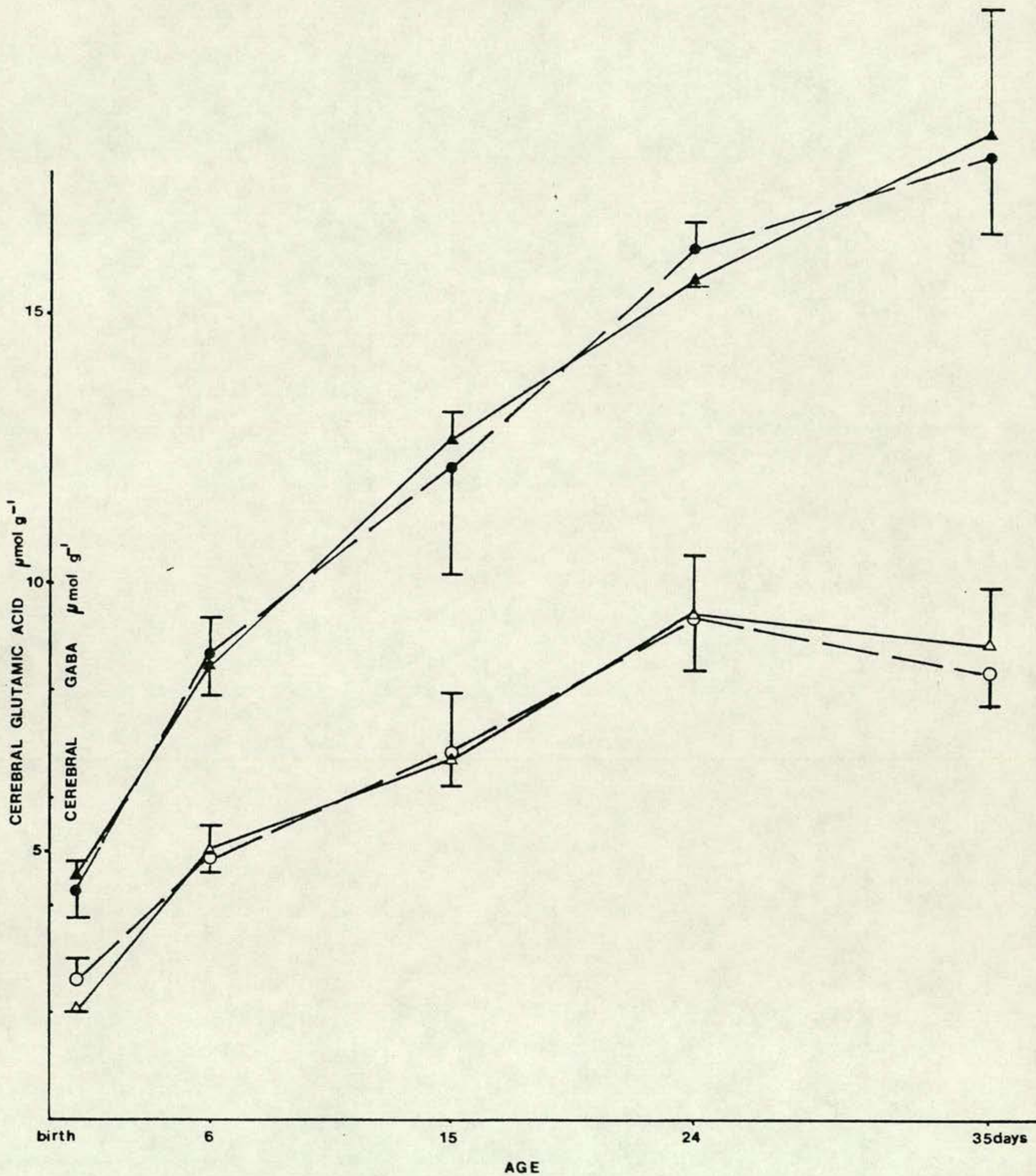


FIG. 4:5 Cerebral glutamic acid (▲, ●) and γ- aminobutyric acid (△, ○) concentrations in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

brain is relatively unrestricted (Table 4:11). The largest concentration difference between the genotypes is found at five weeks when the histidine concentration is increased twentythree-fold (Table 4:11). In the cerebellum, similar increases are found in the mutant (Figure 4:4). The histidine concentration in his/his brain is therefore in the region 1 - 3 μmol/g (about 1 - 3mM) during the brain "growth spurt" compared with 0·1 - 0·3 μmol/g in the wild-type, roughtly a ten-fold difference.

There is no significant difference between the cerebral concentrations of alanine or phenylalanine in +/+ and his/his mice (Table 4:4). Any effect of a raised histidine condentration on the net uptake of these amino acids with overlapping transport specificity (Neame, 1968), is not enough to affect the total pool size. A more subtle effect on quantitatively minor pools cannot be excluded. A tracer experiment would be necessary to examine this possibility.

The cerebral concentration of lysine is significantly increased in his/his mice on the 1st, 6th and 15th days after birth (Table 4:4). Since there is no evidence that lysine and histidine are transported into the brain by a common carrier, this effect may result from an alteration in the metabolism of lysine, induced by the histidine abnormality. A generalised alteration in protein metabolism would presumably be reflected in the pools of other amino acids, such as phenylalanine, which remain the same. The explanation for this interaction is obscure.

There are large changes in the concentration of the putative neurotransmitter amino acids glutamate and GABA during the period of rapid brain growth in all species examined (Davis and Himwich, 1973). A delay or alteration in their rates of accumulation would imply abnormal development. However, there are no significant differences between +/+ and his/his mice in the concentrations of glutamate or GABA at any stage of development, either in cerebrum or cerebellum (Tables 4:4, 4:5 and Figure 4:5). The concentrations of glutamate and GABA both increase about four-fold between 1 and 35 days.

(iii) Water content.

The water content of +/+ and his/his mouse cerebrum was determined during postnatal development. The results are shown in Table 4:6 and

TABLE 4:6

Water content of cerebrum in +/+ and his/his mice during postnatal development.

Genotype	Age[1]	Water Content % Wet Weight	No.
+/+	1	92•23 ± 0•62	4
	6	85•08 ± 0•34	4
	15	82•32 ± 0•26	4
	24	79•01 ± 0•46	2
	35	77•52 ± 0•66	3
his/his[2]	1	90•26 ± 0•21*	3
	6	85•20 ± 0•46	5
	15	82•41 ± 0•52	3
	24	80•55 ± 0•59	3
	35	78•06 ± 0•40	4

*$P < 0{\cdot}05$

[1]Age in days after birth.

[2]Offspring of his/his x his/his crosses.

Values are means ± standard errors.

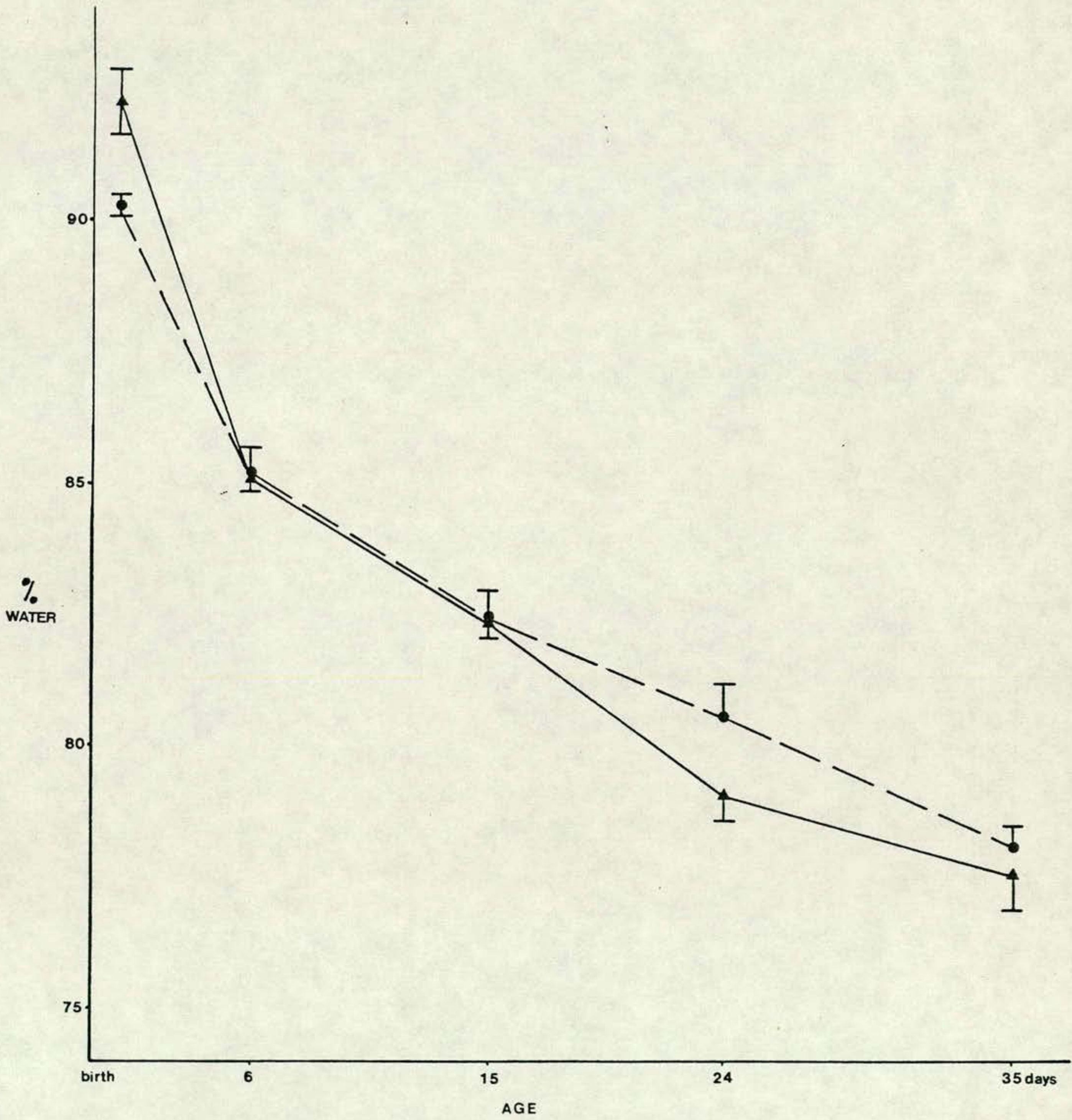


FIG. 4:6 Water content of cerebrum from +/+ (▲——▲) and his/his (●– –●) mice during postnatal development.

Figure 4:6. The water content of the cerebrum falls sharply during the first week, then more gradually until five weeks. On day 1, water represents 90 - 92% of the total wet weight, falling to 77 - 78% at five weeks. This change coincides with an increase in the solid constituents of brain and shrinkage of the extracellular spaces. Comparison of the two genotypes shows that on the first day after birth, the water content of +/+ cerebrum is slightly (2%) higher than that of his/his cerebrum ($P < 0{\cdot}05$). This difference does not persist at later stages of development.

(iv) Cholesterol.

The cholesterol content of +/+ and his/his cerebrum was determined during postnatal development. The results are shown in Table 4:7 and Figures 4:7 and 4:8. There is a four to five-fold increase in the cerebral cholesterol content between the 1st and 35th days after birth (Figure 4:7). The increase is most marked in the second and third weeks during which time myelin is actively being laid down in nerve tracts. At 6 days, the cholesterol content of his/his cerebrum is 19% higher than that of +/+ cerebrum ($P < 0{\cdot}01$). In contrast, at later stages of development the cholesterol content of his/his cerebrum falls below that of the wild-type, although these differences are not statistically significant. While there are no differences in the cholesterol concentration (mg cholesterol/g wet weight) of +/+ and his/his cerebrum at any stage, when expressed in terms of DNA content, there is a small (12% : $P < 0{\cdot}05$) increase in the mutant at 6 days only (Figure 4:7). The cholesterol content per cell is therefore slightly higher in the mutant at the 6 day stage of development but the difference disappears subsequently.

(v) Nucleic Acids.

The total DNA content of cerebrum and cerebellum was determined in +/+ and his/his mice during postnatal development. The results are shown in Table 4:8 and Figures 4:9-10. The total DNA content of mouse cerebrum doubles between the 1st and 24th days after birth (Figure 4:9). By 35 days, it has fallen some 2 - 6% suggesting that cell degeneration has occurred. The latter finding has been noted by other workers (Uzman and Rumley, 1958; Howard, 1968). The DNA content of mouse cerebellum increases six to eight-fold between the 6th and 35th days,

TABLE 4:7

Cholesterol content of cerebrum in +/+ and his/his mice during postnatal development.

Genotype	Age[1]	Total cholesterol mg	Cholesterol Wet weight^{-1} mg g^{-1}	Cholesterol DNA^{-1} mg µg^{-1}	No.
+/+	6	0·998 ± 0·038	6·319 ± 0·109	3·815 ± 0·115	6
	15	3·120 ± 0·276	10·995 ± 0·619	7·637 ± 0·389	4
	24	4·606 ± 0·160	14·587 ± 0·260	11·754 ± 0·432	4
	35	5·000 ± 0·427	16·583 ± 1·178	13·421 ± 0·856	3
his/his[2]	6	1·188 ± 0·027**	6·215 ± 0·153	4·276 ± 0·114*	6
	15	2·561 ± 0·275	9·786 ± 1·359	7·471 ± 0·000	3
	24	4·020 ± 0·295	14·288 ± 0·808	10·897 ± 0·841	4
	35	4·669 ± 0·153	16·006 ± 0·356	12·895 ± 0·125	4

*$p < 0·05$: **$p < 0·01$

[1] Age in days after birth

[2] Offspring of his/his x his/his crosses.

Values are means ± standard errors.

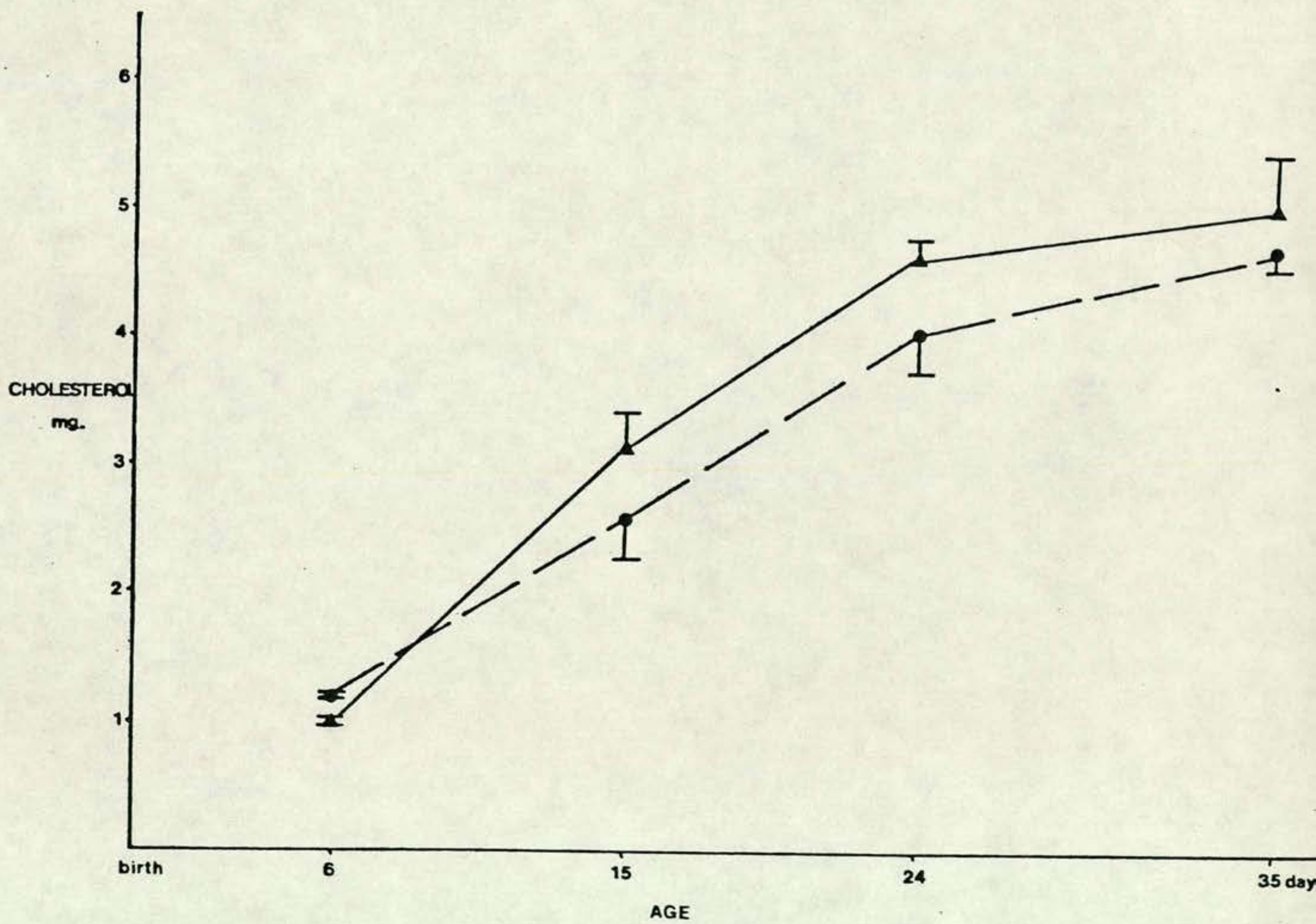


FIG. 4:7 Cholesterol content of cerebrum in +/+ (▲——▲) and his/his (●– –●) mice during postnatal development.

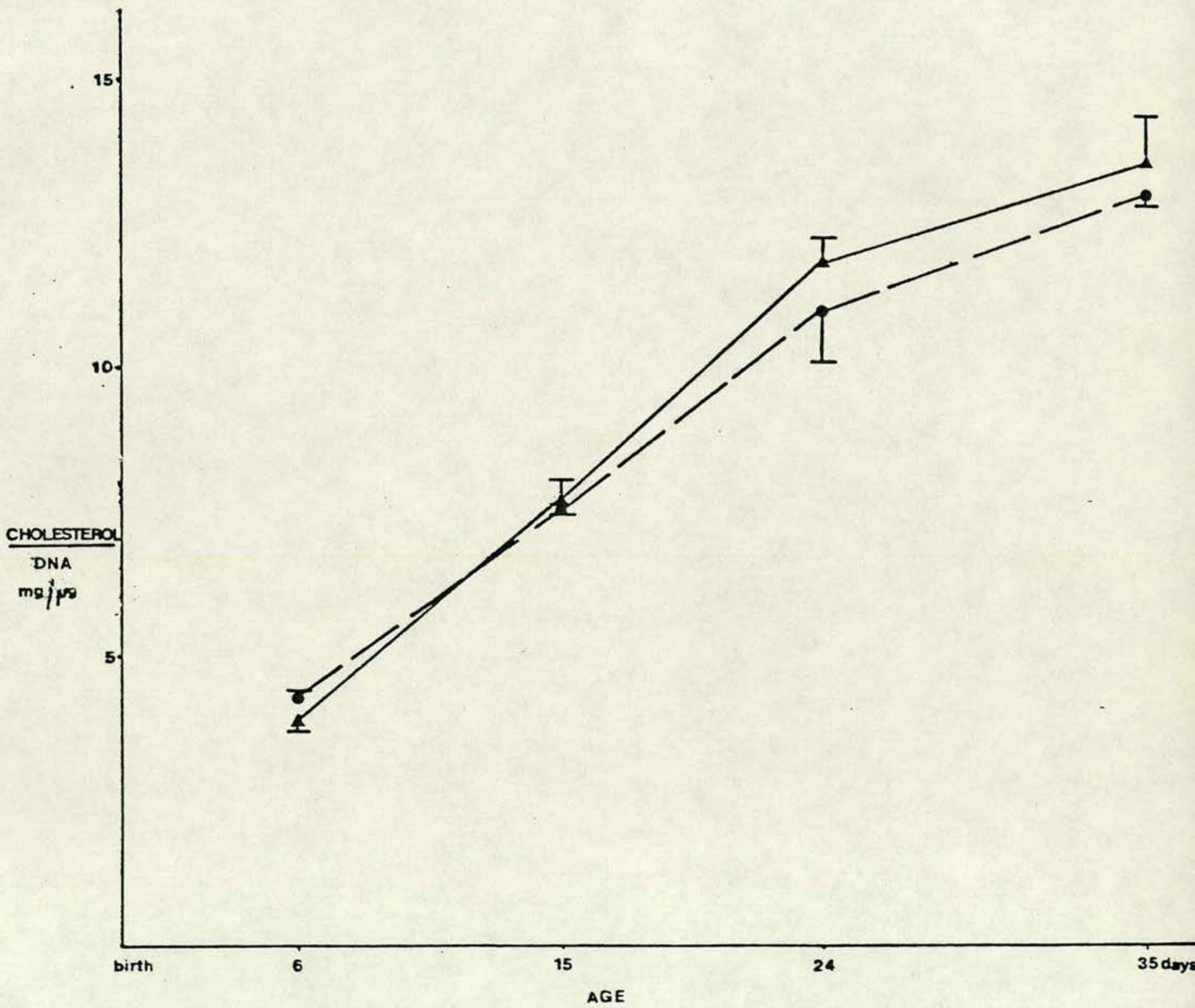


FIG. 4:8 Cholesterol/DNA ratios in cerebrum of +/+ (▲——▲) and his/his (●– –●) mice during postnatal development.

showing that almost 90% of its cells are formed postnatally (Figure 4:9). The increase is most marked during the second week. There are no significant differences in the DNA conrent of cerebrum or cerebellum between +/+ and his/his mice. The total number of brain cells is therefore not altered by pre- and postnatal exposure to a raised histidine concentration. Since there is an estimated 5×10^{-12}g DNA per somatic cell in the mouse (Howard, 1968), the five-week old mouse cerebrum contains approximately 75 million cells. The cerebellum has a similar number of cells at this stage.

The DNA concentration (DNA/wet weight) of mouse cerebrum falls sharply between days 1 and 6, then more gradually during the next three or four weeks (Figure 4:10). This is in keeping with neuro-histological studies showing that the packing density of neurons is high in early cortical development then falls as neuronal processes expand during maturation (Eayrs and Goodhead, 1959). There are no significant differences in cerebral DNA/wet weight between the genotypes at any stage of development. In the cerebellum, the DNA/wet weight rises between 6 and 16 days after birth as cell proliferation proceeds, then falls, as in the cerebrum (Table 4:8). Again there are no differences between the two genotypes at any stage.

The RNA content of cerebrum (Table 4:9, Figure 4:11) increases five to six-fold during the first two weeks after birth, then slowly declines by about 10% over the next three weeks. Similar, but less marked changes in cerebellar RNA content occur during this period. RNA/wet weight increases during the first 1 - 2 weeks in cerebrum and cerebellum then declines slowly. No genotypic differences either in total RNA or RNA/wet weight are found at any stage of development. The raio of RNA/DNA (Figure 4:12) in cerebrum increases dramatically between the 1st and 6th days then remains at about the same level. At the 6 day stage, the ratio is 14% higher in his/his cerebrum ($P < 0{\cdot}05$), but the difference disappears at later stages. In the cerebellum, RNA/DNA falls between 6 and 15 days, then remains relatively constant. The observation that RNA/DNA does not increase between 6 and 15 days in the cerebrum at a time when neuronal processes are expanding enormously, suggests that this ratio cannot be used simply as an index of cyto-

TABLE 4:8

DNA content of cerebrum and cerebellum in +/+

and his/his mice during postnatal development.

I Cerebrum

Genotype	Age[1]	Total DNA µg	DNA Wet weight^{-1} mg g^{-1}	No.
+/+	1	179·5 ± 10·3	2·655 ± 0·079	4
	6	223·5 ± 18·0	1·593 ± 0·058	10
	15	349·6 ± 10·0	1·295 ± 0·012	4
	24	392·5 ± 11·5	1·244 ± 0·029	4
	35	369·6 ± 5·8	1·243 ± 0·017	4
his/his[2]	1	211·4 ± 12·8	2·993 ± 0·130	4
	6	242·9 ± 15·8	1·466 ± 0·023	11
	15	331·2 ± 18·6	1·260 ± 0·022	3
	24	369·4 ± 8·5	1·318 ± 0·033	4
	35	362·4 ± 14·8	1·241 ± 0·017	4

II Cerebellum

Genotype	Age[1]	Total DNA µg	DNA Wet weight^{-1} mg g^{-1}	No.
+/+	6	47·4 ± 11·0	3·070 ± 0·601	6
	15	288·3 ± 14·0	5·597 ± 0·266	4
	24	335·0 ± 25·3	5·590 ± 0·259	4
	35	361·7 ± 3·0	5·455 ± 0·110	3
his/his[2]	6	55·2 ± 13·4	3·117 ± 0·676	9
	15	279·9 ± 5·3	5·552 ± 0·173	4
	24	350·3 ± 19·3	5·685 ± 0·158	4
	35	342·4 ± 12·6	5·320 ± 0·091	3

[1] Age in days after birth. Values are means ± standard errors.

[2] Offspring of his/his x his/his crosses.

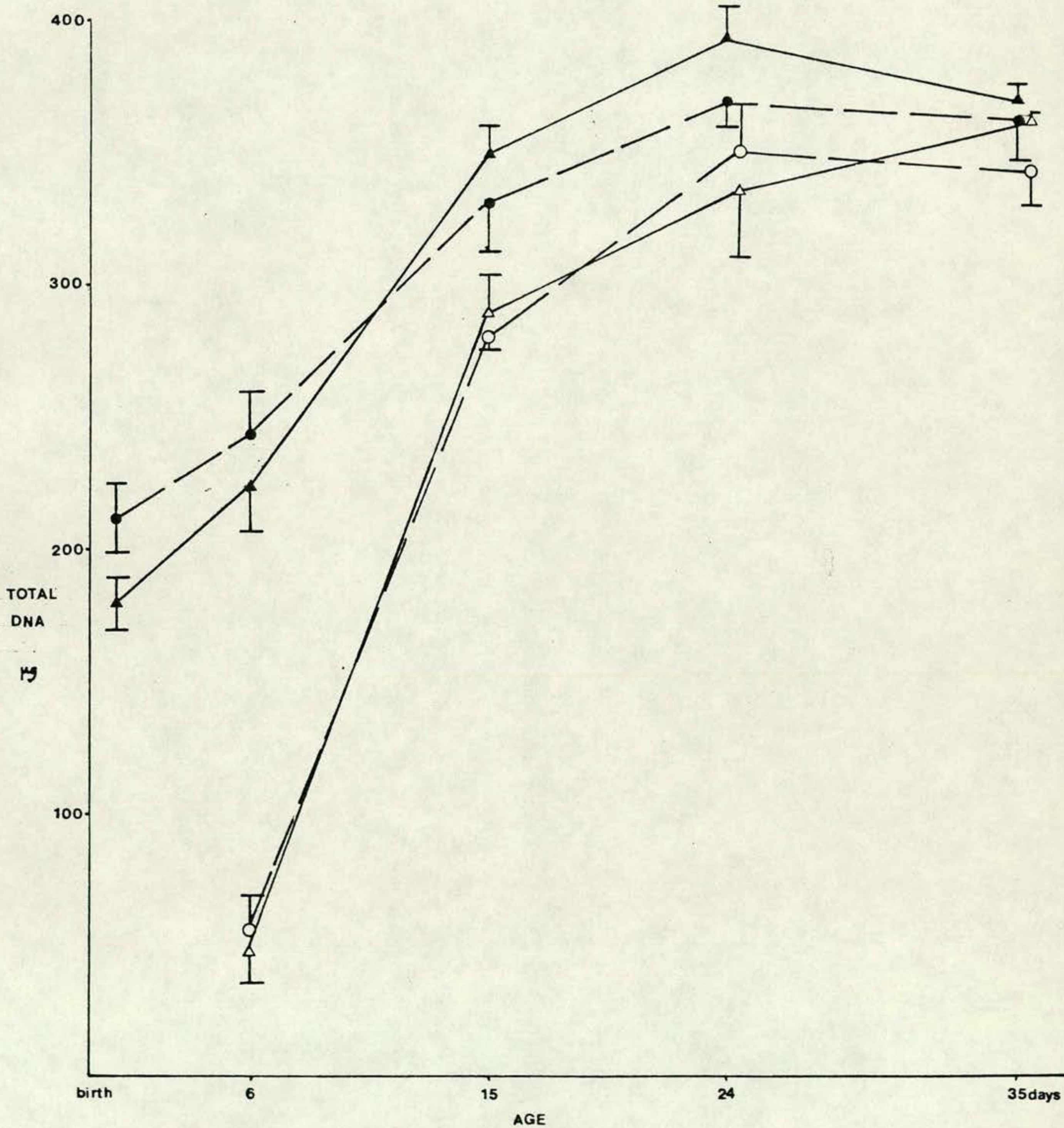


FIG. 4:9 DNA content of cerebrum (▲, ●) and cerebellum (△, ○) in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

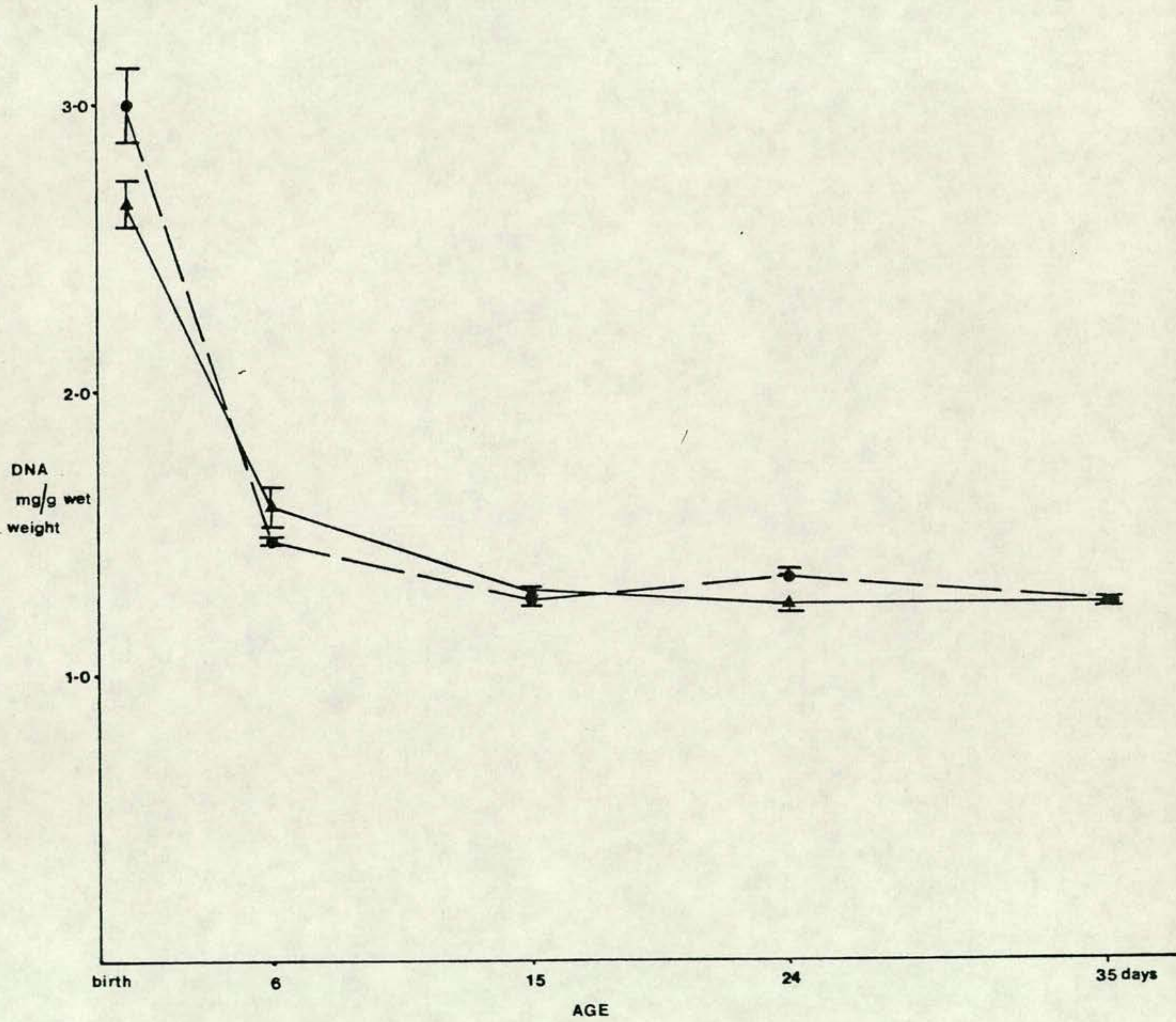


FIG. 4:10 DNA concentration (mg per g wet weight) of cerebrum in +/+ (▲——▲) and his/his (●— —●) mice during postnatal development.

TABLE 4:9

RNA content of cerebrum and cerebellum in +/+ and his/his mice during postnatal development.

I Cerebrum

Genotype	Age[1]	Total RNA µg	RNA Wet weight^{-1} mg g^{-1}	RNA DNA^{-1} µg µg^{-1}	No.
+/+	1	220·8 ± 15·5	3·253 ± 0·030	1·228 ± 0·026	4
	6	687·9 ± 57·1	4·699 ± 0·257	3·109 ± 0·139	10
	15	1307·8 ± 280·0	4·810 ± 0·821	2·755 ± 0·202	6
	24	1231·1 ± 95·8	3·900 ± 0·293	3·143 ± 0·262	4
	35	1233·2 ± 75·8	4·140 ± 0·208	3·339 ± 0·217	4
his/his[2]	1	259·8 ± 20·6	3·672 ± 0·221	1·225 ± 0·032	4
	6	867·6 ± 69·1	5·130 ± 0·112	3·549 ± 0·105*	11
	15	1367·6 ± 324·3	5·374 ± 1·232	2·732 ± 0·252	5
	24	1165·5 ± 111·3	4·172 ± 0·448	3·145 ± 0·261	4
	35	1106·7 ± 72·5	3·854 ± 0·166	3·117 ± 0·161	3

[1] Age in days after birth. Values are means ± standard errors.

II Cerebellum

Genotype	Age[1]	Total RNA µg	RNA Wet weight^{-1} mg g^{-1}	RNA/DNA µg µg^{-1}	No.
+/+	6	64·5 ± 10·5	3·823 ± 0·784	1·650 ± 0·319	6
	15	238·8 ± 41·0	4·691 ± 0·814	0·651 ± 0·119	6
	24	227·9 ± 27·3	3·800 ± 0·386	0·691 ± 0·095	4
	35	233·6 ± 4·0	3·522 ± 0·031	0·646 ± 0·014	3
his/his[2]	6	73·0 ± 14·3	4·099 ± 0·873	1·612 ± 0·220	9
	15	255·2 ± 53·0	4·747 ± 0·907	0·719 ± 0·218	6
	24	239·4 ± 17·5	3·905 ± 0·313	0·689 ± 0·063	4
	35	237·3 ± 16·8	3·657 ± 0·204	0·694 ± 0·042	4

[1] Age in days after birth. Values are means ± standard errors.

[2] Offspring of his/his x his/his crosses.

* $P < 0·05$

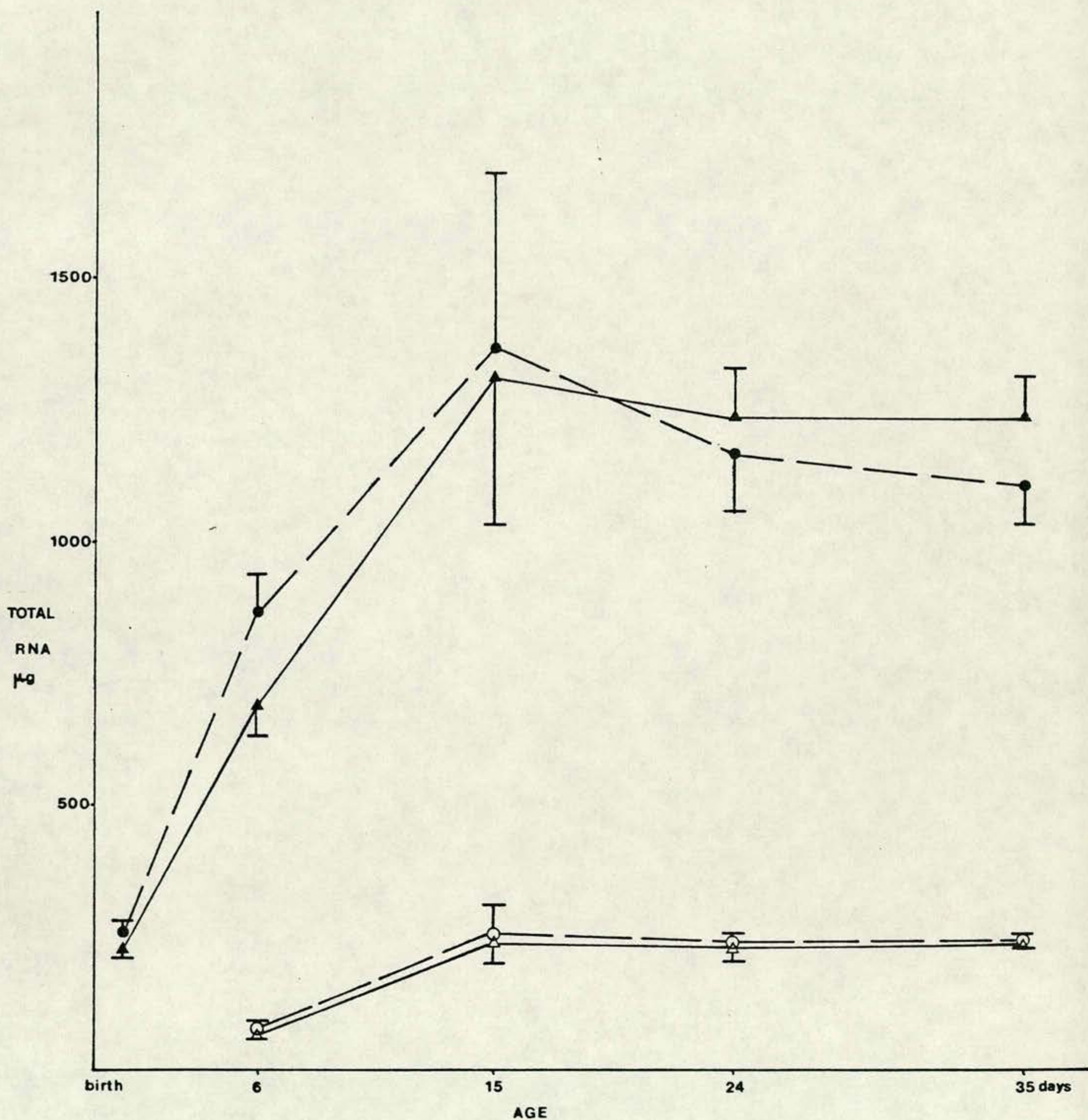


FIG. 4:11 RNA content of cerebrum (▲, ●) and cerebellum (△, ○) in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

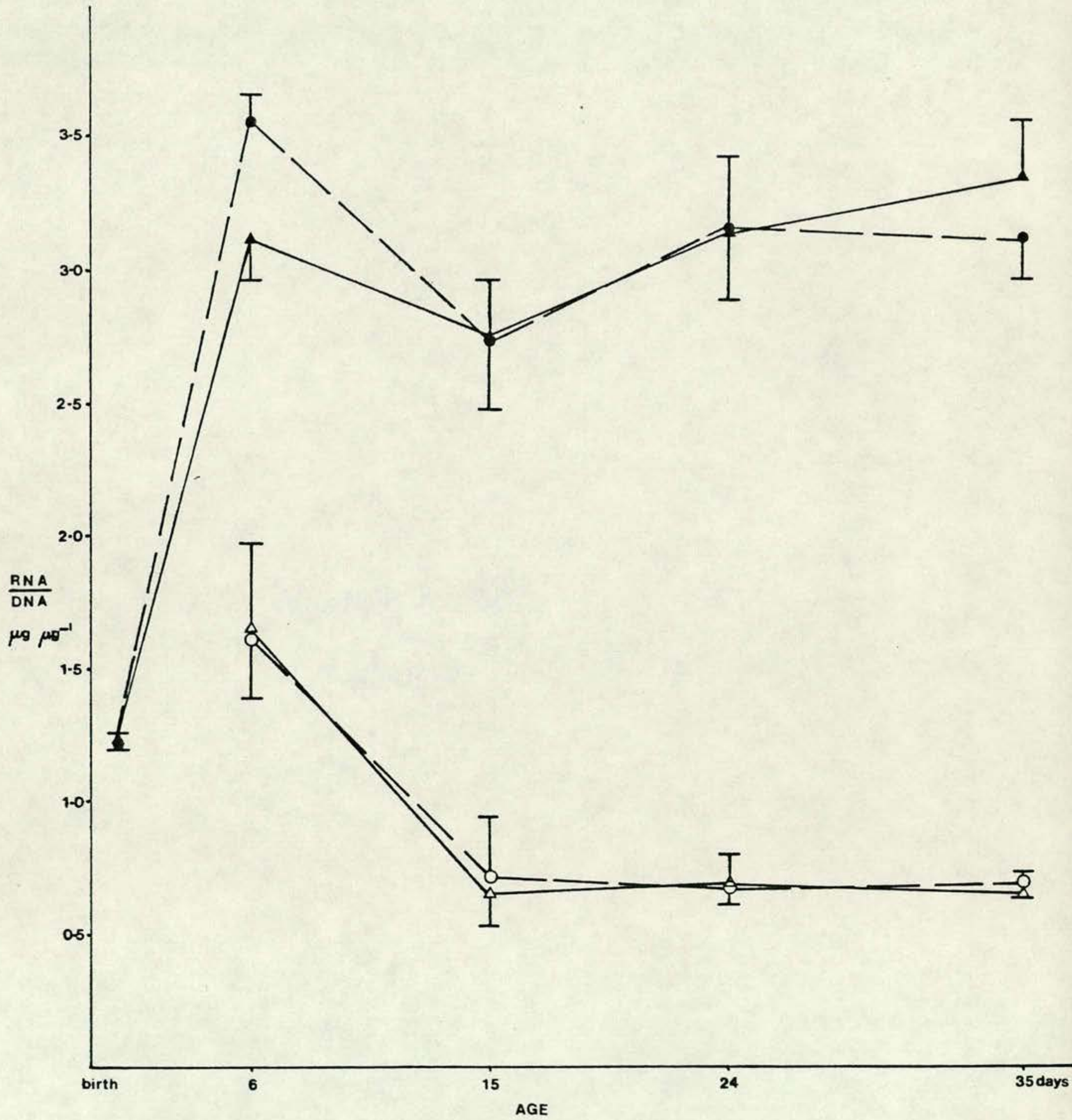


FIG. 4:12 RNA/DNA ratios of cerebrum (▲, ●) and cerebellum (△, ○) in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

TABLE 4:10

Protein content of cerebrum and cerebellum in +/+

and his/his mice during postnatal development.

I cerebrum

Genotype	Age[1]	Total protein mg	Protein Wet weight^{-1} mg g^{-1}	Protein DNA^{-1} µg µg^{-1}	No.
+/+	1	2·782 ± 0·204	40·98 ± 0·77	15·48 ± 0·57	4
	6	8·581 ± 0·619	60·42 ± 1·75	38·80 ± 0·98	10
	15	24·998 ± 0·816	92·69 ± 3·18	71·56 ± 2·08	4
	24	35·495 ± 1·908	112·52 ± 5·86	90·27 ± 2·55	4
	35	34·575 ± 0·645	116·25 ± 1·26	93·56 ± 1·24	4
his/his[2]	1	3·380 ± 0·271	47·80 ± 3·04	15·93 ± 0·41	4
	6	10·647 ± 0·660*	62·82 ± 1·36	44·18 ± 1·24**	11
	15	21·970 ± 1·717	83·51 ± 4·14	66·19 ± 2·12	3
	24	34·025 ± 0·625	107·48 ± 6·69	92·14 ± 0·97	4
	35	34·343 ± 1·910	117·34 ± 2·25	94·58 ± 1·54	4

II cerebellum

Genotype	Age[1]	Total protein mg	Protein Wet weight^{-1} mg g^{-1}	Protein/DNA µg µg^{-1}	No.
+/+	6	0·692 ± 0·214	34·56 ± 8·99	9·36 ± 2·33	3
	15	4·161 ± 0·117	80·57 ± 2·73	13·94 ± 0·39	3
	24	5·893 ± 0·413	98·30 ± 2·92	17·68 ± 0·82	4
	35	6·540 ± 0·181	96·20 ± 1·73	17·81 ± 0·73	4
his/his[2]	6	0·694 ± 0·111	36·47 ± 3·14	9·92 ± 2·23	4
	15	4·378 ± 0·140	86·72 ± 2·26	15·65 ± 0·50	4
	24	6·320 ± 0·336	102·80 ± 4·54	18·11 ± 0·85	4
	35	6·406 ± 0·175	99·03 ± 3·18	18·34 ± 0·86	4

[1] Age in days after birth. Values are means ± standard errors.

[2] Offspring of his/his x his/his crosses. $^{*}p < 0{\cdot}05$: $^{**}p < 0{\cdot}01$

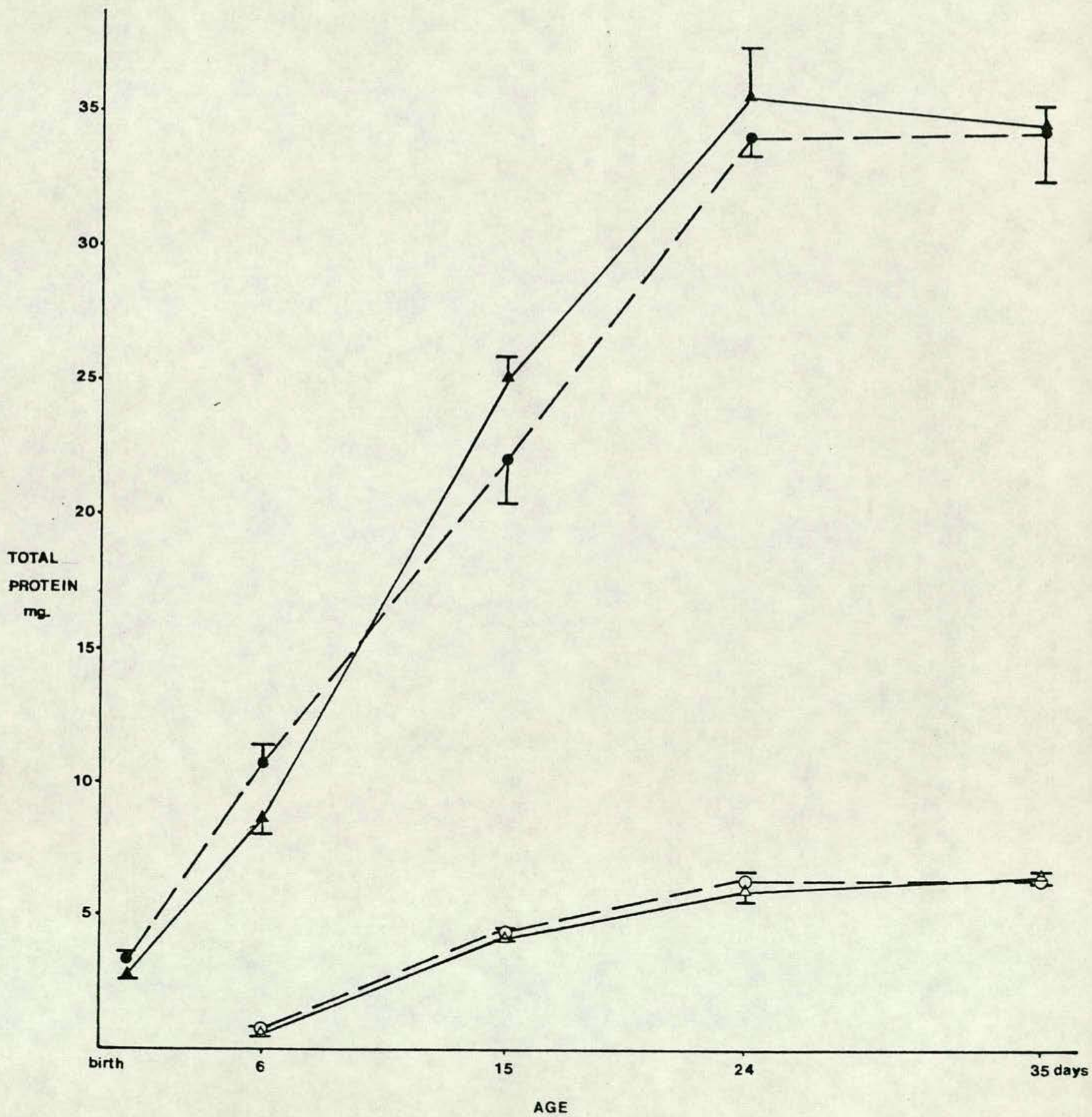


FIG. 4:13 Protein content of cerebrum (▲, ●) and cerebellum (△, ○) in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

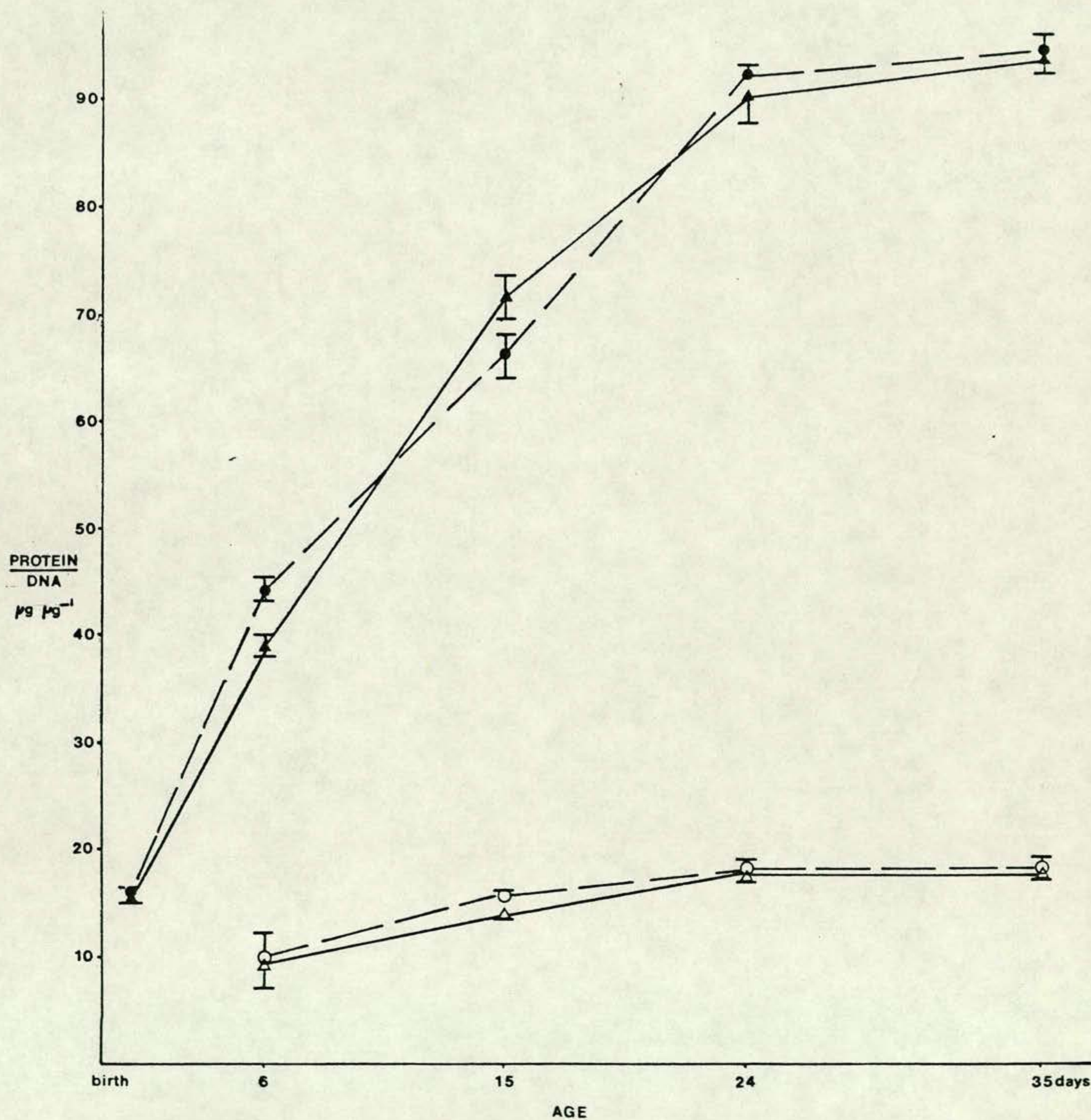


FIG. 4:14 Protein/DNA ratios in cerebrum (▲, ●) and cerebellum (△, ○) in +/+ (▲, △ solid lines) and his/his (●, ○ broken lines) mice during postnatal development.

plasmic nuclear ratio. No difference in cerebellar RNA content, concentration or RNA/DNA ratio is present at any stage.

(vi) Protein.

The protein content of mouse cerebrum increases ten to twelve-fold between the 1st and 24th days, then remains constant (Table 4:10, Figure 4:13). Comparison of the two genotypes shows that at 6 days, his/his mice have a 24% higher ($P < 0 \cdot 05$) protein content than +/+ mice. When expressed as the protein/DNA ratio, this genotypic difference remains ($P < 0 \cdot 01$) (Figure 4:14). The amount of protein per cell is therefore higher in the mutant cerebrum at an early stage of development but the difference disappears subsequently. The protein concentration (mg/g wet weight) of cerebrum increases steadily during the first three weeks after birth in both genotypes and then levels off.

In the cerebellum the total protein increases about nine-fold between 6 and 24 days, then levels off. In contrast to the cerebrum, there is no difference between the genotypes at any stage. Similarly the protein/DNA and protein/wet weight in cerebellum are identical in both genotypes throughout development.

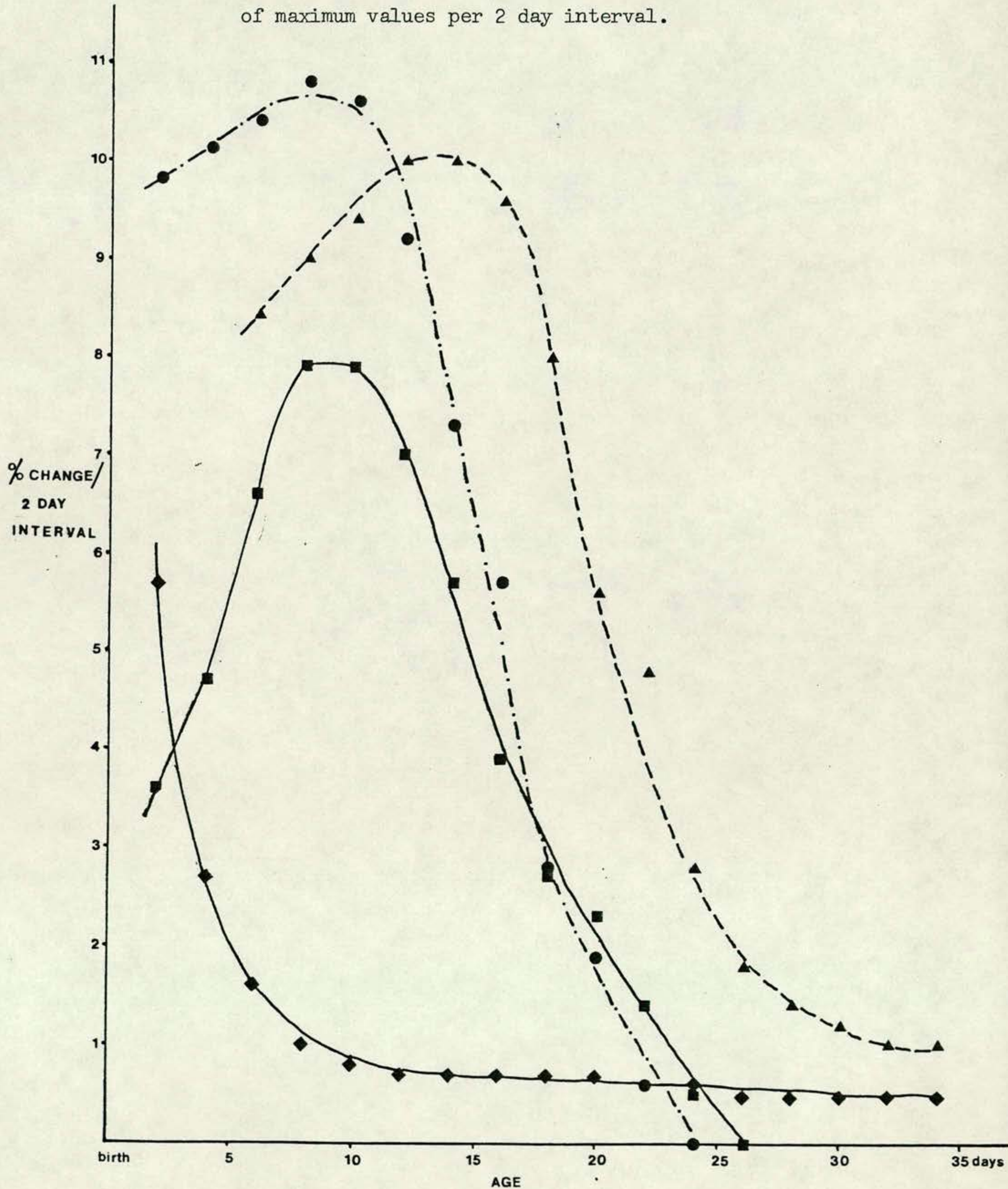
(vii) Discussion.

The biochemical changes accompanying maturation have been well documented in the mouse brain (Roberts et al., 1951; Folch-Pi, 1955; Albrecht, 1956; Uzman and Rumley, 1958; Himwich, 1962; Agrawal et al., 1968; Howard, 1968; Lehrer et al., 1970; Van den Berg, 1970; Lajtha and Toth, 1973). The concentration of most amino acids decreases during postnatal development while those of glutamic acid, glutamine, GABA and aspartic acid increase (Agrawal et al., 1968). The water content declines as total solids, proteins, myelin, proteolipid protein, lipids, cholesterol and phosphatides accumulate (Folch-Pi, 1955). During the third week, the fresh weight of brain actually decreases, because although solids are still accumulating, as seen by the continuing increase in dry weight, this cannot compensate for the rapid loss of water (Uzman and Rumley, 1958). Total brain lipids increase steadily during the first 18 days after birth, then more gradually into adulthood (Uzman and Rumley, 1958). Myelination occurs first in the mouse brain at about ten days after birth and the last areas to begin myelin-

ation do so between 15 and 21 days (Folch-Pi, 1955; Uzman and Rumley, 1958; Jacobson, 1970). Coinciding with this, the maximum rate of cholesterol deposition in the rat and mouse brain occurs between ten and 20 days after birth, although deposition continues into adulthood (Davison, 1970). In the rat, the highest rates of cholesterol synthesis and deposition coincide with maximum myelination as judged by histochemical means. The total DNA content of mouse brain increases two-fold between 1 and 14 days after birth (Uzman and Rumley, 1958), while in the cerebellum it increases by 580% (Howard, 1968). In the cerebellum virtually all the neurons and glial cells are formed after birth (Altman, 1969). In cerebrum, Uzman and Rumley (1958) found a drop in the DNA content during the third week suggesting that cell degeneration occurs. This will contribute to the full in brain weight noted at this stage (Uzman and Rumley, 1958; Howard, 1968). Degeneration of glial cells has been directly observed in the brain of young adult mice, which may account for the DNA loss (Smart and Lebland, 1961). The concentration of DNA (µg/g wet weight) in mouse brain decreases by 37% from birth to adulthood, reflecting the relative increase in other constituents and changes in cell packing density. The RNA content of mouse brain rises progressively until three weeks then falls by about 10% (Uzman and Rumley, 1958).

The results of this study confirm many of these biochemical changes previously reported in the developing mouse brain. The pattern of brain growth, with a slowing and then slight decline after the third week (Figure 4:2) contrasts with the continuing high rate of body growth (Figure 4:1). Changes in the amino acid concentrations of the cerebrum (Figures 4:4-5) show some differences from whole brain (Agrawal et al., 1968; Lajtha and Toth, 1973), but the dramatic increases in glutamic acid and GABA during development are confirmed. The maximum rates of decline in water content and increases in cholesterol, protein and nucleic acid content show differences in timing which reflect the sequence of morphological changes. Rate curves of the changes in cerebral wet weight, DNA, cholesterol and water content are shown in Figure 4:15. This shows that the peak rate of cellular multiplication (change in DNA content) immediately precedes the period of maximum cholesterol deposition, as occurs in the developing rat,

FIG. 4:15 Rate curves of the increases in wet weight (●-·-·●), DNA (■——■) and cholesterol (▲---▲) content, and decrease in water content (◆——◆) in the developing +/+ mouse cerebrum. Changes are shown as a percentage of maximum values per 2 day interval.



guinea pig and pig brains (Davison and Dobbing, 1968). While cell proliferation has fallen to a low level by the end of the third week, cholesterol deposition is continuing at an appreciable rate even at the end of the fifth week, reflecting the continued mye lination of nerve tracts. It is not clear to what extent this postnatal phase of cell proliferation is due to the formation of microneurons as well as glial cells. The latter are almost wholly formed after birth in the cerebrum, while microneurons are only known to be formed postnatally in the cerebellum, hippocampus and olfactory bulb (Altman, 1969). Probably the majority of cells formed during this period are glial, especially the myelin-forming oligodendrocytes (Smart, 1961; Davison and Dobbing, 1968; Altman, 1969). This is consistent with the observed sequence of cell formation preceding myelination. In contrast to the cerebrum, where the DNA content on the day after birth is 49% of the five week value, cerebellar DNA is only 13% of the five week value by 6 days after birth. More than 90% of cerebellar cells are therefore formed postnatally, compared with about half the cerebral cells.

The cerebral "growth spurt" can be seen in Figure 4:15 to reach its peak at 8 - 10 days. The peak rate of water loss occurs before all these events. It falls off almost exponentially during the first week, perhaps indicating the end of the prenatal wave of cell (neuron) formation. Dendritic outgrowth is reported by histologists to be at its highest between 8 and 15 days (Stefanowska, 1898; Ramon y Cajal, 1960). This coincides with the broad peak rate of protein deposition between 9 and 17 days (data not shown).

What effect does a raised histidine concentration have on these indices of growth and differentiation? The developing cerebellum is exposed to seven to seventeen-fold increases in histidine concentration throughout its development and yet there is no measurable effect on any of the indices measured. The cerebrum is also exposed to increases in histidine concentration ranging from four to twentythree-fold (averaging about ten-fold) during the five weeks after birth (Table 4:11). In contrast to the cerebellum, some biochemical differences between _+/+_ and _his/his_ cerebrum do emerge. Before considering these further, a number of difficulties inherent in a study of this nature should be discussed.

TABLE 4:11

Increases in the histidine concentration of blood and brain in *his/his* mice during postnatal development.

Age	Blood Histidine mutant/wild-type ratio	Brain Histidine mutant/wild-type ratio	
		cerebrum	cerebellum
1P	6·0	7·2	–
6P	4·4	4·2	6·9
15P	10·1	14·3	13·9
24P	15·7	15·6	13·2
35P	16·0	23·1	17·3

The suffix P refers to postnatal days

The biggest problem in studies of brain development is the wide variation in maturity among animals of the same chronological age (Himwich, 1962; Davison and Dobbing, 1968). Variation in rates of development among animals of the same age has been attributed to nutritional and genetic factors. Only the former are likely to be important in this study since, apart from the his locus difference, the mice are on the same relatively inbred genetic background. One factor known to affect the rate of brain development, probably via a nutritional mechanism, is litter size (Davison and Dobbing, 1968). In rats, a large litter size (with roughly 10 - 18 young) appears to have much the same effect on brain development as undernourishment during the suckling period (Winick and Rosso, 1973). Chase et al. (1969) compared the effects of large (16) and small (4) litter size on brain development in rats. In the large litter group, cerebral and especially cerebellar weights were reduced, which was mainly attributed to a reduction in cell size (protein/DNA ratio) in the cerebrum and to a reduction in cell size and number (DNA content) in the cerebellum. Undernourishment at a time when cell proliferation is high (especially before birth) leads to a permanent deficit in cell numbers and overall weight: undernourishment at a time when cells are mainly growing or myelinating (especially the post-weaning period) leads to a reversible reduction in indices of cell size or myelination (protein/DNA or cholesterol/DNA) (Altman et al., 1970; Winick and Rosso, 1973). Amino acid imbalance appears to give rise to similar deficits (Chase and O'Brien, 1970) so that the timing of the developmental disturbance may be more important than its nature.

When a large number of litters (n=54) from +/+ and his/his mice were examined, there was no significant difference in litter size between the genotypes. Because smaller numbers of animals were used for the biochemical analyses, sampling error could have resulted in a difference in the size of the litters used, leading to a spurious biochemical difference. Similarly, although no difference in body growth rate was found between +/+ and his/his mice apart from a small difference during the first week (Figure 4:1), sampling error could have led to the analysis of animals from litters receiving inadequate nutrition, leading to a reduced rate of body and brain growth. Table

4:12 provides data on the litters from which the mice used for biochemical analyses were drawn. The most marked difference is in the +/+ litter size at 6 days which is almost double that of the his/his mice. However the mean litter weights at this stage are almost identical in the two genotypes. The most marked differences in body weight are for the animals used for analysis at the 24 day (14% higher in +/+) and 35 day (10% higher in +/+) stages.

The method used to determine the age of animals is another factor to be considered. Because of the rapidity of changes in the developing brain, even a few hours difference in conceptual age can lead to a significant difference in maturity (Himwich, 1962). The mice in these experiments were aged by determining the time of birth. Differences of up to 12 hours are inevitable since the cages were only checked twice daily. Small differences in maturity may become particularly important around the time of birth where slight physiological differences can be exaggerated by the trauma of birth. Changes found only around the time of birth should therefore be viewed cautiously, since they may be temporary fluctuations. Finally, the rapid changes occurring in the composition of the developing mouse brain make it essential both to express each result in terms of different constituents (e.g. absolute amount, per fresh weight, per weight of DNA) and to compare simultaneous changes in several different constituents.

The only genotypic difference found at day 1 is a 2% higher water content in +/+ cerebrum ($P < 0{\cdot}05$) (Figure 4:6). A difference of this magnitude at a stage so soon after birth is probably of little real significance, especially when followed by an essentially identical rate of water loss in the two genotypes at later stages. This conclusion is reinforced by the fact that the one day old samples came from single litters, whose weights were not recorded and which might therefore have been at slightly different stages of maturity. The rate of water loss is also at its highest at this stage of development (Figure 4:15), which will tend to accentuate small differences in maturity.

TABLE 4:12

Details of the mice used for biochemical analyses; number of litters from which animals were drawn, mean litter size and weights, body weights of animals used for analysis.

Genotype	Age[1]	Mean litter size	No. of litters	Mean litter weight, g.	Mean body weight of mice used for analysis, g.	No. of mice analysed
+/+	1	8•0	1	-	-	8
	6	9•5	4	3•49	3•57	14
	15	4•7	3	6•47	7•05	10
	24	6•0	2	12•15	12•60	6
	35	5•0	2	17•56	17•65	7
his/his	1	7•0	1	-	-	7
	6	4•8	4	3•55	3•61	16
	15	7•0	3	6•51	6•68	9
	24	6•0	3	11•25	10•89	7
	35	9•0	1	16•29	15•93	8

[1] Age is given in days after birth

There are a number of differences between +/+ and his/his cerebra at the 6 day stage. Cerebral weight is 13% lower ($P < 0\cdot05$); total cholesterol is 16% lower ($P < 0\cdot01$) and cholesterol/DNA 11% lower ($P < 0\cdot05$); total RNA is 21% lower ($P > 0\cdot05 < 0\cdot1$) and RNA/NDA 12% lower ($P < 0\cdot05$); total protein is 19% lower ($P < 0\cdot05$) and protein/DNA 12% lower ($P < 0\cdot01$) in +/+ mice. Since the total DNA content is not significantly different in the two genotypes, cell growth or differentiation rather than cell numbers is relatively retarded in the +/+ cerebrum. There is no difference in water content however at this stage. Although body growth is slightly depressed in his/his mice at this stage ($P < 0\cdot01$: Table 4:1), there is no difference in the mean body weights of the animals used for analysis (or their littermates) (Table 4:12). However, as mentioned above, the mean size of the litters from which the +/+ mice were drawn was 9·5 (weighted mean 9·3), compared with 4·8 (weighted mean 6·7) for the his/his mice (Table 4:12). This could therefore result in nutritional differences which would be reflected in the biochemical parameters. Against the idea of a nutritional cause for the 6 day differences is the absence of similar changes in the cerebellum. Another possible interpretation is that differences in maturity of this magnitude could arise from the method used to age the mice, allowing differences of up to 12 hours in the timing of birth. These differences would be more prominent at stages soon after birth as discussed above.

A third explanation for the 6 day differences would be that an increased concentration of a neuroactive metabolite of histidine, such as histamine (Schwartz, 1977) or imidazole acetic acid (Phillis et al., 1968; Tunnicliff et al., 1972) temporarily enhances the rate of cerebral development. This possibility, although intriguing, would be more plausible if similar biochemical changes were present at earlier or later stages of development. This is not the case, since no other significant differences emerge other than small (9 - 11%) reductions in his/his cerebral weight at 15 and 24 days. This last finding is partly explained by reductions in cholesterol (13 - 18%) and protein (4 - 12%) content, which do not reach statistical significance.

What effect does a raised blood and brain histidine concentration have on other amino acids in the brain? The concentrations of the

neutral amino acids, phenylalanine and alanine, were the same in mutant and wild-type, so that any inhibitory effect of histidine on the blood-brain transport of these compounds is not revealed by analysis of the total amino acid pools. It may be that histidine is transported into the brain by a histidine-specific as well as a shared carrier mechanism (Sabater et al., 1976), so that it is a less effective inhibitor of uptake than amino acids only using shared systems. The cerebral concentration of lysine is increased in his/his mice during the first two or three weeks after birth (Table 4:4), although this amino acid is not known to utilize a transport system common to histidine. The reason for this change is obscure. The large increases in the glutamic acid and GABA pools that have been reported by others in the developing mouse brain (Agrawal et al., 1968) have been confirmed (Figure 4:5) although no difference between +/+ and his/his mice was found.

In summary, the postnatal growth of the brain has been examined in +/+ and his/his mice. Although small differences in the cerebral fresh weights were found during the third and fourth weeks after birth, these were not accompanied by significant differences in water, cholesterol, nucleic acid or protein content. The only biochemical difference of note was found on the sixth day after birth, when the cerebral cholesterol/DNA; RNA/DNA and protein/DNA ratios were all higher in his/his mice, in the absence of a change in DNA content. These differences disappear at later stages of development and might be due to (1) the 6 day old +/+ mice being reared in larger, nutritionally deficient litters, (2) small differences in chronological age, or (3) a short-lived stimulatory effect of a neuroactive histidine derivative on brain development. There was no change in the cerebral concentrations of alanine, phenylalanine, GABA or glutamic acid in histidinaemic mice, although an unexplained increase in the lysine pool was observed. There were no differences in cerebellar growth or biochemical parameters at any stage of development. Despite an approximately ten-fold increase in brain histidine concentration after birth and similar or greater increases before birth, there is no clear evidence of impaired brain development in histidinaemic mice.

CHAPTER 5

CHARACTERIZATION OF LIVER HISTIDASE IN MURINE HISTIDINAEMIA

(i) Biochemical genetic aspects of histidase deficiency.

It is only in comparatively recent times that the first genetically determined differences in enzyme phenotype were demonstrated (Figge and Strong, 1941; Khanolkar and Chitre, 1942; Sawin and Glick, 1943). Despite the rapidity of subsequent progress in understanding the genetic regulation of enzymes in micro-organisms much less is known of this in higher organisms. As increasing numbers of genetically determined enzyme differences were found in mammals, regulatory analogies drawn from microbial systems were at first too readily applied (Howell, 1970). It has also become amply clear that the distinction between a "regulatory" mutation, in which the protein is present and correctly coded but not fully expressed, and a "structural" mutation where the protein is absent or coded incorrectly, is not easy to make (Paigen, 1971). Interpretation is further complicated by the existence of mutations outside the enzyme structural locus concerned with the post-translational "processing" of enzymes into their final form. So far, relatively few mutations have satisfied the criteria for a regulatory mutation in higher organisms. If most enzyme deficiencies turn out to be structural locus mutations, there are few that fail to highlight some novel aspect of enzyme structure or function. The first reason for studying the biochemical genetics of a histidase deficiency is therefore to characterise the mutant enzyme in terms indicating either a structural, processing or regulatory locus mutation.

As increasing numbers of kinetic and physical studies are carried out on enzyme variants, the importance of genetic heterogeneity in human metabolic illness has become apparent (Childs and DerKaloustian, 1968). This implies that treatment will have to be tailored in many cases to the specific protein variant rather than to the metabolic or clinical expression. Already treatment aimed at increasing the activity of the defective enzyme has led to some surprising successes. For example, there are some fourteen vitamin-responsive inborn errors of metabolism (Scriver, 1973; Brock, 1978). These conditions may show altered coenzyme binding, reduced stability or defective coenzyme

synthesis. The administration of large doses of a vitamin such as pyridoxine in many cases increases enzyme activity sufficiently to restore function. Often only a small (about three-fold) increase in activity is necessary (Mudd et al., 1970). This situation also applies to mouse histidinaemia, where the relationship between histidine concentration and enzyme activity shows that a three or four-fold increase in histidase activity from around 5% could restore the histidine concentration to normal (Kacser et al., 1973). The increasing use of cell culture in mammalian genetics will undoubtedly facilitate the search for activating, stabilising or other therapeutic measures that increase defective enzyme function. A second reason for studying the biochemical genetics of histidase deficiency is therefore to explore enzyme properties, such as stability, substrate or cofactor requirements, which could indicate an appropriate method of treatment.

(ii) Criteria for identifying a mutation at the structural locus of an enzyme.

In order to show that a mutation has occurred in the structural gene coding for an enzyme, a change in some property that depends on the amino acid sequence of the enzyme must be demonstrated (Paigen, 1971). These "structural" properties include kinetic constants, stability to physical denaturation, electrophoretic mobility, immunological specificity and of course direct sequence determination. Fortunately enzyme deficiencies in higher organisms are rarely complete: between 0·1 and 20% of the normal activity often remains (Kirkman, 1972). The residual activity can then be examined for evidence of a structural change. If a difference emerges between the mutant and normal enzyme, this usually implies either a mutation at the structural locus for the enzyme or that the residual activity belongs to some other enzyme. It is not unknown for two enzymes to catalyse the same reaction, one of which only contributes a small proportion of the total activity (Ohno et al., 1966; Srivastava and Beutler, 1969). If the major enzyme activity is lost or diminished through mutation, the residual activity would be largely due to the minor enzyme, (Kirkman, 1972). Guinea pig strains have been described with high and low activity in the hydroxylation of cortisol by the liver (Burstein et al.,

1967). The high activity strain turned out to have two enzymatic components, one with a high Vmax and high Km, the other with a low Vmax and 30 times lower Km. Only the second component was present in the low activity strain.

If the residual activity is not due to another enzyme, does a change in the structural properties of the enzyme necessarily imply a mutation at the structural locus? In most instances, this is the case, but the occurrence of multiple forms of some enzymes showing similar substrate specificity (isozymes) complicates the issue. For example, electrophoresis of the enzyme nucleoside phosphorylase in the red cells of a patient with an unknown haemolytic disease might show an abnormal pattern with a predominance of slower moving isozymes compared with a normal individual (Edwards et al., 1971). In this case, the difference can be simply accounted for by the presence of more young and immature cells in haemolytic blood, together with a tendency of this enzyme to undergo secondary structural modifications in aging red cells. Other possible causes of altered "structural" properties in an enzyme, which may not be due to structural locus mutation include: (1) aggregation or polymerization of the enzyme (Smithies and Connell, 1959; Klee, 1970): (2) stable changes in the tertiary or quaternary structure, induced for example by varying concentrations of substrate, cofactor or other effector molecule (Blume et al., 1971; Smith et al., 1971; Fisher and Harris 1971a, b; Padua et al., 1978): (3) deamidation of amino acids such as asparagine or glutamine in the enzyme molecule which can alter the electrophoretic mobility (Funakoshi and Deutsch, 1969; Midelfort and Mehler, 1972; Karn et al., 1973): (4) artefactual changes arising from extraction, purification or analytical procedures (Hopkinson and Harris, 1969; Hopkinson, 1970; Fisher and Harris, 1972): (5) mixed disulphide formation with glutathione (Muller, 1961): (6) acetylation, methylation, phosphorylation, adenylation or conjugation with carbohydrate moieties (Krebs et al., 1958; Schroeder et al., 1962; Shapiro et al., 1967; Shapiro and Stadtman 1967; Robinson and Stirling, 1968): (7) Schiff's base formation with the N-terminal amino group (Holmquist and Schroeder, 1966): (8) polypeptide cleavage by proteolytic enzymes (Marti et al., 1967; Rubenstein and Steiner, 1970): (9)

sulphydryl oxidation (Klee, 1970). Undoubtedly many other causes exist. It is therefore less surprising that an enzyme known to be coded by the same structural gene is found to differ in urea sensitivity, depending on the source of extraction (Schwartz, 1964; Endo and Schwartz, 1966) or to differ in electrophoretic mobility, depending on the sub-cellular localisation (Ganschow, 1971). Many of these modifying factors are themselves under separate genetic control. An enzyme defect inherited as a single factor, and associated with a non-artefactual change in some "structural" property of the enzyme is therefore quite possibly due to a mutation at another enzyme "processing" locus, secondarily and stably affecting the enzyme conformation or covalent structure. Post-translational processing of an enzyme into its final functional form is probably a common feature in higher organisms. This may involve conjugation or some other form of chemical modification, but can also involve incorporation of the enzyme into a particular subcellular organelle. Paigen et al. (1975) suggested the term "processing" mutant to describe mutations concerned with this aspect of metabolic regulation. Processing mutants have been particularly well characterised in lysosomal enzymes such as β-glucuronidase (Eg and bg loci), acid phosphatase (Apl locus) and α-mannosidase (Map-1, Map-2) (Paigen et al., 1975; Lalley and Shows, 1977; Dizik and Elliott, 1978). Another consequence of post-translational processing is that a structural mutation can lead to loss of the recognition features in the molecule necessary for such processing. It may be difficult to distinguish between a structural mutation and a processing mutation affecting the same enzyme. Loss of a conjugant group or a change in subcellular location would indicate a processing mutant. Both types of mutation can stably affect the three-dimensional structure of the enzyme and hence change its heat stability, kinetic or electrophoretic properties. It may be necessary to sequence the enzyme for a definitive answer although genetic criteria usually provide a simpler means of distinction (see below).

The relative efficacy of properties such as the heat stability or electrophoretic mobility as indicators of a change in amino acid sequence has been discussed by Paigen (1971). In general, a heat stability change is likely to occur after a single amino acid substitution at one-half to three-quarters of all sites (Langridge, 1968a; Lehmann and

Carrell, 1969). About one-third of all amino acid substitutions resulting from a single base change will change the net charge and hence electrophoretic mobility of a protein (Sick et al., 1967; Lehmann and Carrell, 1969). Where mutant enzymes are pre-selected by a change in kinetics, these proportions may be considerably higher. While some change in the velocity constant (Vmax) usually follows a structural locus mutation, only about 5% have an altered affinity for substrate (Km) (Langridge, 1968b). Immùnological methods of disting--uishing protein variants are relatively inefficient (Reichlin, 1972, 1975). Immunoprecipitation or complement fixation techniques are only sensitive to variation at about 3% of the amino acid sites in haemoglobin. A point mutation is therefore unlikely to lead to a loss of antigenicity, but it may reduce it (Brock, 1978). One consequence of this is that such methods are correspondingly efficient means of comparing the amounts of normal and mutant enzyme.

Estimating the amount of immunologically cross-reacting material (CRM) is often an important part of a biochemical genetic analysis. While a reduction in CRM is an essential feature of a regulatory mutation, it is also a common finding in structural mutations. In the latter, this may be due to altered antigenicity, reduced stability or reduced de novo synthesis. What proportion of mutations affecting enzyme function are CRM-positive? Many mutations are likely to lead to a polypeptide that cannot fold into the normal tertiary or quarter-nary structure, in which case CRM is unlikely to be present. Enzymes that are denatured or dissociated into subunits react poorly with antisera (Gelehrter et al., 1970; Celada and Strom, 1972). A reduction in enzyme stability is another property commonly resulting in loss of CRM. Boyer et al., (1973) compared the reported incidence of CRM-positive and CRM-negative mutations in inborn errors of metabolism. They collected data from 260 kindreds in 24 distinct genetic conditions and found a ratio of CRM-positive:CRM-negative of about 3:2. This provides only a rough measure. CRM-negative mutations may only reflect limitations in the immunological techniques, so that the ratio is probably too low (Brock, 1978). Enzyme instability is certainly a frequent consequence of structural mutation. It is reported to be a major problem in about 20% of 123 haemoglobin variants associated with amino acid substitution

(Stamatoyannopoulos, 1972). The unstable haemoglobin variants mainly involve substitution of polar for non-polar amino acids in the hydrophobic interior of the molecule, which distorts the three-dimensional structure (Perutz and Lehmann, 1968). About one-third of all known glucose-6-phosphate dehydrogenase (G6PD) variants have reduced stability to physical denaturation (Beutler, 1972), in some of which the reduced activity is wholly attributable to in vivo denaturation rather than altered catalytic function (Piomelli et al., 1968). Finally, reduced synthesis of a structural variant is not uncommon, although the mechanism is unclear. Virtually all known amino acid substitutions in haemoglobin show a reduced rate of synthesis: on the other hand G6PD structural variants are mostly synthesized at the normal rate (Sutton and Wagner, 1975).

Investigation of an enzyme variant may reach the stage of showing that (1) there is no evidence of a change in the primary structure of the enzyme and (2) there is a change in the amount of identifiable enzyme protein (CRM). This suggests the possibility of a regulatory mutation. It is then necessary to show that the putative regulatory mutation maps separately from the structural gene. Paigen (1971) stated the criteria for identifying a regulatory gene mutation as follows:- (1) the mutation must alter the rate of enzyme synthesis or breakdown, not merely the measurable level of activity:

(2) the mutation must be at a genetic locus separate from the structural gene for the enzyme.

Paigen later classified enzyme degradation mutants among the group of processing rather than regulatory mutants, the distinction being made on whether the mutation affects the enzyme before or after its messenger RNA is translated (Paigen et al., 1975).

The term regulatory mutation should be used cautiously in mammals because of its prokaryotic associations. In bacterial operons, regulatory genes include promoter, operator and repressor sequences (Jacob and Monod, 1961). The cis-active genes (operator and promoter) imply an involvement with the binding of RNA polymerase or initiation of transcription, while trans-active genes (activator or repressor) appear to control protein synthesis via a diffusible product which is generally a protein. In enkaryotes there are a few examples of cis-active genes

which map separately from the structural locus (Swank et al., 1973; Chovnick et al., 1976) but most regulatory genes are of the trans-active type. Here the prokaryotic concept of regulatory gene may be too restrictive since it is often unnecessary to distinguish between a gene coding for a protein that controls gene expression at the level of the genome (transcription) rather than at some other level prior to translation of the messenger RNA. A loss of cytoplasmic testosterone receptors with consequent loss of the hormonal induction of mouse β-glucuronidase in the Tfm mutant could legitimately be regarded as a regulatory mutant, although it is probably also the structural gene for the receptor (Bardin et al., 1973; Lyon et al., 1975; Shire, 1976). Apparent regulatory mutations sometimes turn out to result from deletion of a structural gene when these exist in multiple copies which may be closely linked, (e.g. α-thalassaemia; see White, 1978). The problems of genetically distinguishing between closely linked loci in higher organisms are often insurmountable so that resort to nucleic acid hybridisation may be necessary.

The importance of genetic criteria in identifying regulatory mutations was mentioned above. The regulatory locus must be separate from the structural locus. The few regulatory mutations known in higher organisms are all very closely linked to the structural genes, so that it is difficult to show their physical separation (Paigen et al., 1975). The above criterion implies that there is a clearly defined structural locus variant available for mapping the putative regulatory mutant. This is often not the case. Mapping of a processing mutant will also show it to be at a separate site from the structural locus. In general, these mutants have not been found to be closely linked to their structural genes (Paigen et al., 1975).

A cross between the structural and putative regulatory or processing mutant homozygotes will show whether they are functional allelic, which can be another useful genetic criterion. This is only informative in the case of recessive mutants, where the heterozygote will be mutant if there is allelism. Rarely, allelic mutants affecting a multimeric enzyme show complementation and restoration of activity in the heterozygote.

Another criterion for distinguishing a mutation at the structural locus of an enzyme from a mutation at another locus is that of co-dominance (Paigen, 1971). Most inborn errors of metabolism show recessive inheritance at the clinical or metabolic level and co-dominance at the enzyme level. Heterozygotes therefore express both mutant and normal alleles and show an intermediate level of enzyme activity. Failure to find codominant expression of enzyme activity is strong evidence against there being a mutation at the structural locus, although there are rare exceptions (Morrow et al., 1949, 1950). A processing gene may or may not show codominant expression. While a trans-active regulatory gene is likely to show complete dominance, a cis-active gene will also be codominant. The codominance criterion is therefore most useful when it is absent in the heterozygote.

These considerations show that the distinction between structural processing and regulatory locus mutations cannot be made easily. The problems are even greater where the residual mutant activity is as low as it is in the <u>his/his</u> mutant. Kinetic and physical parameters can be compared between two enzyme forms provided good measurements are possible in both. In the present case, the mutant activity is near the limit of the assay method, so that comparisons using crude extract are subject to some reservations. The work with partially purified extracts largely overcomes this problem, since the specific activity is substantially higher. The instability of the residual (SN-2 extract) activity remains a problem, so that purification and analyses always have to be carried out on the same day otherwise significant loss of activity occurs.

Previous studies of liver histidase in wild-type and histidinaemic mice (Kacser et al., 1973; Bulfield and Kacser, 1974) have shown that histidinaemia is associated with a defective liver histidase (0·00 - 0·05 µmol/min/g liver) with less than 5% of the activity found in wild-type. Heterozygotes show activity that is intermediate between that of the mutant and the wild-type. Recessive inheritance at the metabolic level is therefore associated with codominance at the enzyme level. Since the available evidence from studies of bacterial and mammalian systems suggests that histidase is a multimeric protein made up of identical subunits [Chapter 1 (iv)], it is likely that there is only

one structural locus. If there is more than one copy of this gene then homozygous mutants should retain relatively high residual activity, which is not the case. The activity of the next enzyme in the pathway, urocanase, has been shown to be unchanged in histidinaemic mice (Kacser et al., 1973) and there is nothing in the metabolic profile to suggest a block at a more distal point in the pathway. The only other tissue expressing histidase activity is the skin, which is again found to have reduced activity in young mice (Chapter 3; Bulfield and Kacser, 1974).

The purpose of the experiments described below was firstly to contrast some of the physical or structural properties of liver histidase in mutant and wild-type mice. A difference in one of these properties would support the hypothesis that the _his_ locus is the structural locus for histidase. A failure to find such a difference might suggest the presence of a regulatory gene distinct from the structural locus, or alternatively a gene affecting only the measured activity of the enzyme. Secondly, by investigating the properties of the mutant enzyme, it might be possible to devise a therapeutic approach to increase the residual activity sufficient to reverse the metabolic error.

(iii) Tests for the presence of dissociable activators or inhibitors.

(a) Dialysis. Mutant livers were extracted by homogenisation in Tris/sucrose medium and the 60,000g supernatant dialysed overnight as described in Chapter 2 (xv). An aliquot of undialysed supernatant was retained for histidase assay in comparison with the dialysed sample. The pre-dialysis activity is 0·11 μmol/min/g liver compared with 0·005 μmol/min/g liver after dialysis. Dialysis therefore results in an approximately 50% loss of histidase activity rather than the increase in activity that would be expected in the presence of a dissociable inhibitor of histidase activity. This loss of activity, which does not occur in wild-type extracts, is the first evidence of a difference in stability between mutant and wild-type histidase.

(b) Gel filtration. Mutant liver extracts (60,000g supernatants) were desalted by gel filtration on Sephadex G-25 as described in

Chapter 2(xv). The total histidase activity prior to gel filtration is 0·032 units[1] compared with 0·026 units after gel filtration. The total activity recovered after desalting by this method is therefore about 80% of the initial activity. This again shows that there is no enhancement of mutant histidase activity following removal of dissociable small molecules.

(c) Mixing experiments. Wild-type and mutant liver extracts (60,000g supernatants) were assayed for histidase activity, either alone or in combination with each other. The combined extracts were mixed briefly in a test tube before starting the reaction with histidase/buffer pre-warmed to 30°C. This provides a further test for the presence of dissociable inhibitors or activators of histidase activity. The results are shown in Table 5:1. It can be seen that when extracts from +/+ and his/his mice are mixed, the combined activities are essentially additive. There is no enhancement of mutant activity or inhibition of wild-type activity. This rules out the possibility that wild-type extracts contain a dissociable activator (e.g. a divalent metal) which is lacking in the mutant or that mutant extracts contain a dissociable inhibitor which is absent in the wild-type.

(iv) Inhibition by nitromethane.

Nitromethane has been reported to inactivate bacterial and mammalian histidase irreversibly in vitro and in vivo, probably by reacting with a dehydroalanine residue at the active site (Givot et al., 1969; Wickner, 1969; Givot and Abeles, 1970; Okamura et al., 1974; Hanford and Arfin, 1977). It is a relatively specific inhibitor of histidase. The aim of these experiments was therefore to compare the inhibition of mutant and wild-type histidase by nitromethane. If it proved possible to inactivate mutant and wild-type histidase in vivo, measuring their rates of recovery would provide an estimate of their relative rates of in vivo synthesis and degradation (Rechcigl and Heston, 1967).

Mouse liver histidase activity is reported to be more than 90% inactivated in vivo within 3 - 5 hours of a single intraperitoneal injection of 0·5 ml of 0·5 M nitromethane (Hanford and Arfin, 1977).

[1] Change in absorbance at 277 nm per minute.

TABLE 5:1

Tests for the presence of inhibitors or activators of liver histidase activity by mixing extracts from +/+ and his/his mice. Each value is the mean of triplicate determinations. Histidase units are the change in absorbance at 277 nm per minute.

Liver supernatants (genotype) µl	Histidase activity
50 (+/+)	0·0410
50 (his/his)	0·0006
50 (+/+) 50 (his/his)	0·0403
100 (+/+)	0·0792
100 (his/his)	0·0011
100 (+/+) 100 (his/his)	0·0781

Four +/+ and four his/his five-week old mice were injected intraperitoneally with 0·5 ml of 0·5M nitromethane in 0·9% saline and killed after 12 hours. The livers were removed and extracted for histidase assay. The results are shown in Table 5:2A. Wild-type histidase activity is reduced to 7% of control values ($P < 0{\cdot}001$), while mutant histidase is not significantly different from controls. In a further experiment, four his/his males were injected as before with 0·5 ml 0·5M nitromethane and killed three hours later. Liver histidase activity is again found to be the same as controls. However, since nitromethane is thought to bind to the active site, the twenty-fold higher liver histidine concentration in the mutant could be competing with nitromethane at this site and so preventing it from inhibiting the enzyme. Three male his/his mice were therefore injected intraperitoneally with 0·5 ml of 0·8M nitromethane, 1·0M nitromethane and 0·9% saline respectively. They were killed five hours later and the livers assayed for histidase activity. The results are shown below:-

Treatment	Histidase Activity
0·9% saline	0·012 μmol/min/g liver
0·8M nitromethane	0·016 μmol/min/g liver
1·0M nitromethane	0·010 μmol/min/g liver

Mutant histidase is again not inactivated by nitromethane even at double the concentration required in the wild-type. A single his/his mouse was injected intraperitoneally with 1·0 ml of 1·0M nitromethane and liver histidase assayed after five hours, but no significant change in activity was found. *In vitro* experiments were then carried out to determine the effect of nitromethane on mutant histidase. His/his liver extracts were pre-incubated for 30 minutes at 30°C with or without 4mM or 20mM nitromethane (made up in Tris buffer, pH 9·0) and assayed for histidase activity. The results are shown in Table 5:2B. In this case exposure to nitromethane reduces the activity to 10% of the control (which was not pre-incubated for 30 minutes at 30°C). In order to ensure that the loss of activity is due to specific inhibition

TABLE 5:2

A. Liver histidase activity 12 hours after intraperitoneal injection with 0·5 ml of 0·5M nitromethane in 0·9% saline, in comparison with noninjected controls.

B. Liver histidase activity in his/his extracts following pre-incubation for 30 minutes at $30^{o}C$ with and without nitromethane or nitromethane plus histidine.

A Genotype	Histidase activity, umol/min./g. liver	
	Nitromethane injected	Control
+/+	0·019 ± 0·003	0·284 ± 0·059***
his/his	0·009 ± 0·003	0·005 ± 0·001

B Preincubation	Histidase activity µmol/min./g. liver
None	0·010
4 mM nitromethane	0·001
4 mM nitromethane + 30 mM histidine	0·001
20 mM nitromethane	0·001
20 mM nitromethane + 30 mM histidine	0·002

***P < 0·001

by nitromethane rather than a non-specific mechanism such as enzyme denaturation, samples were pre-incubated with nitromethane in the presence of 30 mM histidine, which should compete with the nitromethane and reduce or abolish any specific inhibition. Histidase activity under these conditions is still reduced to about 10% of control values (Table 5:2B) suggesting a non-specific mechanism for the loss of activity.

The above evidence suggests that while the wild-type histidase is inactivated in vivo by nitromethane injection, this does not occur in the mutant. Mutant histidase may be protected as a result of the considerably higher endogenous histidine concentration, although higher concentrations of nitromethane still failed to inactivate the enzyme. Pre-incubation of mutant liver extracts with nitromethane in vitro, where the histidine concentration is very low (circa 0·4 mM) compared with the nitromethane concentration (4 mM or 20 mM), results in a significant loss of activity. This inactivation still occurs in the presence of a seventyfive-fold higher histidine concentration, suggesting that the mechanism of inactivation is non-specific.

(v) Divalent metals.

A divalent metal ion requirement has been reported for bacterial and mammalian histidase (Klee, 1972; Okamura et al., 1974). The effect of a divalent metal on wild-type and mutant histidase was therefore examined in crude liver extracts (60,000g supernatants). Extracts were assayed in the presence and absence of manganous chloride at a concentration of 10^{-3}M. The results are shown in Table 5:3. Addition of Mn^{+2} did not result in a significant change in histidase activity in either genotype.

(vi) Sulphydryl reagents.

The effect of sulphydryl reagents on histidase activity was examined in crude liver extracts (60,000g supernatant) in +/+ and his/his mice. The liver of a single +/+ or his/his mouse was extracted in Tris/sucrose medium in the presence and absence of dithiothreitol 10^{-3}M. Histidase activity was then determined as before. The results are shown in Table 5:4. The histidase activity in both genotypes is unaffected by the presence of thiol reagent. The effect of thiol reagents on partially purified histidase was not determined.

TABLE 5:3

Effect of a divalent metal on liver histidase activity in crude +/+ and his/his liver extracts. Values are the means of duplicate determinations, using a discontinuous assay method.

Genotype	Histidase activity [1]	
	$-MnCl_2$ $10^{-3}M$	$+MnCl_2$ $10^{-3}M$
+/+	0·110	0·102
his/his	0·007	0·009

TABLE 5:4

The effect of dithiothreitol (DTT) in the extraction medium at $10^{-3}M$ on crude liver histidase activity (μmol/min./g. liver) of +/+ and his/his mice.

Genotype	Histidase activity	
	- DTT	+ DTT
+/+	0·345	0·343
his/his	0·004	0·005

(vii) Heat stability.

A change in the heat stability of an enzyme variant provides evidence for a mutation at the structural locus. Dr G. Bulfield carried out a preliminary examination of the relative heat stabilities of mutant and wild-type histidase activities between 35°C and 82°C. This showed that the mutant loses about 50% of its activity in 30 minutes in the region 40 - 42°C compared with 70 - 72°C in wild-type. Four +/+ and seven his/his mice were therefore killed and the livers extracted and dialysed overnight in the presence of 10^{-5}M glutathione, as described in Chapter 2 (xv). An aliquot of +/+ enzyme extract was then incubated in pH 9·2 phosphate buffer at 70°C for varying periods of time, then returned to ice before assay for histidase activity. His/his extracts were extracted and dialysed in the same way, but pre-incubated for varying periods of time at 40°C before assay. The assays were carried out in quadruplicate, using a discontinuous assay method with a 15 minute incubation at 30°C. The results are shown in Figure 5:1 and Table 5:5. In +/+ extracts, half of the initial activity is lost ($t_{\frac{1}{2}}$) after 16 minutes at 70°C compared with the his/his enzyme which has a $t_{\frac{1}{2}}$ of 19·5 minutes at 40°C. When log histidase activity is plotted against pre-incubation time, the +/+ enzyme appears to show a biphasic decay, with a more rapid component initially, followed by a slower, linear decay. This might suggest the presence of more than one enzyme species or alternatively interference by urocanase. On the other hand, the mutant enzyme shows a linear decay (Figure 5:1) which makes the latter explanation unlikely.

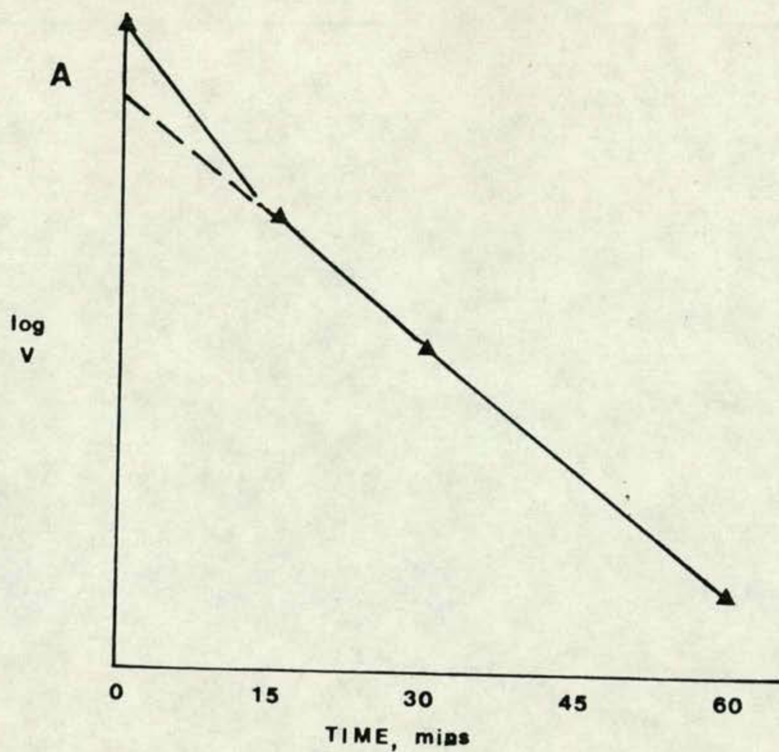
The heat stability of partially purified liver histidase was examined. The low ionic strength extract (SN-2) following protamine sulphate precipitation was compared in +/+ and his/his mice. Aliquots of SN-2 enzyme were incubated in pH 7·2 Pipes buffer for 2 - 30 minutes in a 40°C waterbath, then returned to ice before assay. The results are shown in Table 5:6 and Figure 5:2A. The first experiment was carried out on his/his extracts only and shows a markedly heat labile SN-2 activity with a $t_{\frac{1}{2}}$ of 9 minutes at 40°C. The second experiment examined the heat inactivation of SN-2 extracts from +/+ mice and shows an almost identical inactivation curve (Figure 5:2A). When the

TABLE 5:5

Heat inactivation of liver histidase at

70°C and 40°C in crude +/+ and his/his extracts.

Genotype	Fraction	Temp. °C	Time mins.	Residual histidase activity µmol/min./g. liver	% maximum
+/+	60,000g supernatant	70	0	0·262	100·0
			15	0·133	50·8
			30	0·084	32·1
			60	0·036	13·7
his/his	60,000g supernatant	40	0	0·0076	100·0
			15	0·0043	56·6
			30	0·0026	34·2
			64	0·00	0·0



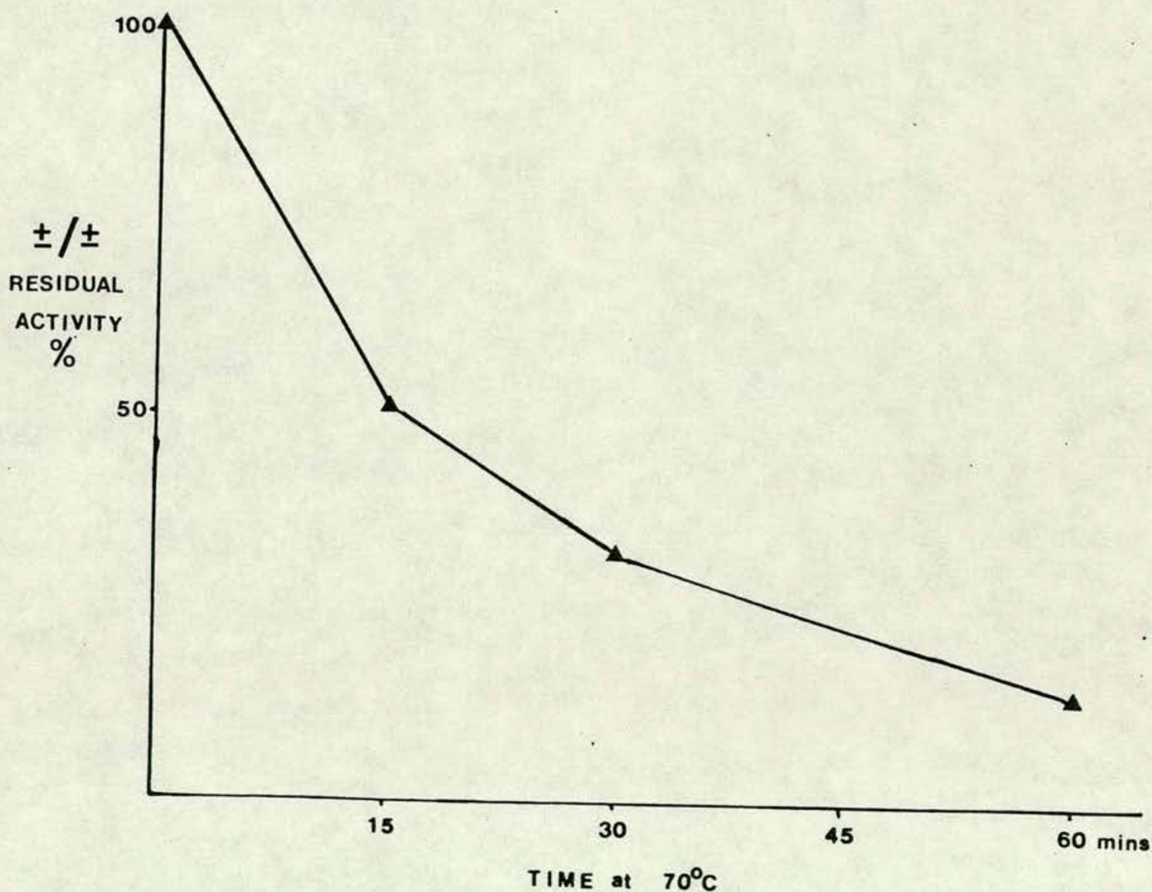
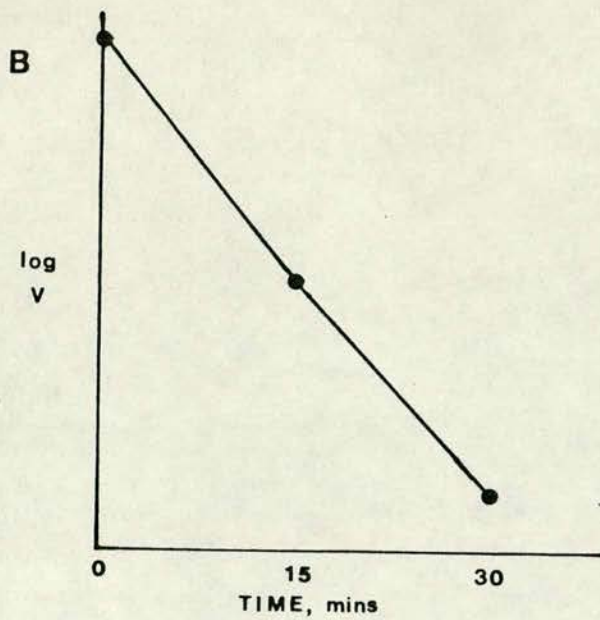


FIG. 5:1 A Heat inactivation of +/+ liver histidase in crude extracts at 70°C.

B Heat inactivation of *his*/*his* liver histidase in crude extracts at 40°C. The log plots of histidase residual activity (V) against preincubation time are shown above, in each case.


B
log
V
0
15
30
TIME, mins

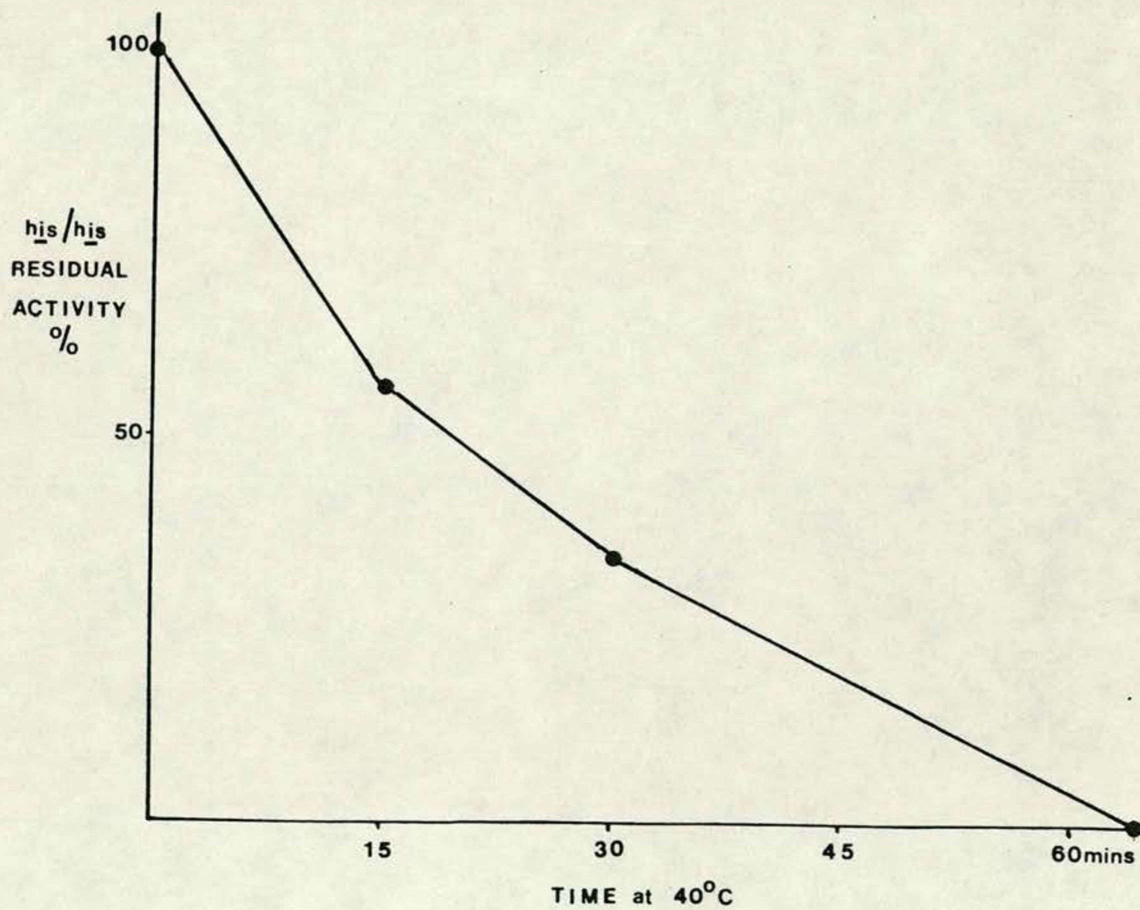

100
50
his/his
RESIDUAL
ACTIVITY
%
15
30
45
60mins
TIME at 40°C

TABLE 5:6

Heat inactivation of partially purified liver histidase at 40°C, in +/+ and his/his extracts.

Genotype	Fraction	Pre-incubation time, mins.	Residual histidase activity nmol/min/mg. protein	% maximum
+/+	SN-2	0	5·004	94
		2	5·323	100
		5	3·726	70
		10	1·411	27
		15	0·772	15
		20	1·384	26
		30	1·224	23
his/his	SN-2	0	1·516	100
		2	1·087	72
		3·75	1·172	77
		5·5	0·850	56
		7·5	0·719	47
		11	0·595	39
		15	0·566	37
		18·5	0·404	27
		22·5	0·336	22
		30	0·254	17
+/+	SN-3	0	51·38	84
		2	48·13	79
		5	59·84	98
		10	60·49	99
		15	55·93	91
		30	61·14	100

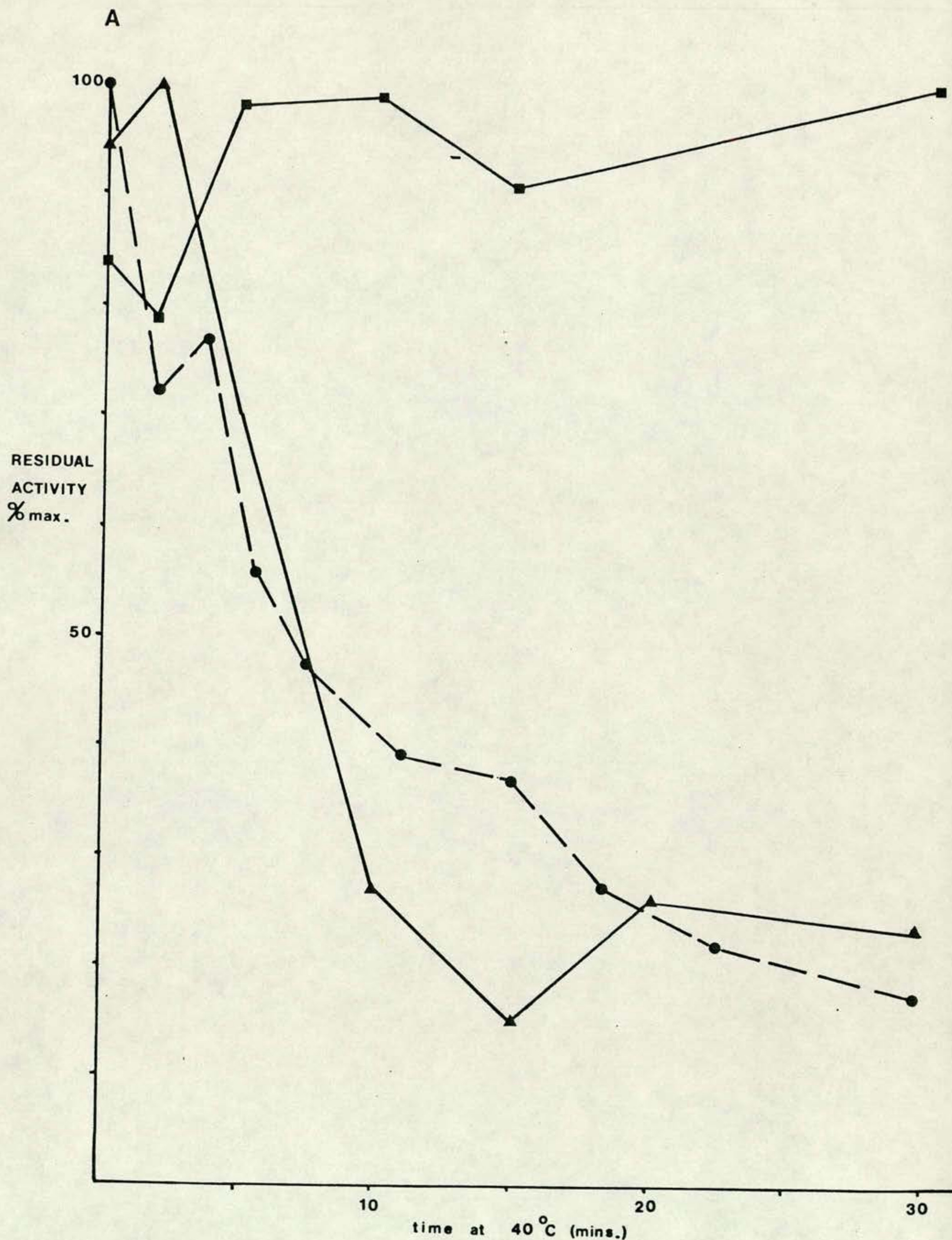
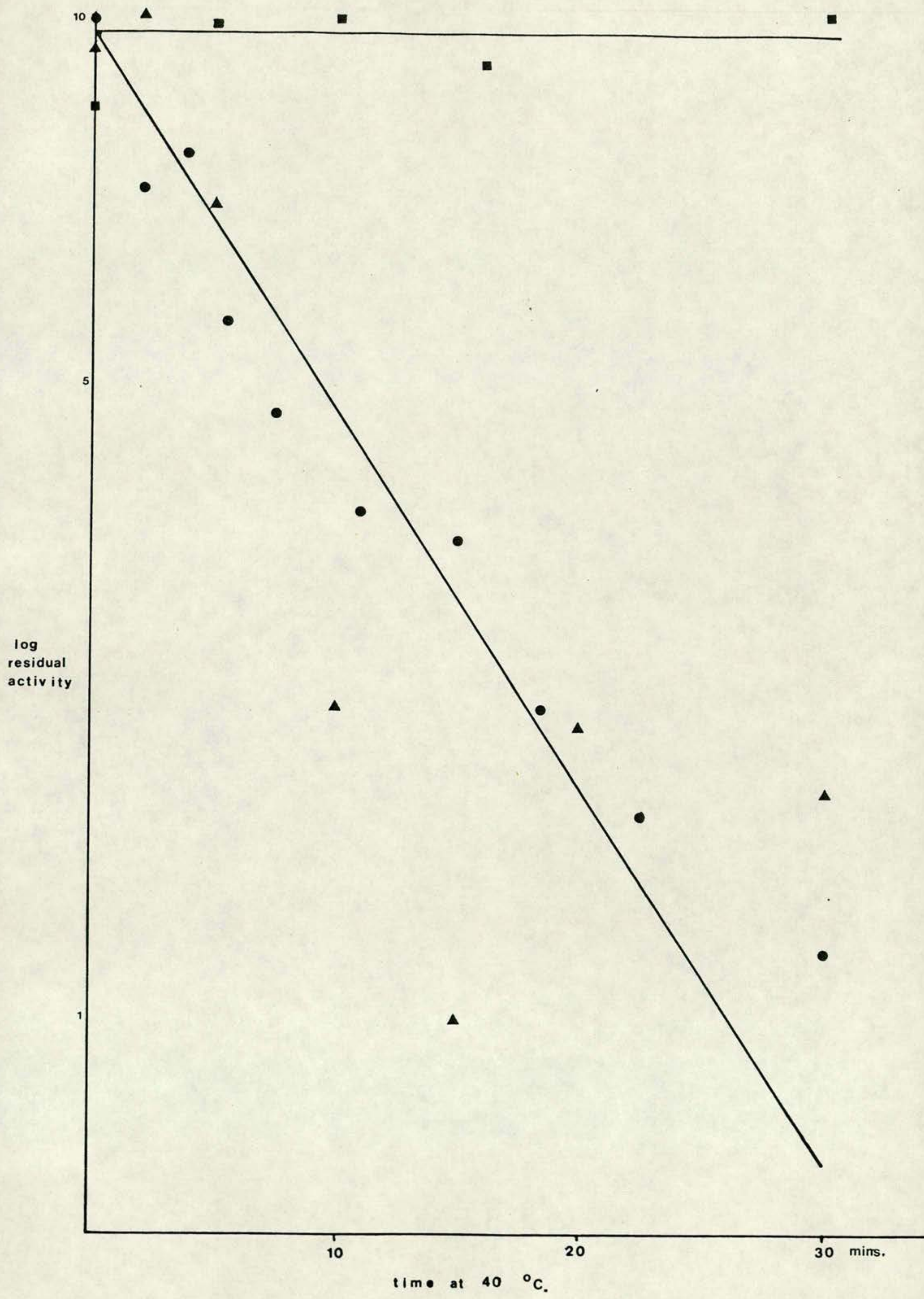


FIG. 5:2 Heat inactivation of partially purified liver histidase at 40°C. The low ionic strength extract activity (SN-2) is shown for +/+ (▲——▲) and his/his (●- - -●) mice. The high ionic strength activity (SN-3) is shown for the +/+ (■——■) genotype.

A The % residual activity is plotted against incubation time at 40°C.

B Log residual activity against preincubation time. The lines were fitted by eye.



B
10
5
1
log
residual
activ ity
10
20
30 mins.
time at 40 °C.

log residual activity was plotted against pre-incubation time, an identical linear relationship is obtained with the SN-2 enzyme from +/+ and his/his livers (Figure 5:2B). In +/+ samples, most of the histidase activity is recovered in the high ionic strength extract (SN-3) following protamine sulphate precipitation. The heat inactivation of SN-3 extracts from +/+ mice was then examined. This activity is found to be heat stable at 40°C (Figure 5:2A and B). The heat stability of SN-3 extracts from his/his mice was not tested. Although the +/+ SN-3 enzyme is in a higher ionic strength medium than the SN-2 enzyme, this difference is eliminated in the assay mixture so that two clearly distinguishable enzyme species with histidase activity appear to be present in the wild-type. The heat labile component is also found in SN-2 extracts from his/his livers. The heat stable component in +/+ samples can be identified with the major histidase species, which is known to be stable at this temperature [see Chapter 1 (iv) and crude extract results above]. In crude his/his extracts, histidase is heat labile with a $t_{\frac{1}{2}}$ of 19·5 minutes at 40°C compared with the heat labile component in SN-2 fractions with a $t_{\frac{1}{2}}$ of 7 minutes at 40°C. This relatively small difference could result from the removal of adsorbed material on purification or from the fact that crude extracts were incubated at pH 9·2 compared with pH 7·2. Although the heat stability of SN-3 extracts from his/his mice has yet to be determined, the above results suggest that the residual mutant activity could be due to a distinct histidase isozyme unaffected by mutation at the his locus and capable of deaminating histidine to urocanic acid at a low rate in both genotypes.

(vii) Kinetic constants.

The effect of substrate concentration on histidase activity has been investigated in crude and partially purified liver preparations. Because of the twenty to twentyfive-fold difference in liver histidine concentration between +/+ and his/his mice (Bulfield and Kacser, 1974), it was necessary to desalt the samples by dialysis or gel filtration beforehand. Livers from wild-type females were extracted to obtain a 60,000g supernatant. This was dialysed overnight to remove endogenous histidine as described in Chapter 2 (xv). Dialysed samples were then

TABLE 5:7

Comparison of apparent Michaelis constants (Km) for histidine and velocity constants (Vmax) in crude liver extracts from 3 strains of mice. The constants were determined from double reciprocal plots.

Inbred strain	Km for histidine Molar	Vmax µmol/min./g. liver
PERU	$0\cdot68 \times 10^{-3}$	0·341
C57BL/6J	$0\cdot64 \times 10^{-3}$	0·358
DBA/2J	$0\cdot58 \times 10^{-3}$	0·372

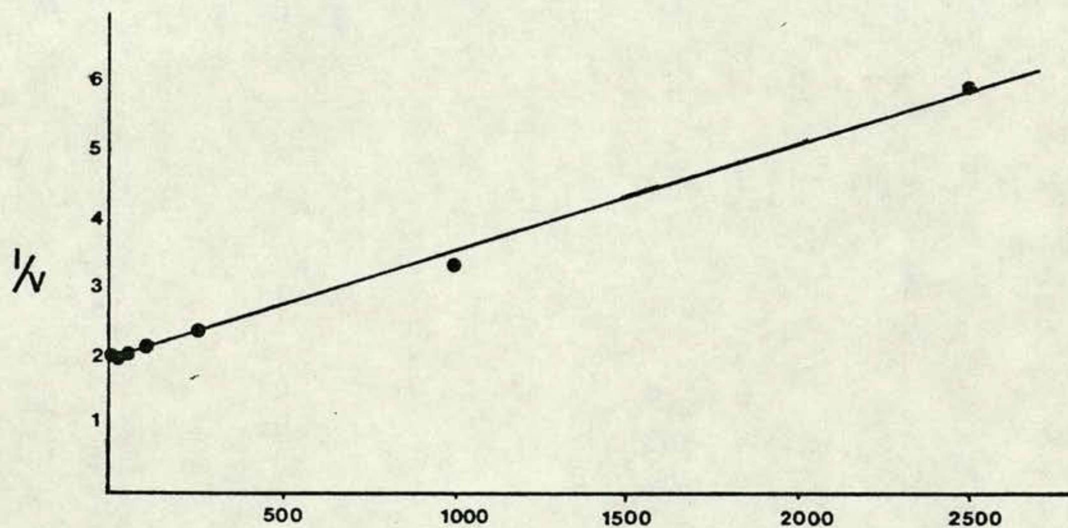
TABLE 5:8

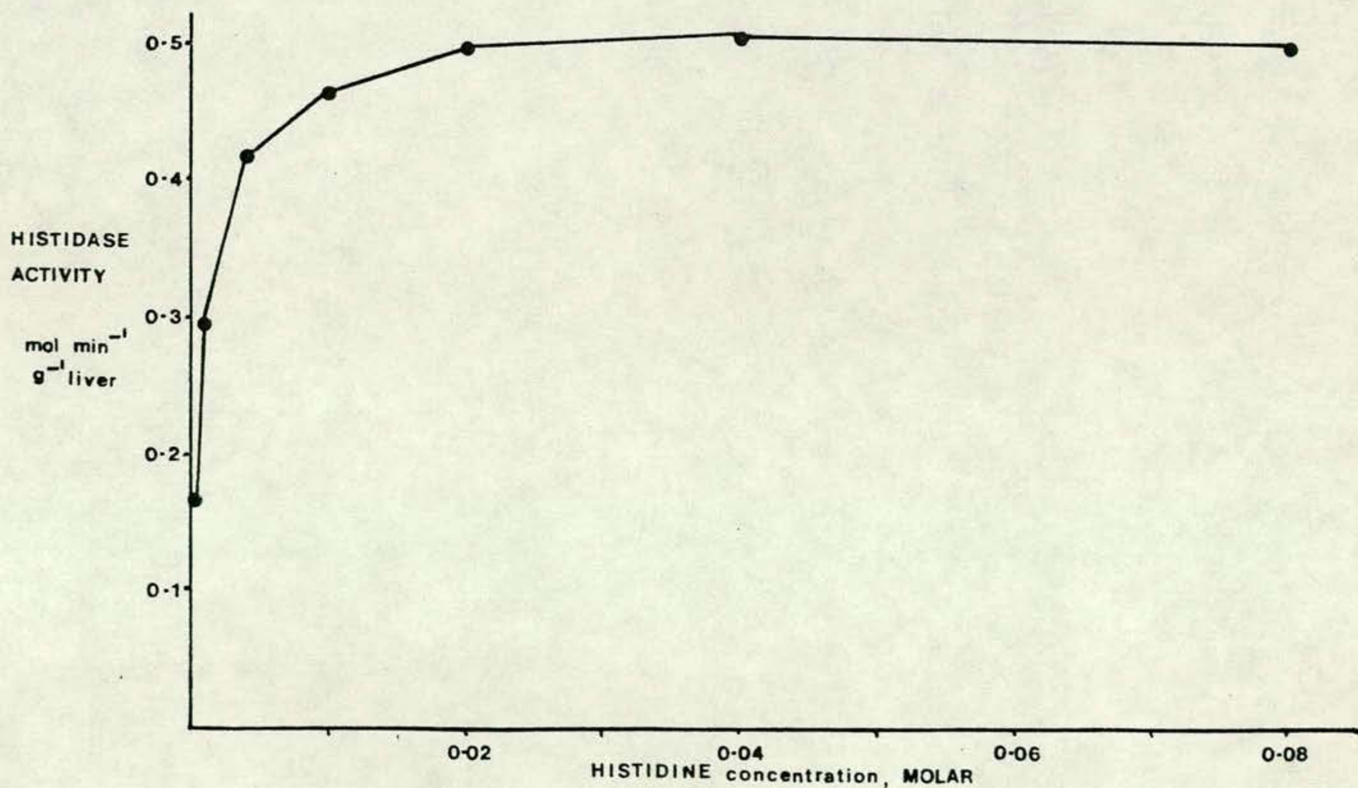
The effect of histidine concentration on liver histidase activity in crude (60,000g supernatant) extract from +/+ mice.

Genotype	Histidine mM	Histidase activity µmol/min./g. liver
+/+	0·4	0·168
	1·0	0·296
	4·0	0·417
	10·0	0·464
	20·0	0·497
	40·0	0·504
	80·0	0·497

FIG. 5:3 A The effect of histidine concentration (S) on histidase activity (V) of dialysed liver extracts from +/+ mice.

B Double reciprocal plot of histidine concentration against histidase activity.





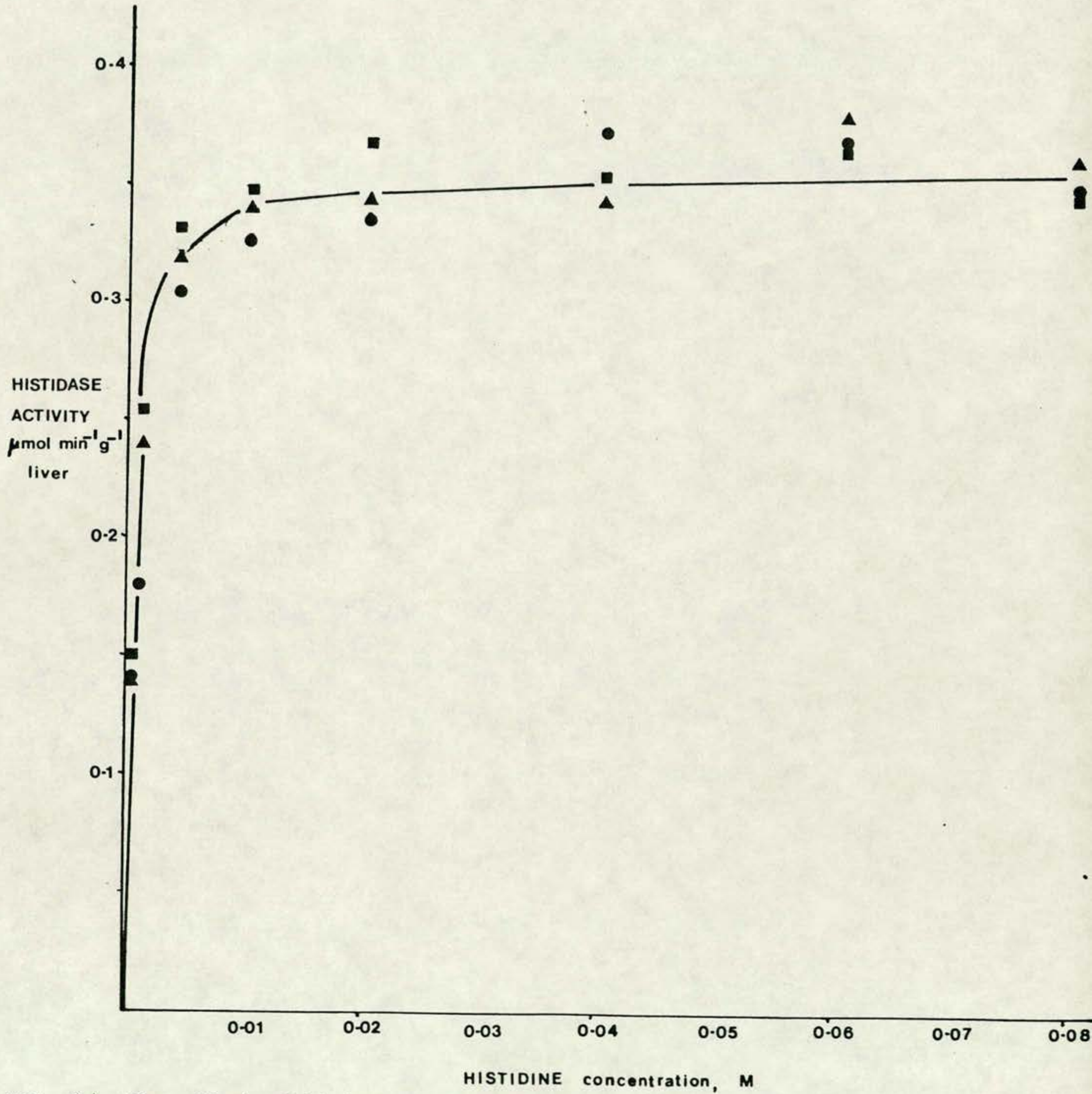


FIG. 5:4 The effect of histidine concentration on histidase activity in three inbred strains of mouse; Peru (●), C57BL/6J (▲) and DBA/2J (■).

assayed in the presence of varying concentrations of histidine. The result of one experiment is shown in Table 5:8 and Figure 5:3. A double reciprocal plot of histidine concentration against histidase activity was used to estimate the Km for histidine and velocity constant (Vmax). The Km for histidine was $0{\cdot}81 \times 10^{-3}$M and Vmax was 0·512 µmol/min/g liver. The experiment was repeated using +/+ males from three different strains of mice to examine the possibility of kinetic variation between strains. Samples were not dialysed in this experiment. The strains examined were Peru (genetic background of the his mutant), C57BL/6J and DBA/2J. The kinetic difference in histidase activity between inbred strains reported by Hanford and Arfin (1977) was not known at this time. All three strains have closely similar kinetic constants, as estimated from double reciprocal plots. (Table 5:7 and Figure 5:4). These data provide no evidence for heterogeneity between these strains of mice and show that the Peru strain is in the "high" histidase activity group reported by Hanford and Arfin (1977), since C57BL/6J and DBA/2J are both found in this group [see Chapter 1 (v)].

The substrate affinity of +/+ and his/his histidase was compared. Livers from +/+ and his/his female mice were extracted and dialysed overnight. Dialysed samples were then assayed in the presence of varying concentrations of histidine. Assays were carried out using both the discontinuous method modified from Kacser et al., (1973) with a ten minute incubation time and the continuous method used routinely. The results are shown in Table 5:9 and Figure 5:5. The two assay methods give closely similar results. +/+ histidase shows a very different kinetic response to changes in histidine concentration compared with the his/his enzyme. The his/his enzyme does not show typical Michaelis-Menten kinetics, since the activity falls off sharply at higher histidine concentrations. The Km for histidine appears to be higher in the his/his enzyme than the +/+ enzyme, although it is not possible to measure it accurately in the mutant since the activity may or may not be saturating in the region 20 - 30 mM, above which the activity falls off. The decrease in activity with increasing histidine concentration suggests that substrate inhibition is occurring. There is no evidence of this with the +/+ enzyme.

TABLE 5:9

The effect of substrate concentration on histidase activity in crude liver extracts from +/+ and his/his mice.

Assay method	Genotype	Histidine mM	Histidase activity % maximum
Discontinuous	+/+	0·5	10·9
		1·0	23·4
		2·5	67·6
		10·0	86·5
		20·0	96·2
		50·0	100·0
		100·0	99·0
Continuous	+/+	0·25	11·9
		1·0	21·7
		2·5	59·9
		5·0	75·5
		10·0	81·5
		20·0	97·1
		50·0	100·0
Discontinuous	his/his	1·0	0·0
		2·5	17·7
		5·0	35·5
		10·0	42·6
		20·0	100·0
		50·0	28·4
Continuous	his/his	1·0	0·0
		5·0	20·5
		10·0	38·5
		20·0	100·0
		50·0	51·3

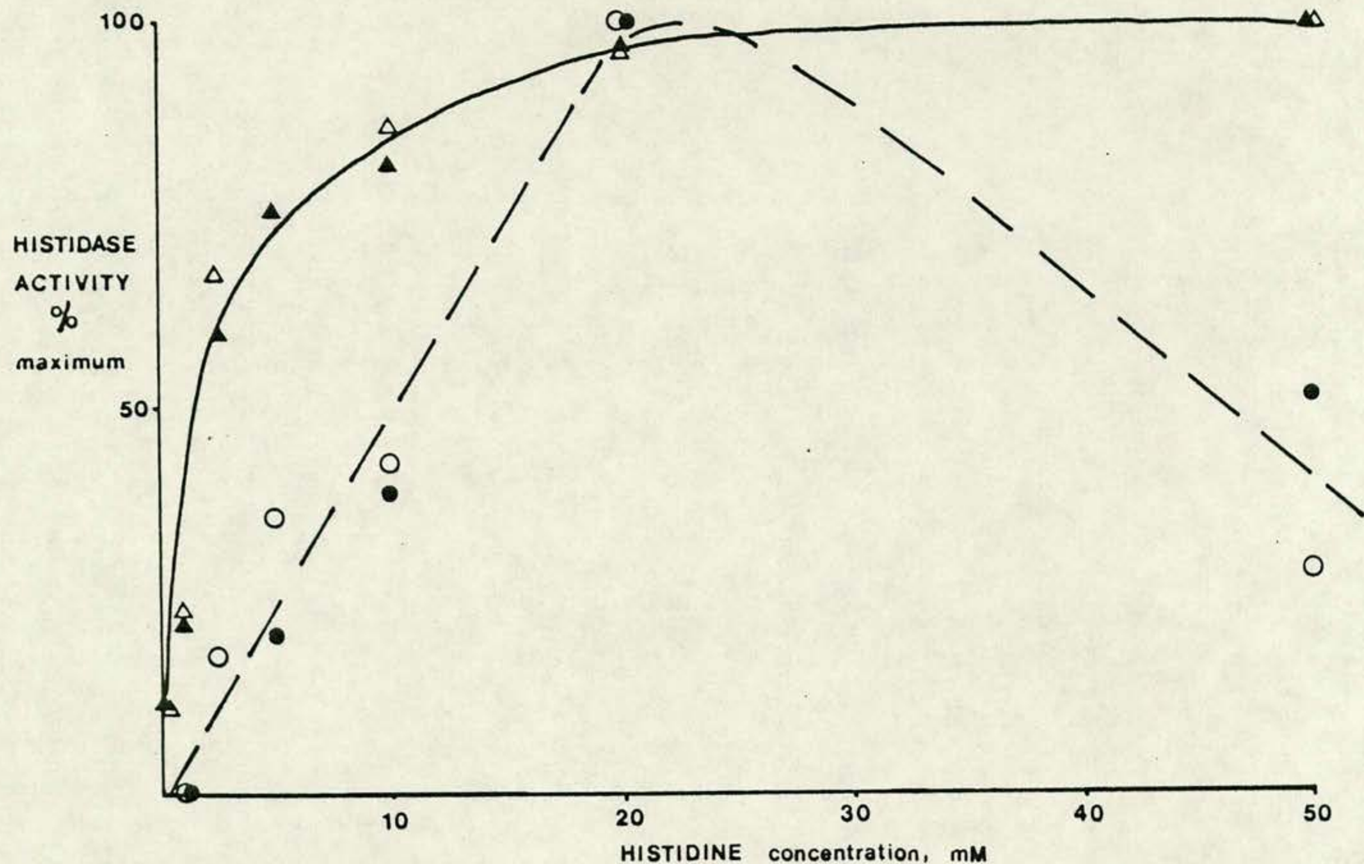


FIG. 5:5 The effect of histidine concentration on histidase activity in dialysed liver extracts from +/+ (▲, continuous assay; △, discontinuous assay) and his/his (●, continuous assay; ○, discontinuous assay) mice.

TABLE 5:10

The effect of histidine concentration on his/his liver histidase

(1) after desalting by fel filtration on Sephadex G25,

(2) after partial purification by protamine sulphate precipitation (SN-2 fraction).

	Genotype	Fraction	Histidine Concentration mM	Histidase activity % maximum
(1)	his/his	60,000g supernatant, desalted on Sephadex G25	3	73·5
			6	93·0
			18	100·0
			30	61·3
			60	52·3
(2)	his/his	SN-2	0·02	26·0
			9·02	95·9
			15·02	94·5
			30·02	100·0
			36·02	90·4
			45·02	45·2
			60·02	24·7

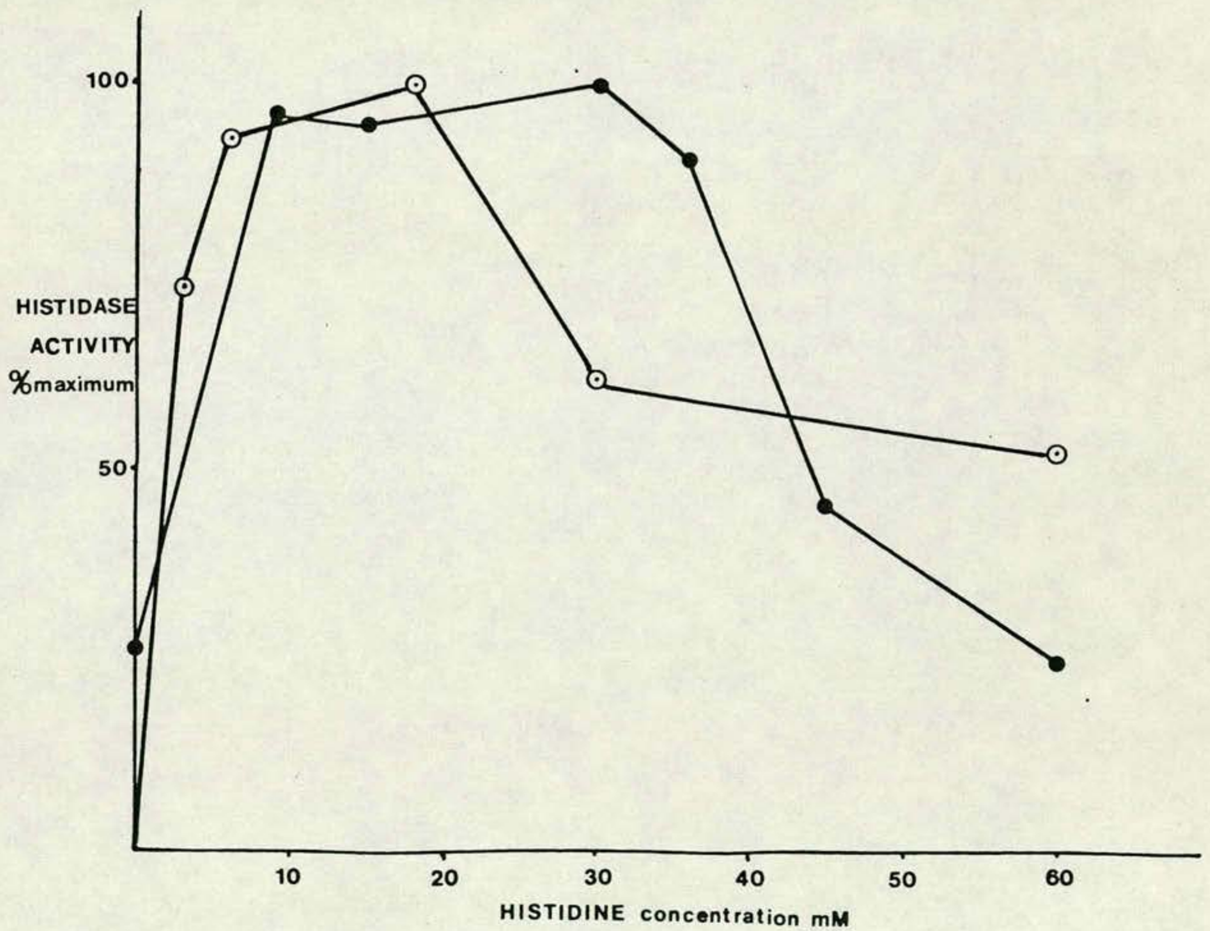


FIG. 5:6 The effect of histidine concentration on his/his liver histidase (A) after desalting by gel filtration on Sephadex G-25 (⊙), (B) partially purified by protamine sulphate precipitation (SN-2 fraction) (●).

The kinetics of the histidase response to changes in substrate concentration were examined further in crude his/his extracts desalted by gel filtration on Sephadex G-25. This method allowed the enzyme to be assayed on the day of extraction, so reducing the likelihood of changes in the enzyme due to ageing. The results are shown in Table 5:10(1) and Figure 5:6A. The affinity for histidine appears to be higher than observed in dialysed extracts and saturation starts to occur in the region 10 - 20 mM histidine. The Km for histidine therefore appears to be in the region of 2 mM. Again the activity falls off at histidine concentrations above 20 mM in a manner suggesting substrate inhibition.

The effect of substrate concentration on mutant histidase activity was then determined after partial purification by protamine sulphate precipitation. The initial experiment was carried out on SN-2 extracts from his/his livers. The endogenous histidine concentration of SN-2 extracts was found by amino acid analysis to be relatively low at 0·29 μmol/ml (cf. 60,000g supernatant from +/+ mice with a histidine concentration of 0·22 μmol/ml). The effect of histidine concentration on activity was therefore examined directly in SN-2 extracts without further desalting. The results are shown in Table 5:10(2) and Figure 5:6B. A similar kinetic response to that seen in crude his/his extracts is found. Unfortunately the lack of activity readings between 0·02 mM and 9 mM histidine make estimation of the Km unreliable. The fall in activity with increasing histidine concentration occurs rather later (> 30 mM) than seen in the gel filtration experiments.

The activity of SN-2 histidase in the absence of added histidine was found to be measurable (about 20% of maximum) presumably as a result of the endogenous histidine concentration of SN-2 extracts (ca. 0·02 mM in the final reaction mixture). In an attempt to reduce the endogenous histidine altogether, the SN-2 extract was dialysed overnight against Tris 0·02M pH 7·4/sucrose 0·25M. The activity on the following morning was reduced to 18% of the pre-dialysis level. There was insufficient activity to repeat the Km experiment. It was not possible to examine the effect of substrate concentration on histidase activity in SN-3 extracts from his/his mice, SN-2 or SN-3 extracts from +/+ mice in the time available.

(ix) Ionic strength.

The effect of ionic strength on histidase activity was examined in an attempt to characterise the residual histidase activity further in his/his mice. This experiment was suggested by the observation that mutant SN-2 histidase activity decreases markedly at high substrate concentrations (see above). It is possible that the activity decreases not because of substrate inhibition but because of an accompanying change in the ionic strength of the assay medium. This was tested by adding increasing amounts of 2M NaCl to the standard assay mixture, keeping everything else constant. The results are shown in Table 5:11 and Figure 5:7. SN-2 histidase activity decreases by a factor of 10 as the salt concentration is increased from 0·01M to 0·1 M in both +/+ and his/his extracts. This again suggests that the residual histidase activity in the mutant is not a product of the major histidase structural locus. Histidase activity with an almost identical sensitivity both to thermal denaturation and to ionic strength is present in mutant and wild-type extracts, suggesting that "unmasking" of a quantitatively minor histidase activity has occurred following loss or reduction of the major activity component by mutation. It was not possible to examine the sensitivity of SN-3 extracts to ionic strength in the time available.

(x) pH optimum.

The pH optimum of liver histidase was determined in crude extracts from +/+ and his/his mice. In the first experiment, the histidine solution was made up in 0·1M sodium phosphate buffer and adjusted to pH's between 7·5 and 10·5. Although sodium phosphate (pKa 7·2) is only an effective buffer in the region of 6·2 - 8·2, histidine itself is an effective buffer at the higher pH's used (pKa_3 9·2). The results are shown in Table 5:12 and Figure 5:8A. Histidase activity in both genotypes shows a broad pH optimum lying between 8·5 and 9·0. Mutant histidase activity falls off more sharply on the alkaline side and less sharply on the acidic side of the optimum compared with wild-type enzyme. The experiment was later repeated using Tris HCl buffer (pKa 8·1) in the pH range 7·8 - 8·9 and diethanolamine HCl buffer (pKa 8·9) in the pH range 9·3 - 10·2. With these buffers the pH optimum occurs at around pH 8·9 (Figure 5:8B). The activity falls off more

TABLE 5:11

Effect of ionic strength on partially purified liver histidase activity of +/+ and his/his mice.

The assay mixture contained 100 µl SN-2 (500-600 µg protein), 0·02M Tris HCl pH 9·0, 0·03M Histidine HCl pH 9·0 and NaCl as indicated.

Genotype	Fraction	NaCl concentration Molar	Histidase activity % maximum
+/+	SN-2	0	78
		0·003	100
		0·037	46
		0·070	23
		0·103	12
his/his	SN-2	0	93
		0·003	100
		0·020	81
		0·037	50
		0·070	9
		0·103	6

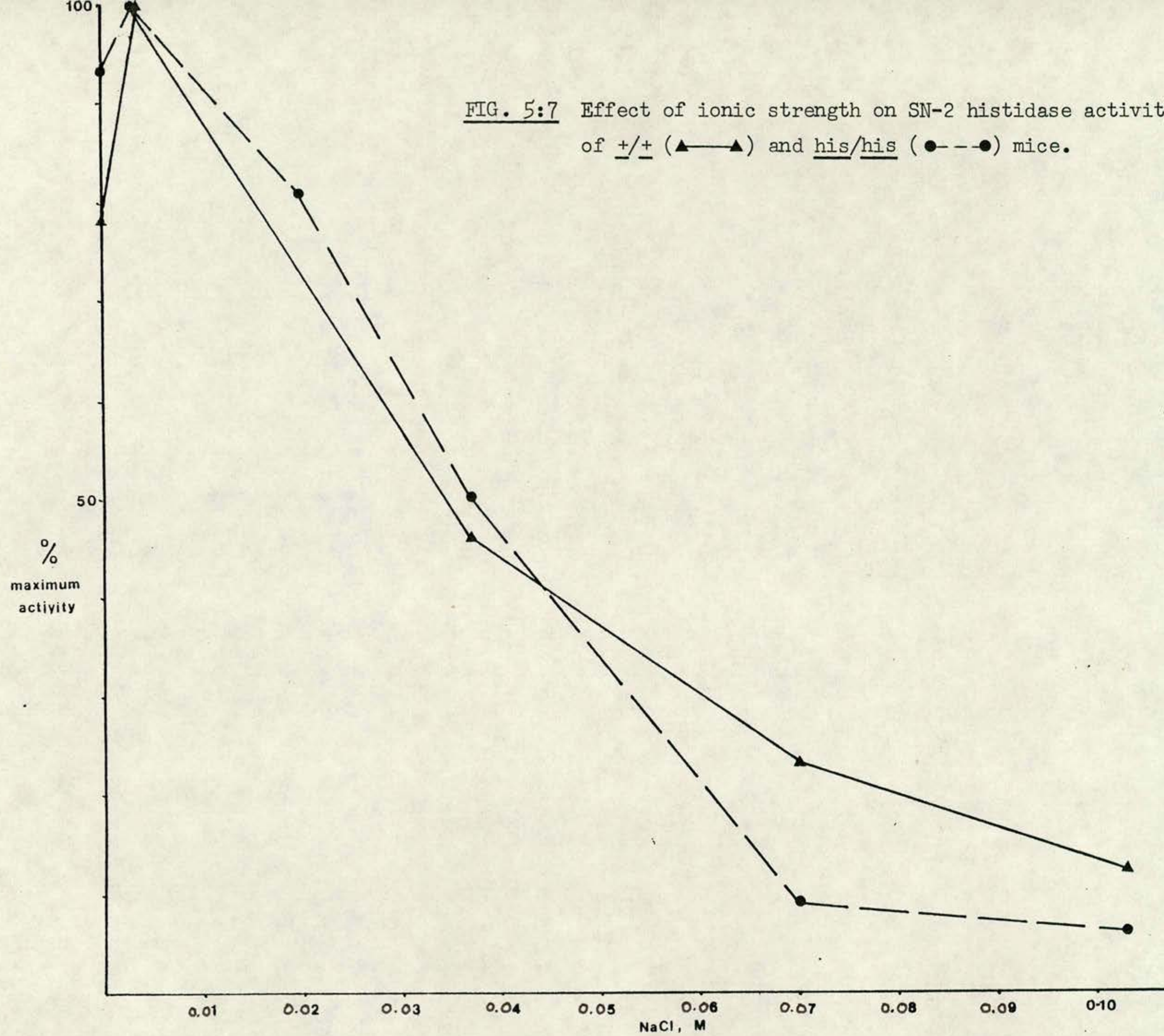


FIG. 5:7 Effect of ionic strength on SN-2 histidase activity of +/+ (▲——▲) and his/his (●- - -●) mice.

TABLE 5:12

The effect of pH on histidase activity in crude liver extracts from +/+ and his/his mice.

Buffer	pH	Histidase Activity % maximum +/+	his/his
phosphate/ histidine	7·5	22·2	71·5
	8·0	68·9	86·2
	8·5	95·6	100·0
	9·0	100·0	97·6
	9·5	85·8	72·2
	10·0	61·8	-
	10·5	46·5	26·8
Tris/ diethanol-amine	7·8	8·7	0
	8·3	60·9	0
	8·5	76·1	46·9
	8·9	100·0	100·0
	9·3	89·1	65·6
	9·7	63·0	37·5
	10·2	47·8	0

A

100

HISTIDASE
ACTIVITY
%maximum

50

7·5 8·0 8·5 9·0 9·5 10·0 10·5

pH

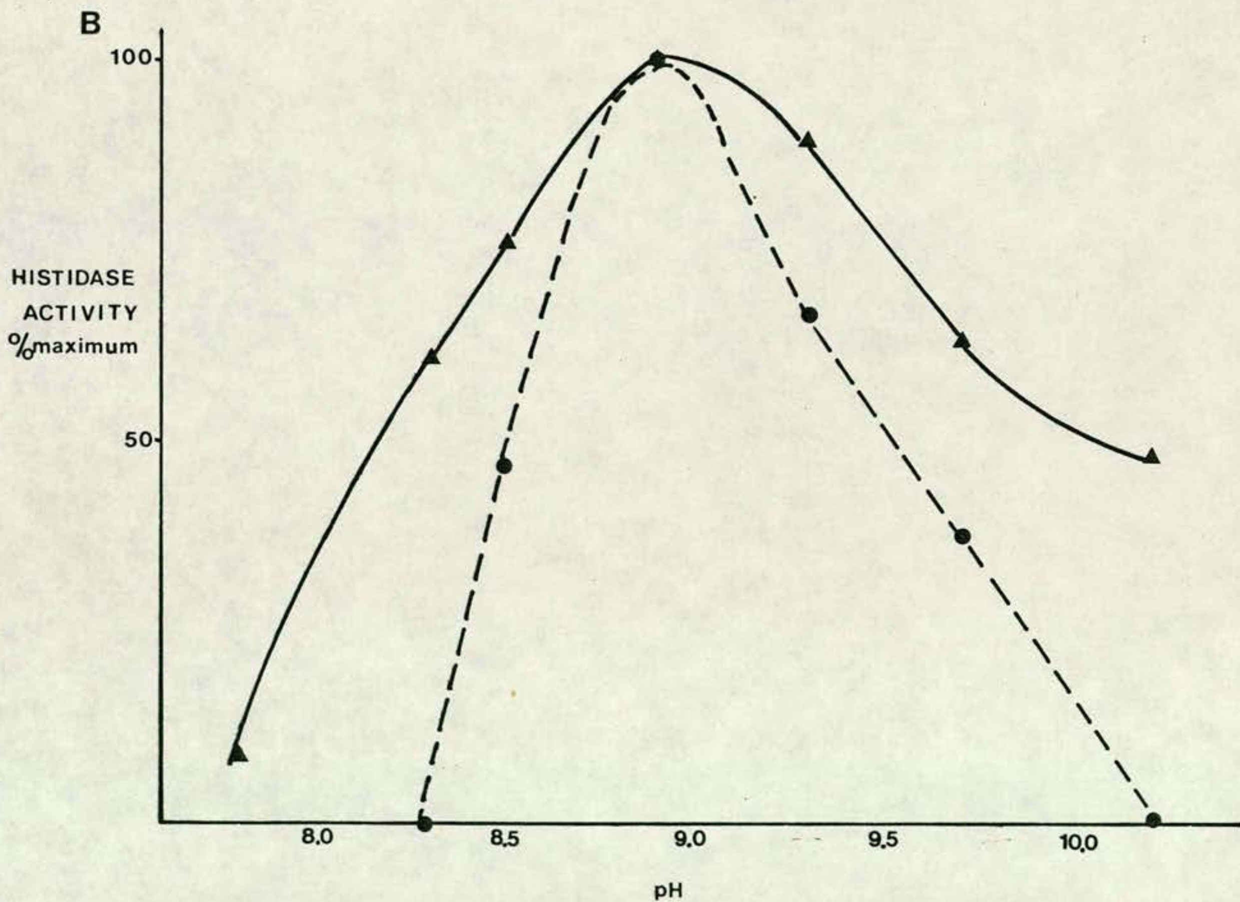


FIG. 5:8 The effect of pH on crude liver histidase activity in +/+ (▲——▲) and his/his (●— —●) mice.

A pH-activity curve in the presence of phosphate/histidine buffer.

B pH-activity curve in the presence of Tris or deithanolamine buffer.

steeply on either side of this pH compared with the phosphate buffer, particularly in his/his samples.

There is therefore no difference in the optimum pH of +/+ and his/his histidase, which lies in the region 8·5 - 9·0, although there are minor differences in the pH profiles of the two genotypes.

(xi) Electrophoretic mobility.

The electrophoretic mobility of crude +/+ and his/his liver histidase was examined as described in Chapter 2 (xvii). In a preliminary experiment, the liver from a +/+ C57BL/6J mouse was extracted as before and 50 µl or 100 µl aliquots of the 60,000g supernatant applied to polyacrylamide gels. These were run until the marker dye was close to the bottom of the tube. The position of the dye was marked and the gels were frozen and cut into 1 mm slices. Groups of three adjacent slices were each put into test tubes containing 0·3 ml Tris HCl 0·025M pH 7·4 on ice. The gels were crushed with a glass rod and left overnight at 4°C. They were then assayed for histidase activity by adding a 0·2 ml aliquot to 2·8 ml of the routine assay mixture. The assay tubes were incubated at 30°C for one hour and the reaction stopped by adding 0·5 ml of ice-cold 20% trichloroacetic acid. The samples were frozen until the absorbance at 277 nm was read. The results are shown in Figure 5:9. Histidase activity is found in corresponding positions on both gels with a mobility of 0·44 relative to the bromophenol blue marker dye. Another +/+ C57BL/6J mouse was sacrificed and the liver extracted and subjected to electrophoresis as before. The whole gel was then repeatedly scanned directly with a Joyce Loebl UV Chromoscan after immersion in assay medium containing histidine, buffer and sucrose to reduce diffraction at the gel-solution interface. A single, strongly UV absorbing band can be identified and increases linearly with time (Figures 5:10-11). The position of this band corresponds to that identified in the previous experiment.

When the 60,000g supernatant from a +/+ Peru mouse is subjected to polyacrylamide gel electrophoresis and assayed for histidase activity with the chromoscan, a single UV absorbing band is again found. The mobility relative to the bromophenol blue (BPB) marker is 0·46. Gels loaded with 20 µl crude extract containing about 600 µg protein, give

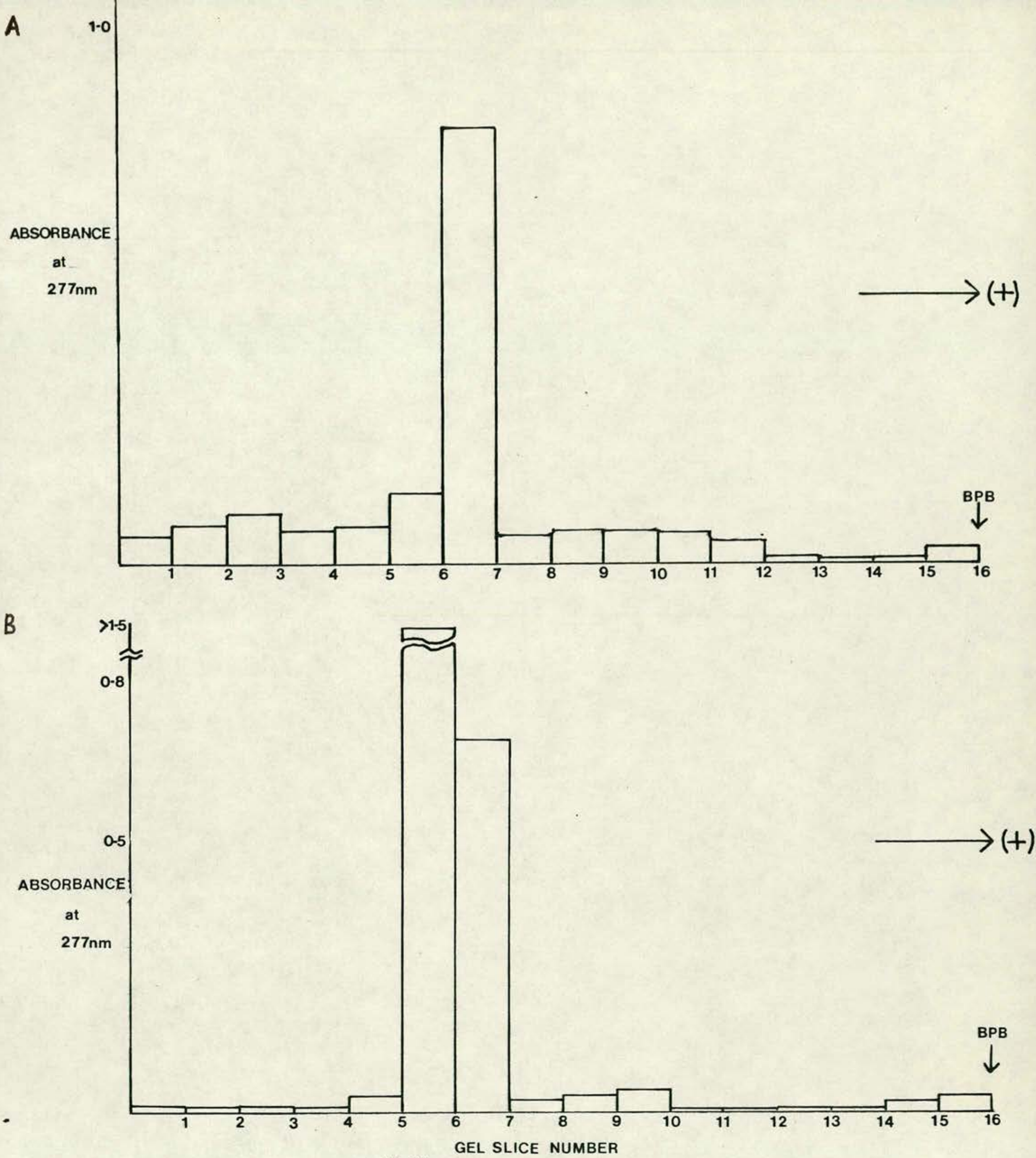


FIG. 5:9 Histidase activity eluted from polyacrylamide gel slices after electrophoresis of (A) 50 µl crude liver extract and (B) 100 µl crude liver extract from a +/+ mouse. The position of the bromophenol blue (BPB) marker dye is shown.

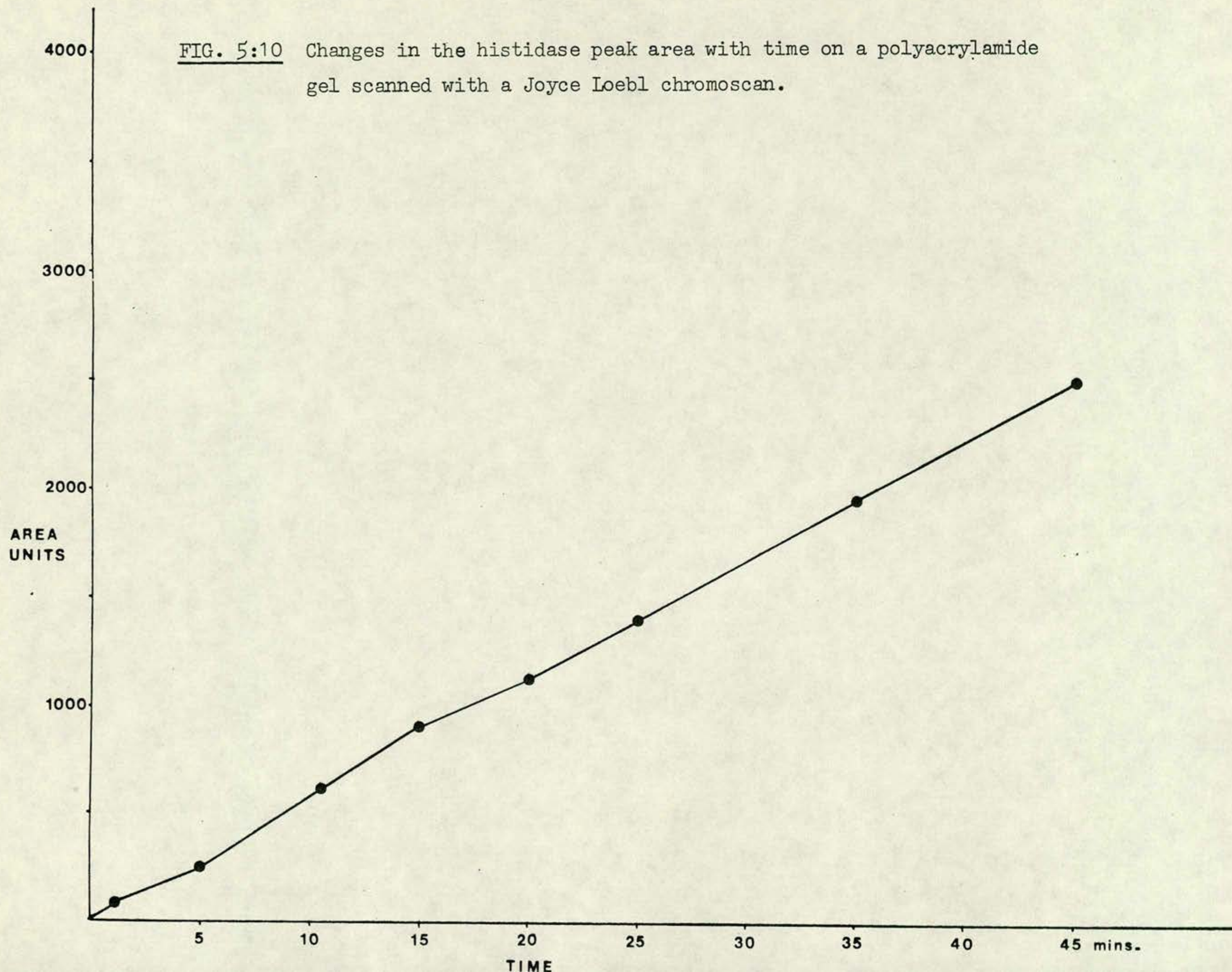
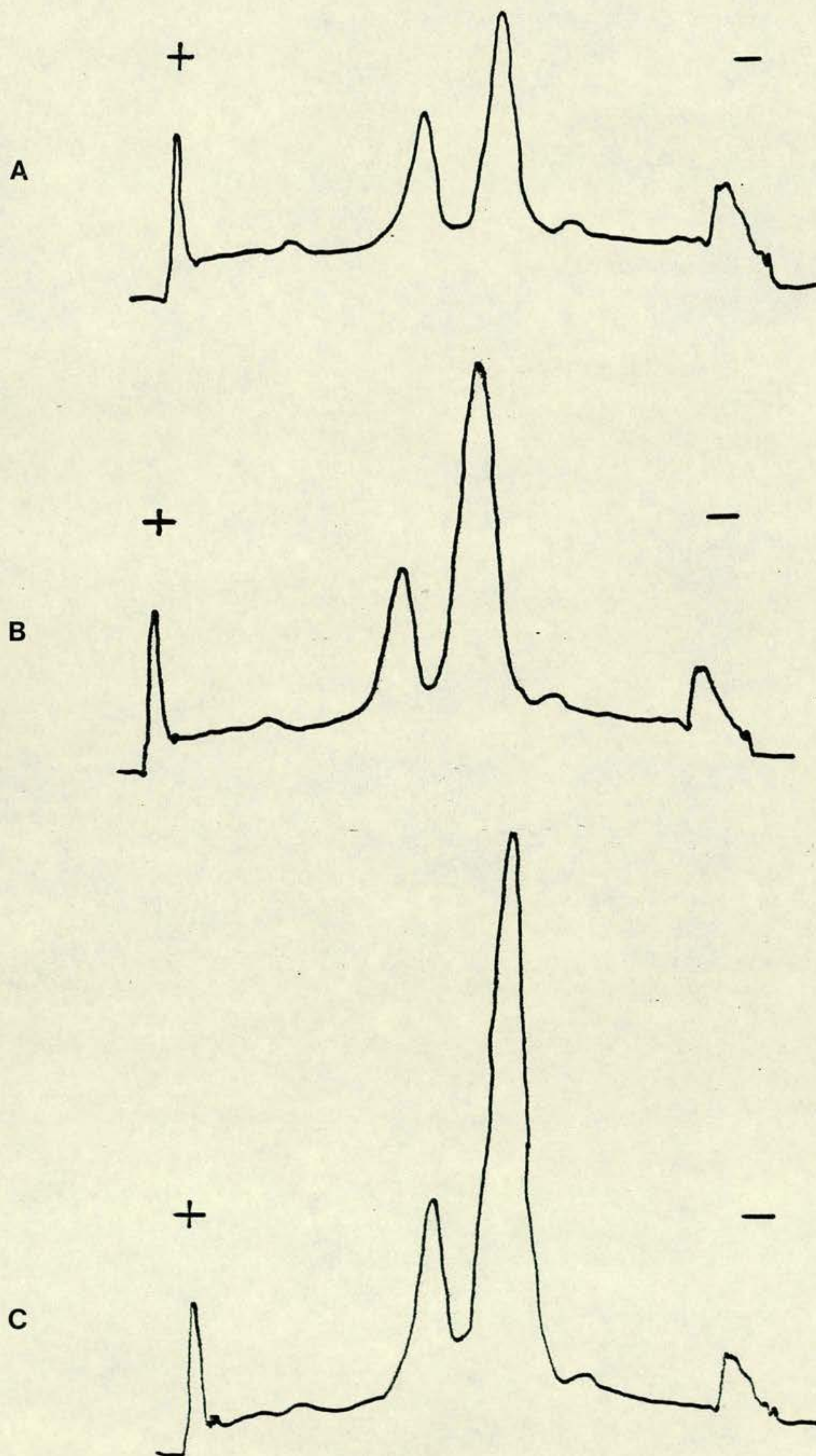


FIG. 5:10 Changes in the histidase peak area with time on a polyacrylamide gel scanned with a Joyce Loebl chromoscan.

FIG. 5:11 Gel scans of crude +/+ liver supernatant (20 µl) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal. (A) - (C) 15, 29 and 47 minutes after addition of histidine to the gel (10 volts sensitivity).



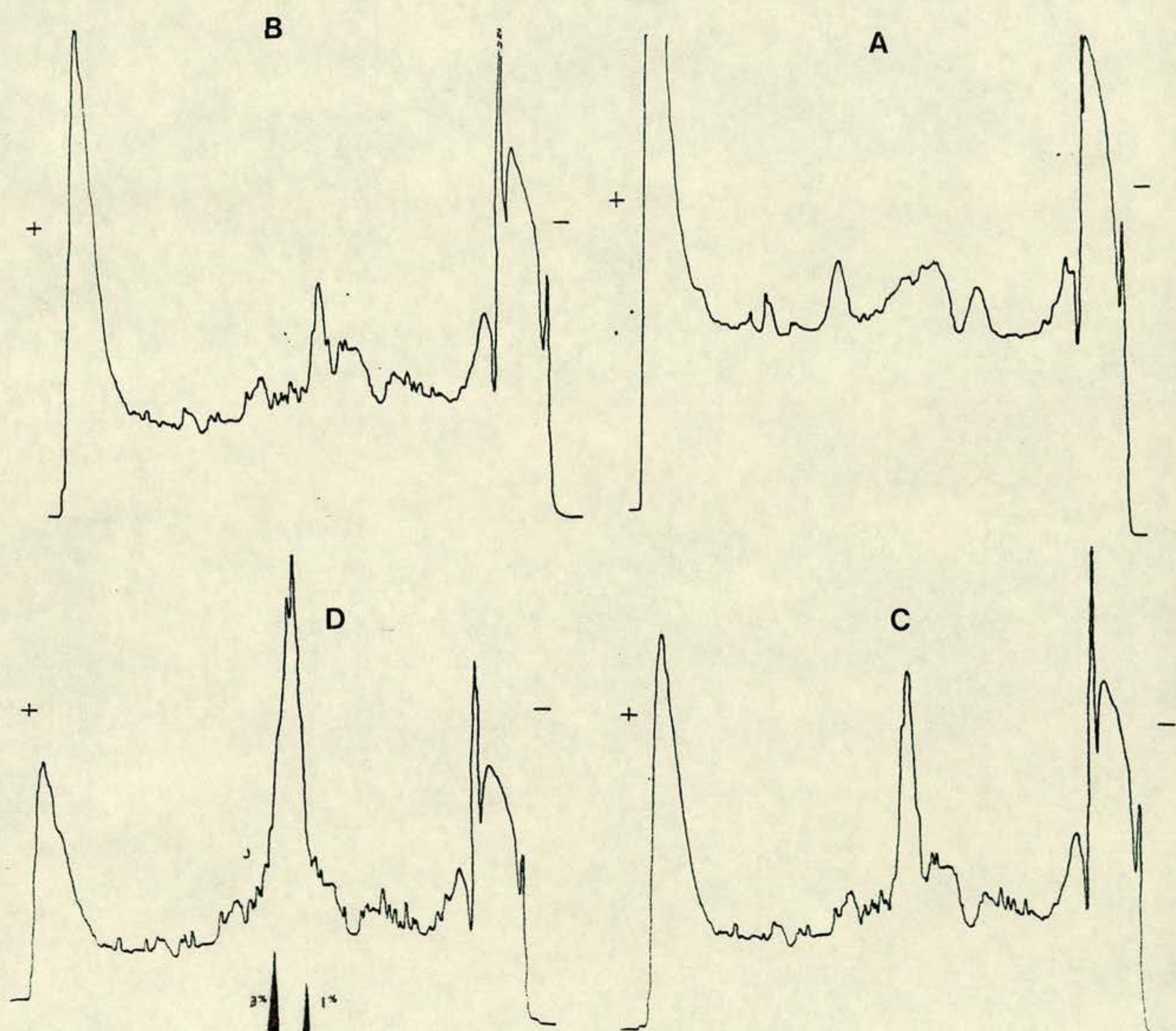
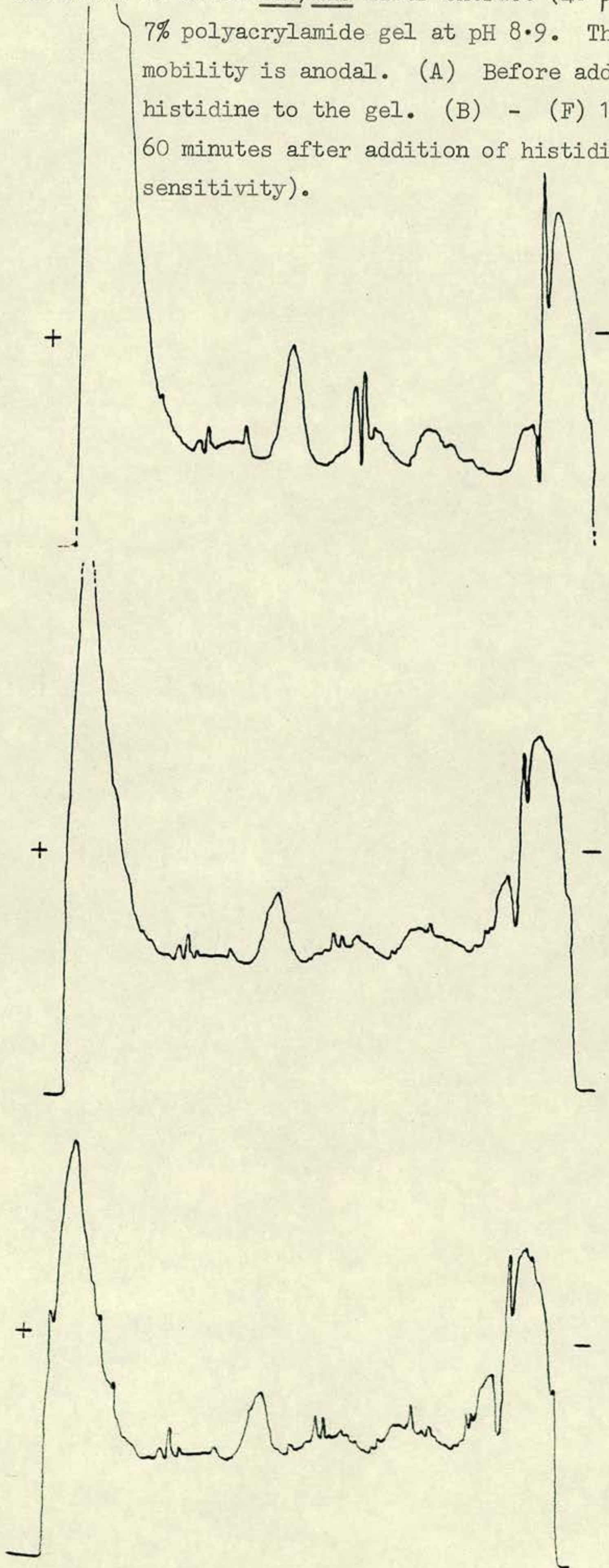


FIG. 5:12 Gel scans of crude +/+ liver extract (20 µl) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal. (A) Before addition of histidine to the gel. (B) - (D) 3, 7 and 23 minutes after addition of histidine (2·5 volts sensitivity). The estimated size of a peak with 1% or 3% of +/+ histidase activity is shown inset in D.

FIG. 5:13 Gel scans of crude his/his liver extract (40 µl) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal. (A) Before addition of histidine to the gel. (B) - (F) 1, 11, 20, 40 and 60 minutes after addition of histidine (2·5 volts sensitivity).



D
+
−

E
+
−

F
+
−

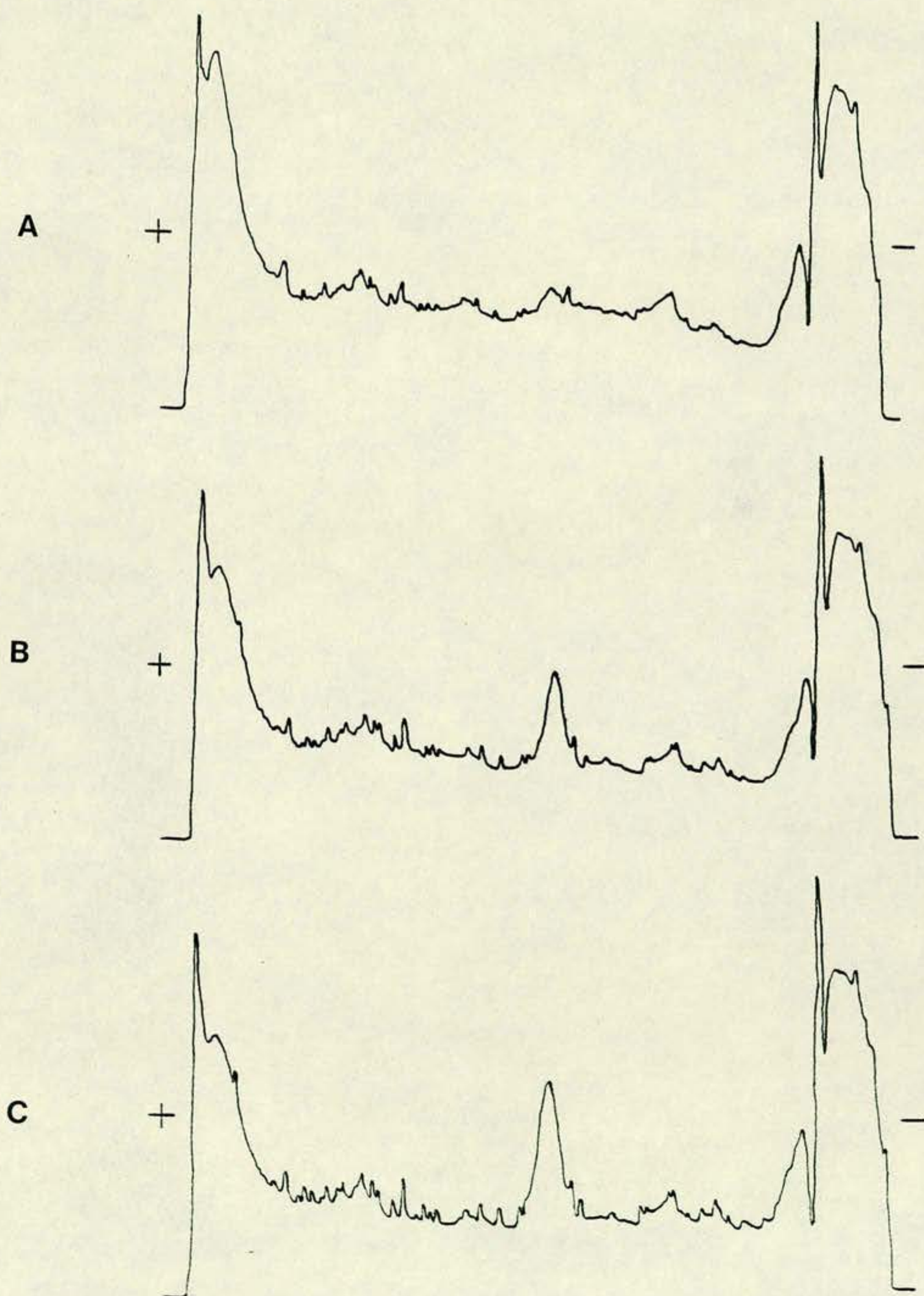
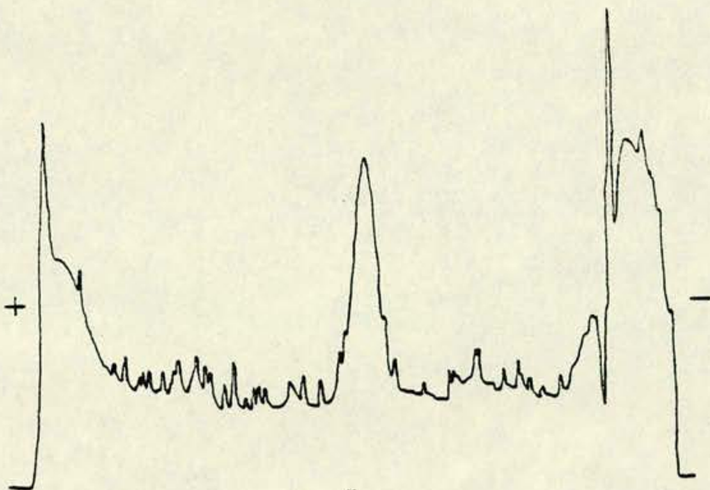
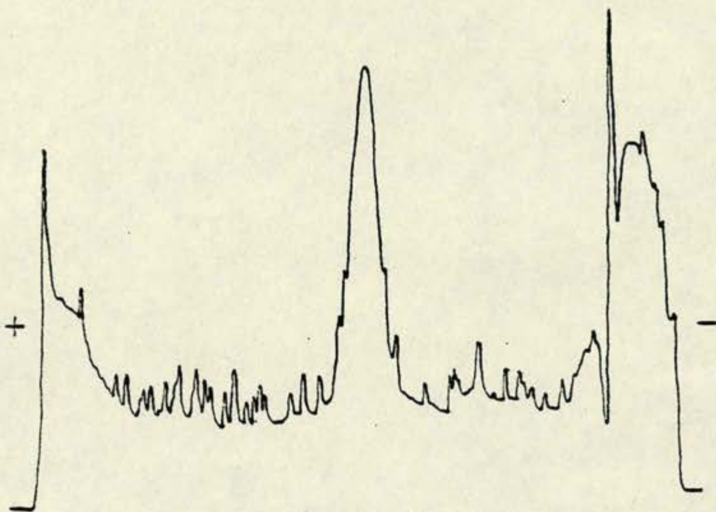


FIG. 5:14 Gel scans of crude +/his liver extract (20 µl) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal. (A) - (F) 1, 4, 8, 20, 30 and 45 minutes after addition of histidine to the gel (2·5 volts sensitivity).

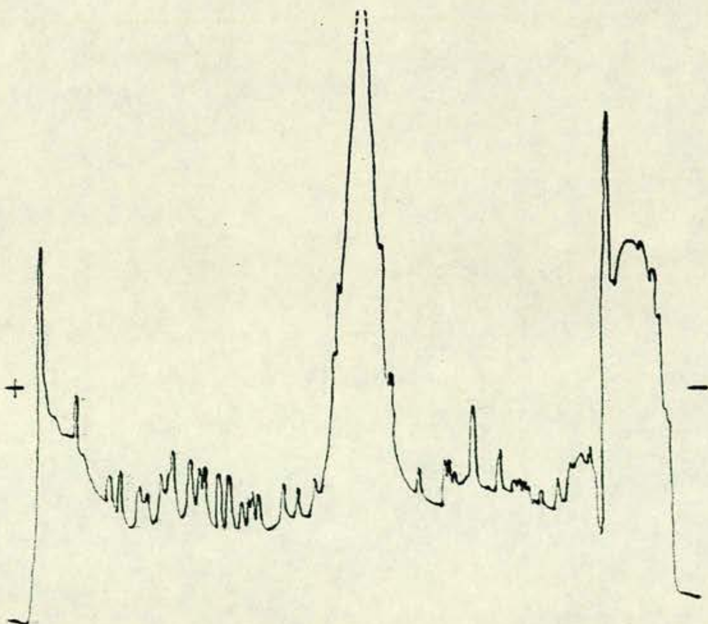
D



E



F



a peak area after 20 - 30 minutes which is large enough for even 3 - 5% of this activity to be distinguishable from background, (Figure 5:12). When 20 or 40 µl of mutant liver supernatant is applied under identical conditions, there is no clear evidence of a histidase peak at any point in the gel (Figure 5:13). The identification of small UV absorbing peaks corresponding to the mutant activity is complicated by the fact that increases in the size of all peaks occurs to a limited extent with increasing assay time (see Figures 5:13-14), probably due to diffusion of proteins within the gel. Loading larger amounts of enzyme extract leads to loss of resolution. Electrophoresis was carried out at 4°C using a very low current (1 - 2 mA per tube) so that sample heating was minimised (Davis, 1964) but in view of the lability of his/his histidase some loss of activity during electrophoresis could not be ruled out.

Samples containing 20 µl of liver extract from +/his mice were run under the same conditions (Figure 5:14). A single UV absorbing peak is found with the same mobility relative to BPB as found in +/+ extracts, but no additional peaks. Since mammalian histidase is likely to be a tetrameric protein, the existence of a mutant structural gene could lead to formation of hybrid proteins in heterozygotes (Harris, 1975). Five distinct tetrameric enzymes might therefore be expected in a heterozygote if the mutant and wild-type polypeptides were able to associate into a tetrametic structure. Some, or all, of the three hybrid forms could retain histidase activity and all five could be electrophoretically distinguishable. This possibility is ruled out by the above finding with +/his extract, since no additional UV absorbing peaks are distinguishable. Mixing of +/+ and his/his extracts might give rise to hybrid enzyme formation *in vitro*, even if this does not occur *in vivo*. 10 µl of +/+ extract was therefore mixed with 10 µl of his/his extract prior to electrophoresis and then run in parallel with +/+ and his/his samples. A single histidase peak can be identified with identical mobility to that in +/+ extracts but no additional peaks are present.

The problem of determining the significance of small UV absorbing bands led to the attempts to purify mutant histidase. It seemed to be impossible to distinguish a band with low histidase activity from

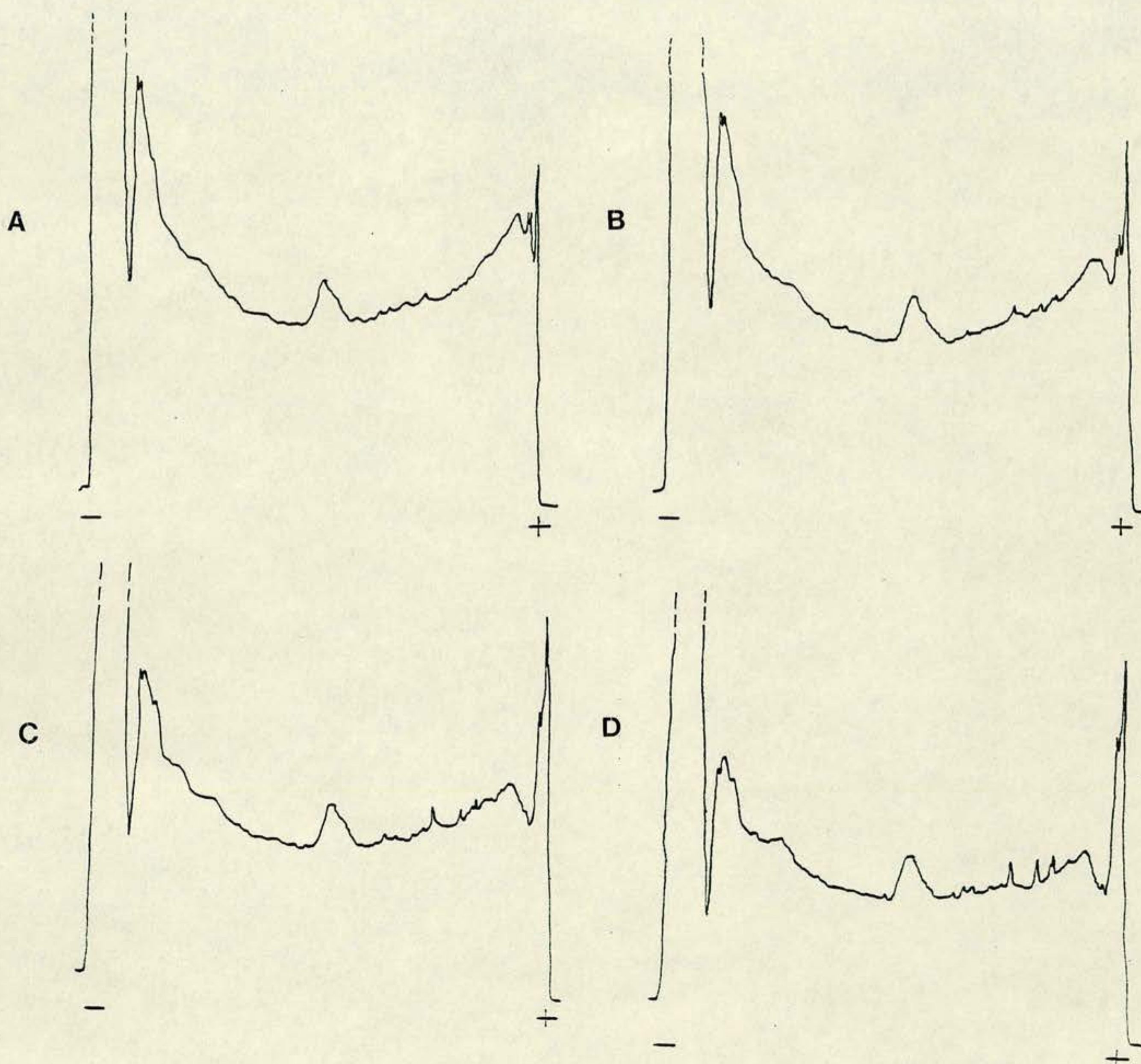


FIG. 5:15 Gel scans of partially purified his/his liver histidase (60 µl : SN-2 fraction) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal. (A) - (D) 6, 16, 30 and 52 minutes after addition of histidase to the gel (2·5 volts sensitivity).

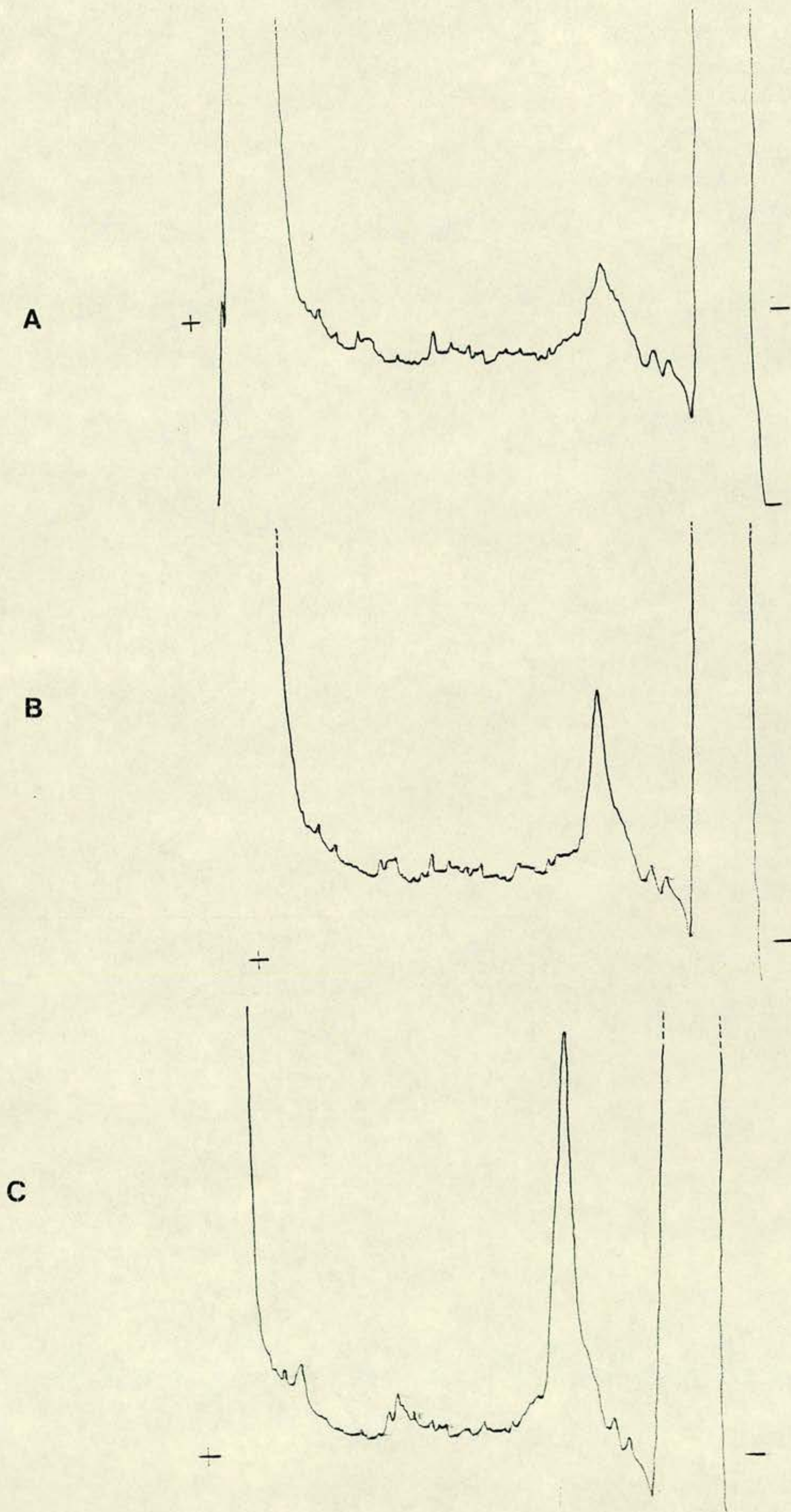


FIG. 5:16 Gel scans of partially purified +/+ liver extract (10 μl : SN-3) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal (A) - (C) 1·5, 3·5 and 7·75 minutes after addition of histidine to the gel (2·5 volts sensitivity).

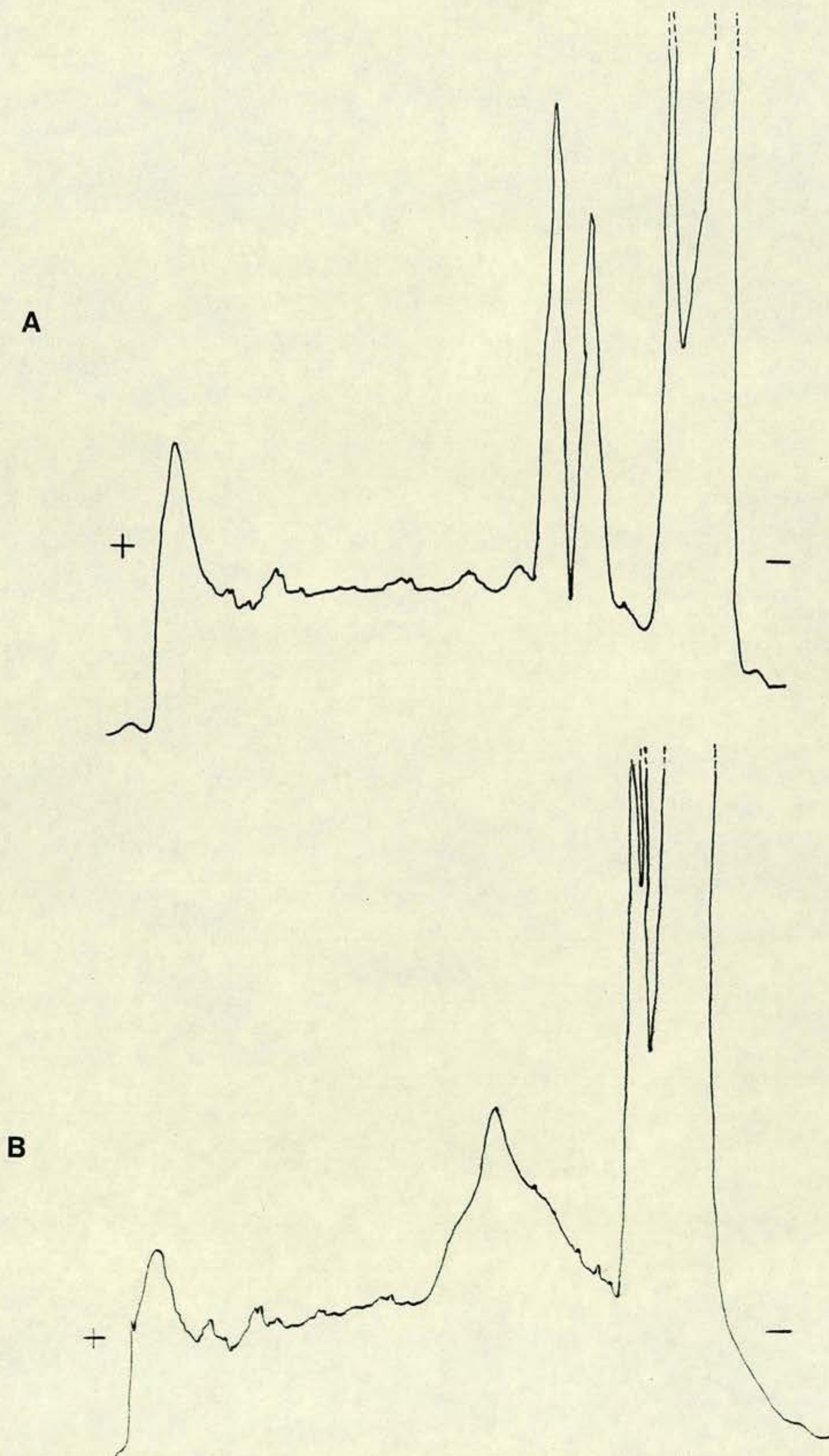


FIG. 5:17 Gel scans of partially purified +/+ liver extract (50 µl : SN-3 fraction) run on a 7% polyacrylamide gel at pH 8·9. The direction of mobility is anodal (A) - (B) 10 and 20 minutes after addition of histidine to the gel (10 volts sensitivity).

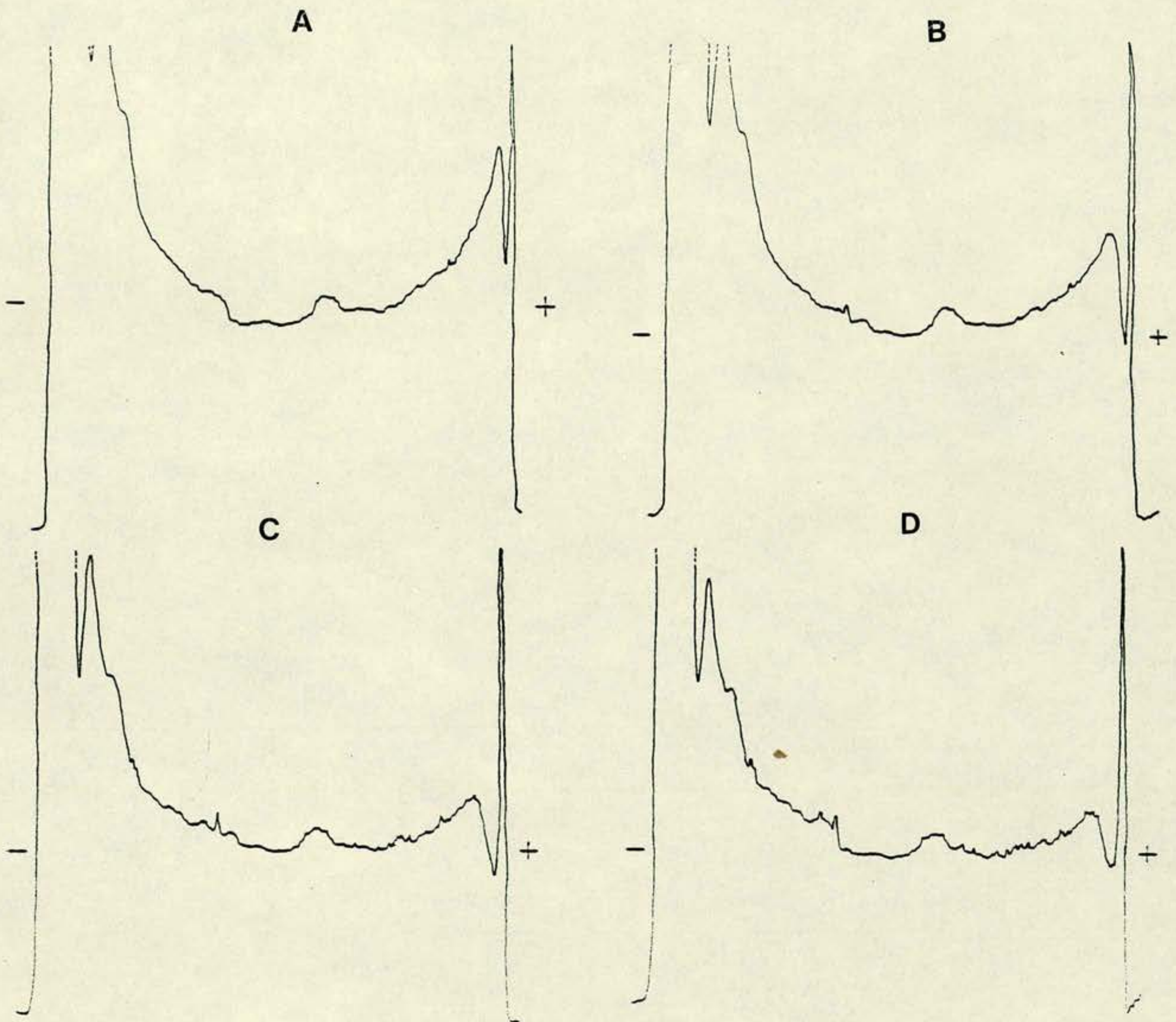


FIG. 5:18 Gel scans of his/his liver extract (20 µl : SN-3 fraction) run on an 8% polyacrylamide gel. The direction of mobility is anodal. (A) - (D) 2, 14, 32 and 44 minutes after addition of histidine to the gel (2·5 volts sensitivity).

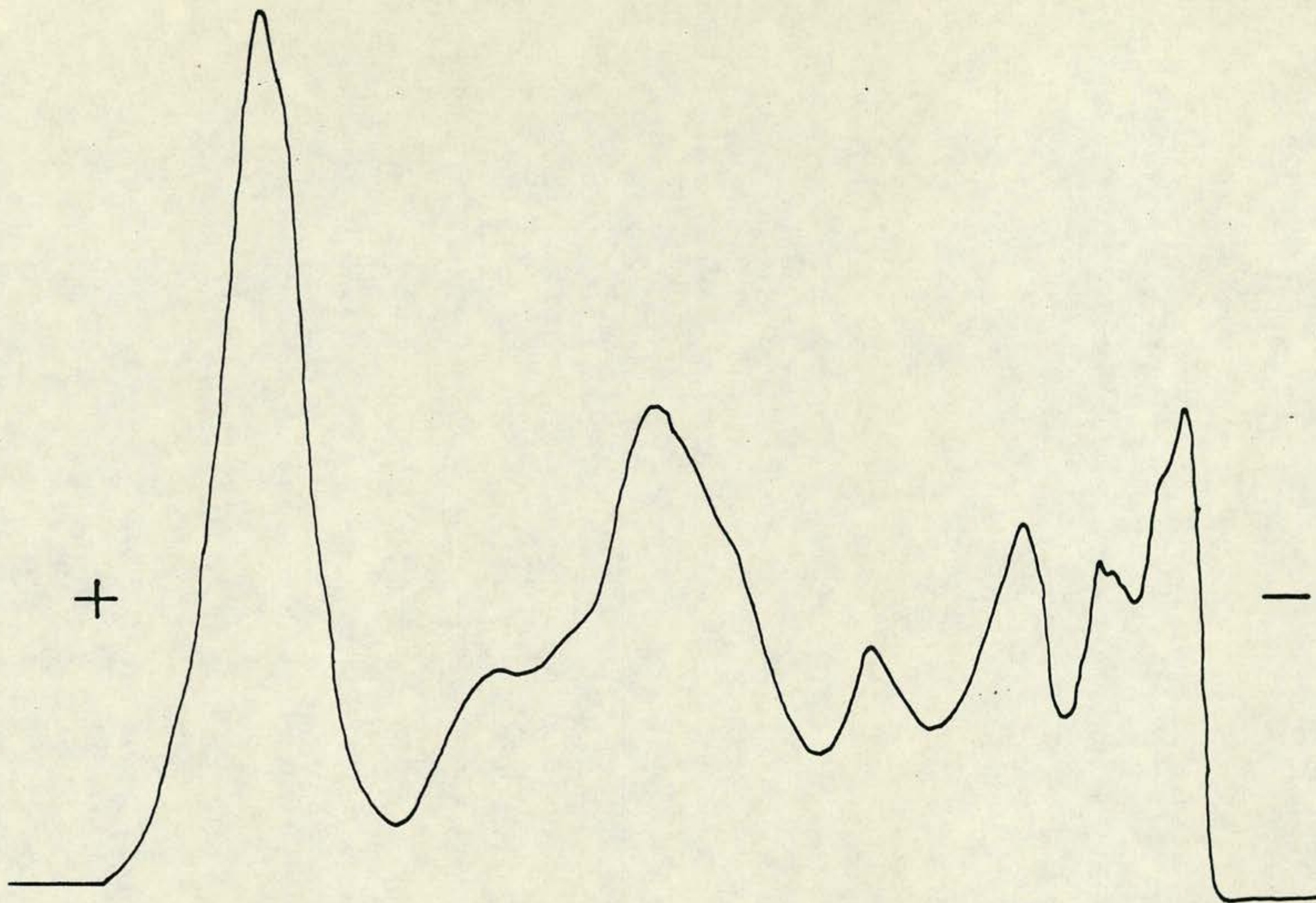


FIG. 5:19 Densitometer tracing of crude *his/his* liver extract (20 ul) run on a 7% polyacrylamide gel, fixed and stained with Amido Black. The anodal peak is probably due to retained bromophenol blue marker.

one in which diffusion of protein was causing the peak to change. Partially purified histidase extracts were therefore subjected to polyacrylamide gel electrophoresis after protamine sulphate precipitation. SN-2 extracts containing 120 - 360 μg protein in a 20 - 60 μl volume were loaded on the gels and run as before. SN-3 extracts containing 170 - 340 μg protein in a 10 - 20 μl volume were also run. When samples of the SN-2 fraction from his/his mice are subjected to electrophoresis and assayed by UV scanning, there is no clearcut histidase band that increases linearly with time, although three peaks running close together in an anodal position are suggestive (Figure 5:15). The latter peaks could equally have been due to diffusion of protein as seen in Figures 5:13-14 after prolonged incubations. SN-2 extracts from +/+ mice were not examined electrophoretically because of time limitations. When SN-3 fractions from +/+ mice are run and the gels assayed as before, a histidase peak is clearly seen, with a mobility of 0·24 relative to BPB (Figure 5:16). In one experiment, two histidase peaks can be seen with mobilities of 0·24 and 0·16 respectively (Figure 5:17). The slower migrating band probably results from aggregation of the enzyme, as observed by others (Okamura et al., 1974). A sample of the SN-3 fraction from his/his mice was also subjected to electrophoresis. The gel was assayed by UV-scanning and no histidase band could be detected in a 45 minute incubation (Figure 5:18).

Polyacrylamide gels containing samples of crude liver extract were stained with Amido Black and destained as described in Chapter 2 (xvii). A microdensitometer tracing from such a stained gel is shown in Figure 5:19. The gels containing partially purified histidase were not stained for protein.

(xii) Discussion.

The difference in liver histidase activity between wild-type and histidinaemic mice is not due to enzyme activation or inhibition. This is shown by the mixing, dialysis and gel filtration experiments. For example, a deficiency of metal cofactor (perhaps due to loss of the binding protein) could cause a histidase deficiency, but activity should be restored by mixing the two extracts, assuming the presence of free metal in the wild-type. Also, codominant expression of histidase

activity in the heterozygote is unlikely to occur if the his mutant is due to loss of an activator or formation of an inhibitor product. The above conclusion is supported by experiments carried out during the course of the thesis in collaboration with Professor S.M. Arfin and coworkers (unpublished). These workers had previously raised monospecific antibodies to 70% pure preparations of mouse liver histidase, which they used to immunoprecipitate liver histidase in +/+ and his/his liver extracts. The mouse livers used in these experiments were frozen immediately after dissection in Edinburgh and sent by air in dry ice to the Department of Biological Chemistry, California College of Medicine. Two experiments were carried out to determine the amount of cross-reacting material (CRM) in the two genotypes.

In a constant antigen experiment, three volumes of +/+ liver extract, one volume of +/+ extract plus two volumes of his/his extract, and one volume of +/+ extract plus two volumes of mouse kidney extract (i.e. a tissue lacking histidase activity) were each titrated with anti-histidase serum. The unprecipitated histidase activity was then measured using their standard assay procedure. If the his/his extract contained the same amount of CRM as the +/+ extract, the end point of the titration of one volume of +/+ plus two volumes of his/his extract would be the same as that for three volumes of +/+ extract. If there was no CRM in his/his extracts, the end point would equal that of one volume +/+ extract plus two volumes of kidney extract. Any intermediate end point would provide an estimate of the amount of CRM present. The result was clear-cut, showing that there is no CRM in his/his extracts. In the second experiment, a constant amount of anti-histidase antibody was pre-incubated for 30 minutes at 37°C with his/his liver or mouse kidney extract, then increasing amounts of +/+ extract were added. Any his/his CRM present would therefore use up antibody leading to the appearance of +/+ histidase activity at lower antigen concentrations than in the presence of the kidney extract. The result was that +/+ histidase activity appeared at the same point whether the antibody had been pre-incubated with his/his extract, kidney extract or nothing at all. Again, mutant histidase appears to be CRM-negative.

Arfin and coworkers concluded that if there is any CRM in his/his livers it must be less than 10% of that in wild-type. The sensitivity of the techniques could not exclude the possibility of 5% CRM. However they were unable to detect any histidase activity in the frozen his/his livers received from Edinburgh so that the low CRM could have resulted from damage to the enzyme sustained in transit. It had previously been found that mutant histidase activity is retained (although reduced) after rapid freezing and storage in liquid nitrogen. Since antigenicity is thought to reside in the three-dimensional structure, enzymes that are denatured or dissociated into subunits may react poorly or not at all with antisera (Gelehrter et al., 1970; Celada and Strom, 1972; Brock, 1978). This possibility can only be excluded by the use of liver extracts taken directly from live his/his mice. It would also be useful to compare the amount of CRM in all three genotypes, +/+, +/his and his/his, since there is a report of CRM-negative homozygotes with sucrase-isomaltase deficiency, while heterozygotes with 50% of normal enzyme activity have virtually 100% CRM (Gray et al., 1976). If confirmed, the finding of absent CRM in mutants suggests either that the enzyme is so structurally altered by mutation that it is no longer antigenically active or more likely (see ii, above) that there is no (or less than 10%) histidase present at all. The latter could result from rapid degradation due to molecular instability (structural variant), increased enzymic degradation (processing variant) or alternatively from reduced synthesis of normal (regulatory variant) or altered (structural variant) enzyme. The above results do tend to exclude the possibility of activators or inhibitors as causes of the difference in histidase activity.

The difference in heat stability between crude +/+ and his/his histidase is evidence against a regulatory gene mutation. The +/+ enzyme has a $t_{\frac{1}{2}}$ of 16 minutes at 70°C compared with 19·5 minutes at 40°C in his/his extracts. This unambiguous difference suggests three possibilities; (1) mutation at the structural locus for histidase: (2) mutation at a histidase processing locus: (3) "unmasking" of activity in a distinct histidase isozyme, due to mutational loss of of the major enzyme species.

The first possibility, mutation at the histidase structural locus, has not been confirmed by the electrophoretic studies since no histidase electrophoretic band could be clearly identified either in crude or partially purified extracts of his/his liver. The unstable mutant enzyme is presumably denatured during electrophoresis or during the prolonged gel assays. The sensitivity of the gel assays should have been sufficient to detect mutant activity in partially purified extracts since the specific activity in SN-2 extracts was 1 - 2 nmol/min/mg protein compared with about 3 nmol/min/mg protein in crude +/+ extracts. The latter were easily identified on gels using an identical assay. For example, in the gel shown in Figure 5:15, 0·5 units of mutant SN-2 histidase (in 408 µg protein) were loaded on to the gel compared with 2·2 units (in 660 µg protein) of crude +/+ histidase, shown in Figure 5:12. The mutant histidase peak in Figure 5:15 should therefore have been the same size as that in Figure 5:12B by 13 minutes, while in fact there was no major peaks visible even after 30 minutes (Figure 5:15C). These experiments again exclude the possibility of a reduced amount of normal enzyme as would be expected with a regulatory gene mutation. A regulatory mutant could however lead to complete loss of the major enzyme species with "unmasking" of a previously unrecognized form of histidase. The absence of hybrid enzyme bands with histidase activity in heterozygotes or mixed +/+ and his/his liver extracts can be interpreted in a number of ways. First, a structural locus variant could be so altered that it is unable to polymerise with the normal subunits. Second, the hybrid enzymes could be unstable under the electrophoretic or assay conditions, so that the activity is lost. Third, the his mutation could represent a null allele which produces no enzyme product, perhaps due to a deletion or frameshift mutation. This last possibility is supported by the finding that the mutant is CRM-negative. Fourth, it has not been shown conclusively that the native mammalian enzyme exists in multimeric form [see Chapter 1 (iv)].

A mutation at the structural locus for histidase might lead to a change in the response to activators, cofactors or inhibitors. However neither a sulphydryl reagent nor a divalent metal ion at 10^{-3}M has any effect on histidase activity in crude liver extracts of either

genotype. Nitromethane is a relatively specific, irreversible inhibitor of histidase. Intraperitoneal injection of nitromethane reduces +/+ histidase activity to 7% of control values while having no effect on his/his histidase. Since the twenty-fold higher histidine concentration could competitively inhibit nitromethane inactivation in the mutant, higher concentrations of nitromethane were injected, still without effect. In vitro, nitromethane inhibits his/his liver histidase by 90% but this is unchanged by the presence of 30 mM histidine, which shows that inhibition is occurring by some means other than covalent binding to an active site residue. These experiments therefore do not distinguish between a structurally altered histidase and another enzyme with low histidase activity. A histidase structural variant would probably be inhibited by nitromethane even if with altered kinetics. An enzyme able to convert histidine to urocanic acid at a low rate due to cross-specificity is less likely to be inhibited by nitromethane.

The kinetics of the histidase response to substrate is strikingly different in +/+ and his/his mice (Figures 5:5-6). The marked reduction in activity occurring at high substrate concentrations is only found in the mutant. The subsequent finding that mutant activity declines with increasing ionic strength could explain this result, so the kinetics should be re-examined under conditions of low or constant ionic strength. An alternative explanation would be that substrate inhibition is occurring.

The pH optimum of crude liver histidase activity has been compared in +/+ and his/his mice. An identical pH optimum in the region 8·5 - 9·0 is found using two buffer systems, although there are differences in the activity changes on either side of the optimum (Figure 5:8). This finding does not help to clarify the molecular nature of the residual histidase activity.

Two observations made in partially purified liver histidase extracts suggest that the third possibility mentioned above may be correct, namely that the residual mutant activity is due to "unmasking" of another enzyme by a null allele at the major histidase locus. First, when the heat stability of the low ionic strength (SN-2) extracts from +/+ and his/his mice are compared, the activity in both extracts shows

an identical lability to heating at 40°C ($t_{\frac{1}{2}}$ = 9 minutes) (Figure 5:2). The major histidase activity of +/+ livers is recovered in the high ionic strength (SN-3) extract which is heat stable at 40°C (Figure 5:2). The presence of two enzyme species with histidase activity in +/+ mice is supported by the heat inactivation studies carried out in crude liver extracts at 70°C (Figure 5:1A). A bi-phasic rate of inactivation was found, with a faster rate during the first 15 minutes than between 15 and 60 minutes of pre-incubation at 70°C. This could be due to rapid loss of the heat labile component followed by a slow decay of the major species. In contrast, the mutant enzyme decayed in accordance with a single enzyme species (Figure 5:1B).

The second observation suggesting the unmasking of a minor histidase species, is the effect of ionic strength on activity. When the activity of SN-2 extracts from +/+ and his/his livers is compared in the presence of increasing salt concentrations, both sources of enzyme show the same marked loss of activity with increasing salt concentration (Figure 5:7). The nature of this inactivation has not been investigated. It could be due to the effect of ionic environment on enzyme conformation leading to impaired catalysis, dissociation into subunits or denaturation. Alternatively it could be due simply to salting-out the enzyme from solution.

Further support for this interpretation of the findings comes from the study of developmental changes in skin histidase activity [Chapter 3 (ii)]. Although +/+ skin histidase activity is markedly higher than the mutant from birth to ten days, by fifteen days the activity is identical in the two genotypes. This would suggest either that the his/his enzyme is associated with a loss of inductive responsiveness but normal catalytic function or that only one of two histidase isozymes is lost in the mutant. The 15-day +/+ activity would, in the latter case, correspond to the minor activity component and show the same properties as the mutant enzyme.

Although further confirmation is required with samples taken directly from intact mice. the finding of absent or less than 10% CRM in his/his livers is also compatible with the unmasking of a

distinct enzyme with histidase activity. The evidence relating to the absent (or diminished) major histidase activity does not distinguish between a null allele at the histidase structural locus or at a regulatory locus.

An alternative, more complex explanation for the findings discussed above can be made. There is good evidence from the study of bacterial and mammalian histidase that the enzyme can exist in different states of aggregation (Klee, 1970; Okamura et al., 1974). While the native Pseudomonas enzyme is thought to be a tetramer made up of identical subunits, these tend to aggregate into polymers which are all enzymatically active. The relative stability of these different molecular forms to denaturation has not been studied, but Roth and Hug (1971) report the disappearance of a slow (polymerized) histidase electrophoretic band after heating the crude extract at 80°C while the fast (unpolymerized) band was stable to this treatment. Mutation at the structural locus for histidase could alter the rate at which the native enzyme aggregates into larger units. If the enzyme also becomes increasingly labile as it polymerises, the residual mutant activity could result from a predominance of these unstable forms which would also be present in the wild-type, although in a smaller ratio to the native form. The low ionic strength (SN-2) extract would contain the more polymerised forms, while the high ionic strength (SN-3) extract would contain predominantly native enzyme. This would be consistant with the reduced CRM and instability of the his/his enzyme. It might also explain the failure to demonstrate SN-2 histidase band(s) on electrophoresis. If the residual activity is split between three or more different polymeric forms of the enzyme, the sensitivity of the gel assays is probably too low to detect these components with confidence. The existence of such multiple forms of wild-type histidase is suggested by the electrophoretic demonstration of activity with R_f values of 0·44 (crude extracts), 0·24 and 0·16 (SN-3 extracts). This could be confirmed by showing that these convert back to a single peak (R_f = 0·44), corresponding to the unpolymerised enzyme, on prior incubation with thiol reagent (see Klee, 1970; Okamura et al., 1974; Bhargava and Feigelson, 1976a). It is therefore possible that mutant histidase is a structural locus allele which has similar catalytic properties to the wild-type but tends to aggregate into polymerised

forms more readily than the wild-type enzyme. These aggregates have reduced in vitro and in vivo stability, but specific activity similar to the native enzyme (Klee, 1970), so that the amount of CRM is similar to the level of mutant activity.

The hypothesis discussed above is only tentatively put forward since it requires careful examination of the enzyme properties in both genotypes in the presence and the absence of thiol reagents after finding the optimal concentration and incubation conditions for conversion of polymerised to native forms of the enzyme. Furthermore it is difficult to explain the similar histidase activity in the 15-day old skin of +/+ and his/his mice on this hypothesis.

These results help to clarify the nature of the his mutation although they do not provide an unambiguous answer. A change only in the amount of measured activity due to the presence of activators or inhibitors is very unlikely. A processing gene mutation is also made improbable by the finding of an enzyme with similar properties to the mutant in wild-type SN-2 extracts. The results suggest three possibilities. First, a structural locus mutation alters the ratio of stable to unstable forms of histidase (perhaps related to its state of polymerization), so that there is a predominance of the latter in the mutant. Second, there is a null allele at the "major" histidase structural locus so that no detectable CRM is present. The residual mutant activity is in this case due to a low activity isozyme whose presence is normally masked by the more active species. This would be coded by a distinct structural locus whose product does not associate into a heteropolymer with the product of the "major" histidase locus. Third, there is a defective regulatory locus so that again no, or very little, identifiable histidase is present and the residual activity is explained on the same basis as above.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

More than 30 different aminoacidopathies are associated with mental deficiency (Woolf, 1970). Despite this our knowledge of pathogenetic mechanisms is insubstantial, despite numerous theories and a large body of experimental work. In some aminoacidopathies, mental deficiency can be averted by restricting the dietary intake of the offending amino acid(s) during childhood. This implies that the developing organ is particularly susceptible to metabolic interference. The way in which amino acid excess interferes with the developing brain has not been established in any aminoacidopathy.

Neuropathological findings are often misleading and only provide the barest indication of possible pathogenetic mechanisms (Crome and Stern, 1967; Martin and Schlote, 1972a and b). These include abnormalities in myelination, glial cell changes and the occurrence of scattered, fluid filled spaces (spongy change). These are all non-specific changes, common to numerous metabolic and other conditions. In PKU, which has an obvious biochemical similarity to histidinaemia, myelin is often present in reduced amounts although its composition seems to be qualitatively normal (Gaull et al., 1975). Glial cell changes include both diffuse and focal areas of fibrillary gliosis - often a non-specific response to abnormal function or damage. Spongy change reflects a loss of brain substance but provides no indication as to the cause. There are only two reports of histidinaemic brains seen at autopsy (Waisman, 1967; Corner et al., 1968). Although both gliosis and a reduction in myelin are present, such isolated reports are of little value, especially since the tendency is to report only the more severely affected.

A large body of experimental evidence has accumulated from the use of animal models on the relationship between amino acid excess and brain dysfunction. Many of these models have serious limitations (Karrer and Cahilly, 1965; Gerritsen and Siegel, 1972; Gaull et al., 1975). This field has been comprehensively reviewed by Gaull et al., 1975. The extensive literature has tended to focus on five main ideas. (1) A raised concentration of one amino acid may competitively inhibit the transport of other amino acids into the brain or into specific

brain sites, as a result of a shared carrier system. For example, the plasma histidine concentration required to halve the influx of tryptophan into the rat brain in vivo is 1·0 - 1·5 mM (Daniel et al., 1976), which is in the same region as the plasma histidine concentration in histidinaemia (1 - 3 mM in the mouse; ca. 0·7 mM in man). The ensuing restriction of essential amino acids could be sufficient to interfere with the proper function or development of the brain. (2) Several amino acids at concentrations $\geqslant$ 2 mM, inhibit the apparent incorporation of other, radioactively-labelled amino acids into immature brain proteins in vivo and in vitro. If the de novo synthesis of proteins is inhibited, as this might suggest, brain development could be permanently retarded. (3) The synthesis of neurotransmitter substances could be reduced either by inhibition of precursor amino acid entry or by inhibition of transmitter-synthesizing enzymes. (4) Certain amino acids may inhibit the activity of enzymes concerned with energy metabolism in the immature brain, when their activity is already low, which would have widespread effects on cell division, migration and differentiation. (5) Impaired synthesis of myelin lipids or proteolipids, either by inhibition of enzymes concerned with lipid metabolism or by some of the mechanisms mentioned above, could lead to impaired myelination and transmission of nerve impulses.

None of these hypotheses has been found adequate to explain the occurrence of mental deficiency in an aminoacidopathy. It is likely that a combination of such mechanisms, some specific to one amino acid or its metabolites others common to many, each acting on the developing brain are required to produce a lasting cognitive defect.

Rather than investigating such pathogenetic hypotheses in murine histidinaemia, it was felt that the more fundamental question as to whether brain development or function is abnormal at all should be investigated. The mutant mice could not be consistently distinguished from controls using the rather insensitive behavioural tests available[1]. In human histidinaemia, the plasma histidine concentration is about ten

[1] MacKenzie and Kacser, unpublished.

times normal and only 1 in 6 or 7 subjects are mentally retarded. Murine histidinaemia is more akin to PKU in that the adult plasma histidine is about thirty times increased. In classical PKU 96 - 98% of subjects are severely retarded (IQ< 50) (Tourian and Sidbury, 1978). The experiments undertaken were therefore designed to provide a quantitative comparison of brain development in mutant and normal mice using indices of cell growth, cell proliferation and myelination.

The results of this study allow a firm conclusion. Although small (generally 10 - 15%) differences were found in some parameters at some stages of development, these can be attributed to the problems of assessing maturity, of differences in litter size and in some cases to the small number of animals studied. When each of the indices is examined in the two genotypes over the whole five week period, no consistent difference emerges. The two genotypes show near identical profiles despite large metabolic differences before and after birth. This result does not of course exclude changes in the more subtle, functional aspects of development or in all-important morphological events such as synapse formation. Nevertheless, in maternal PKU a thirtyfold increase in maternal phenylalanine concentration is sufficient to produce severe mental defect with accompanying features such as microcephaly suggesting severe impairment of cell growth or differentiation in virtually all cases (MacCready and Levy, 1972; Tourian and Sidbury, 1978). The twenty to thirty-fold increase in maternal plasma histidine concentrations in murine histidinaemia could therefore be expected to produce similar changes in the offspring. This is made more likely by the finding of a high foetal:maternal concentration ratio for histidine. Any metabolically induced brain damage in utero might also be accentuated by an average ten-fold increase in the histidine concentration of the litters during the postnatal growth spurt. Despite these factors, brain cell numbers and growth during the first five weeks after birth are essentially normal in histidinaemic mutants. The very high intrauterine histidine concentration (about 2·5 µmol/ml could perhaps lead to retardation of brain growth with postnatal recovery in the presence of a less extreme histidine elevation. There is no indication of this in the data. Also, as discussed in Chapter 4, neuronal multiplication and morpho-

genesis are the predominant events occurring before birth, which do not usually recover from metabolic or toxic interference (see Altman et al., 1970). In contrast, cell differentiation or histogenesis which occurs to the greatest extent during the postnatal growth spurt, shows considerable powers of recovery.

These results are consistent with the data on maternal histidinaemia, where the few reports available show offspring of normal intelligence. Possible reasons for the striking differences between PKU and histidinaemia with respect to intelligence include; (1) the two to three-fold higher plasma amino acid concentration in PKU, (2) differences in the ability of the two amino acids to interfere with processes such as transport or myelination discussed above; (3) differences in the concentrations or toxicity of their metabolites. The first possibility could be examined further in this mutant by a similar analysis of brain development in mutants raised on a 2% histidine supplemented diet. As far as the metabolites of histidine are concerned, they appear to be rapidly excreted both in utero and after birth, so that tissue concentrations are very low in molar terms (0·01 - 0·1mM). Although there are one or two examples of amino acids or their metabolites inhibiting enzymes or processes in this concentration range, usually such effects are found at a concentration 1 or 2 orders of magnitude higher (Gaull et al., 1975). The tissue concentration of phenylketones in PKU has not been determined, but animal studies suggest that it is little more than 0·01 mM (Edwards and Blau, 1972).

If there is no major effect of histidinaemia on the overall growth and development of the mouse brain what, if any, other aspects of brain function should be investigated? During the course of investigations into the penetrance of the balance defect, it was noticed that some $11\frac{1}{2}$ day embryos had abnormal hindbrain morphology (Kacser et al., 1979b). Since the hindbrain may induce the formation of the ear vesicle during embryogenesis (Deol, 1964a, b), it has been suggested that disturbance of this inductive effect in histidinaemia could distort inner ear development and cause the balance defect (Kacser et al., 1979b). The available evidence, while not sufficient to sustain this view, is not inconsistent with it. The experiments described in this thesis were carried out on a stock with only 7% penetrant balance defect, while the hindbrain abnormality was noted in a 73% penetrant (CAM) stock. If the hindbrain defect is confirmed,

it would be useful to extend the biochemical characterisation to embryonic brains in high and low penetrant stocks and to the hind-brains of mice taken after birth. One further observation may be relevant in this context. In the five years since the introduction of the his/his mutant in this laboratory, the penetrance of the balance defect has fallen from 80% to less than 10% (Kacser et al., 1979b). Bulfield and Kacser (1974) noted, at a time when the penetrance was still relatively high (> 50%), significant maternal effects in his/his mice on litter size (33% reduction) and growth (measured by the three-week weight). The three-week weights were reduced by 34% in balance defective mice and by only 15% in behaviourally normal mice. In contrast, there is only a very small (9%) effect on litter size and no effect on three-week weight in this study, at a time when the penetrance is only 7%. This might suggest that there is a relationship between the penetrance of the balance defect and growth impairment. Brain growth may be particularly important in this respect. The fall in penetrance is not due to a change in the plasma histidine concentration and so far no biochemical difference has emerged between high and low penetrant stocks (Kacser et al., 1979b). Further studies of brain growth in these stocks would therefore be useful. The dependence of the balance defect on the genetic background as well as the raised maternal histidine concentration also illustrates the general point that the effect of an aminoacidopathy will depend on the genetic as well as the environmental background on which it occurs. Both factors are likely to contribute to the variable expressivity of human histidinaemia with regard to mental defect.

The latter part of this thesis is concerned with the nature of the enzyme defect. The results of these experiments are less clear-cut. The difference in histidase activity between wild-type and mutant does not appear to be due to stimulation or inhibition of histidase activity. Preliminary immunological studies suggest that the mutant is CRM negative. The striking difference in heat stability between crude mutant and wild-type liver extracts initially favoured a structural or processing locus mutation rather than a regulatory mutation affecting only the quantity of enzyme protein. This was

supported by studies on partially purified liver extracts, which led to the unexpected finding that two forms of histidase are present in wild-type extracts, only one of which is present in the mutant. The two forms differ markedly in their heat stability and sensitivity to ionic strength. While the possibility still exists that the two forms of histidase represent stable and unstable forms of a single enzyme (perhaps related to the state of aggregation), it seems more likely that they represent distinct isozymes, one of which is normally masked by the major, heat stable component. The low activity or instability of the minor component would result in its being destroyed or discarded at an early stage of most purification procedures, which is another reason for it not being previously recognised. The existence of distinct histidase isozymes is also suggested by the findings in the skin, where the early difference in activity between wild-type and mutant disappears completely at 15 days, suggesting that one component, that is present in both genotypes, is unaffected by mutation. If a low activity isozyme is uncovered by loss of the more active enzyme, what is the nature of the his locus mutation? This could be either a structural or regulatory locus mutation, resulting in the formation of little or no active enzyme. If it is a structural mutation, the low activity could arise from reduced catalytic function or instability and synthesis may also be diminished. A grossly abnormal structural gene product might lose its catalytic and antigenic properties or be rapidly degraded. Complete absence of the structural gene, due to a deletion, could also have occurred, in which case it would be hard to distinguish from a null regulatory mutant except by genetic mapping. A regulatory mutant could cause a complete absence of enzyme protein or a greatly diminished amount of normal enzyme. Analysis of the SN-3 extract of his/his liver would help to distinguish between these alternatives by showing whether or not it has the same properties as the wild-type SN-3 extract.

Recently it has been suggested that multilocus enzymes are very common in higher organisms and frequently show a marked degree of tissue differentiation in the expression of the different loci (Harris, 1979). For example, lactate dehydrogenase is controlled by three loci (A,B,C),

two of which (A,B) are expressed in most tissues, but in a proportion varying from 10:1 in some tissues to 1:10 in others. Similarly, histidase may be controlled by two loci represented by the heat stable and heat labile components. The low, but measurable activity reported in many tissues in the guinea pig (Zannoni and La Du, 1963) may represent a predominance of the heat labile isozyme (which may also act on other substrates), while the heat stable activity is predominant in the liver. This could be confirmed by comparing the heat stability of histidase in crude extracts from different tissues or by comparing the ratio of heat stable to heat labile forms in partially purified tissue extracts.

Finally, these results do not suggest any obvious therapeutic approach to increasing mutant histidase activity, so that preliminary attempts to inject purified histidase in a stable form are under way. Although no clear answer to the genetic nature of the his locus mutation has emerged yet, the lack of any other histidase structural locus variant means that genetic mapping is not yet a feasible alternative approach. Further studies of SN-2 and SN-3 extracts in both genotypes may clarify the problem further.

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APPENDIX

Summary of Reported Cases of Histidinaemia [1]

Reference	Case	Sex	Age	IQ	speech defect	Other	plasma histidine (mg/100ml)	urine histidine	histidase activity in skin
				Clinical findings			Laboratory findings		
Ghadimi et al. 1961a and 1962	1	F	13 yr	93	+	frequent infections, temper tantrums, behaviour problems, failed one year in school, had 5 years speech therapy and still did not speak clearly, hearing defect	9·0	616 mg/24 hr	absent
MacMillan 1970	2	F	14 yr	normal	-	sister of case 1. Retarded physical development	7·1	819 mg/24 hr	absent
Auerbach, et al. 1962 and 1966	3	F	4y,4m	83	+	small stature, history of seizures; elevated plasma alanine	15·8	506 mg/24 hr	absent
La Du et al., 1963	4	F	6 yr	normal	+	mirror writing	17·3	378 mg/24 hr	absent
	5	M	5 yr	normal	+	brother of case 4	13·4	850 mg/24 hr	absent
Andrews et al., 1962	6	F	5y,6m	85	+	small stature	elevated	NR	NR
	7	F	7 yr	normal	-	sister of case 6	elevated	NR	NR
Hudson et al., 1963	8	M	8 yr	retarded	+	seizures	NR	elevated	NR
	9	NR	1 mo	NR	NR		NR	elevated	NR
Davies and Robinson 1963	10	M	5 yr	retarded	-	physical development below 3rd percentile, episode of anaemia treated by transfusion, febrile convulsions, frequent infections	9	312-578 mg/24 hr	NR

[1] Nos. 1-70 from Ghadimi (1974).

APPENDIX cont...

Snyderman et al., 1963; Berlow et al., 1965; Kilhara et al., 1968	11	F	11 yr	65	+	precocious puberty, hemi-vertebrae, dislocated patella and genu valgum, scoliosis, delayed developmental milestones, internal strabismus, poor muscular coordination, poor comprehension, minimal perceptual efficiency, distractable, frequent infection	15	709 mg/24 hr	absent
Shaw et al., 1963; Kilhara et al., 1968	12	F	13 yr	50	+	intention tremor, mild progressive ataxia	4·9-5·5	340-680 mg/g creat	very low
Gerritsen, 1964;	13	F	17 yr	76	+	severe emotional problems	11	NR	absent
Waisman, 1967	14	M	3y,9mo	severely retarded	+	very obese, dull expression hyperreflexia, failure to stand, failure to cry on painful stimuli, brain degeneration	7·8-9·5	600 mg/24 hr	absent
	15	F	1 yr	severely retarded	?	delayed developmental milestones, dull expression, sister of case 14	9·11	570-617 mg/24 hr	absent
	16	F	3 yr	84	NR	small stature, retarded growth, sister of case 14	5·3-15·0	elevated	¼ of normal
Holton, 1964, 1965; Holton et al., 1964	17	M	3y,5mo	86	-	seizures, frequent infections, cerebral maldevelopment, postepileptic encephalopathy	5-14	218 mg/24 hr	absent
Corner et al., 1968									
Clarence and Bowman, 1966	18	F	13 yr	54	+	height and weight below 3rd percentile, encorpresis	11·2	599 mg/24 hr	absent

APPENDIX cont...

Galamon and Szulc-Kuberska, 1966	19 to 24	5M 1F	9 yr	NR	+	history of stammering in 3 generations of one family	approx 14	approx 238 ug/ml	NR
			16 yr	NR	+				
			35 yr	NR	+				
			37 yr	NR	+				
			43 yr	NR	+				
			65 yr	NR	+				
Wadman et al., 1967	25	F	8 yr	retarded	+	delayed developmental milestones	8·1-10·3	495-590 mg/g creat.	NR
	26	F	10 yr	retarded	NR	presence of pubic hair	7·4	771 mg/g creat.	NR
	27	F	1 yr	mildly retarded	?	jaundice in early life, did not cry or react to sounds in early life, wt. below 3rd percentile, mild anaemia	12·6-18·0	1409-2627 mg/g creat.	NR
Wadman et al., 1971	28	F	14y,8mo	retarded	NR		7·91	590 mg/g creat.	NR
	29	F	4y,8mo	retarded	NR		8·38	3250 mg/g creat.	NR
	30	M	7y,5mo	retarded	NR		13·65	3840 mg/g creat.	NR
	31	F	10y,5mo	retarded	NR		14·90	1216 mg/g creat.	NR
Gilman and Howell, 1969	32	F	12 yr	normal	-	small stature, congenital hypoplastic anaemia	8·2	824 mg/24 hr	absent
Gatfield et al., 1969	33	F	5 yr	23	+	severe behaviour problem in earlier years, self-destructive, ht. 10th percentile, delayed motor development, hyperreflexia, seizures, EEG dysrhythmic	13·8-17·1	elevated	absent
	34	F	11 yr	107	-	bad temper, short attention span, failed one year in school, sister of case 33	8·5-12·6	elevated	absent

APPENDIX cont...

	35	M	6 yr	88	-	bad temper, short attention span, failed 1 yr in school, encopresis, brother of case 33	17·4-18·0	elevated	absent
	36	F	19 mo	97	-	sister of case 33	11·6-14·9	elevated	absent
Bruckman et al., 1970	37	M	6y,9m	92	+	short stature, left esotropia, short attention span, abnormal EEG, dysarthria, dyslalia	19·5	5.10 gm/ 100 ml	absent
	38	F	8y,6m	74	+	abnormal EEG, visual motor coordination impaired, sister of case 37	10·1	685 mg/100 ml	absent
	39	F	12y,9m	85	-	abnormal EEG, sister of case 37	8·0	13·20 mg/ 100 ml	markedly decreased
	40	F	37 yr	83	+	diabetic, multinodular goiter, enlarged liver, iron deficiency, anaemia, reduced auditory retention span, mother of case 37	12·3	8·90 mg/ 100 ml	absent
	41	F	40 yr	normal	-	stuttering during childhood, aunt of case 37	4·2	10·00 mg/ 100 ml	NR
	42	F	44 yr	NR	-	progressive deafness, optic atrophy, abnormal EEG, aunt of case 37	7·1	7·25 mg/ 100 ml	absent
	43 to45	1M 2F	NR	NR(3)	NR(3)	2 aunts, 1 cousin of case 37	2-4	3·50-5·75 mg/100 ml	NR(3)
Baden et al., 1969	46	M	2 mo	NR	-		elevated	elevated	NR
Baden 1972	47	M	5y,8m	87	-		14·7	elevated	NR

APPENDIX cont...

Rosenblatt et al., 1970	48	F	17 yr	normal	NR	single transplanted kidney; Cushing-like appearance	4·8-6·2	elevated	absent
Lott et al., 1970	49	M	5y,4m	100	-	hypogammaglobulinaemia	10	elevated	NR
	50	M	8y,11m	70	-	motor seizures, absence attacks, otitis media	9	elevated	NR
	51	F	8y,7m	96	-	tonsillar hypertrophy, hypospadias	13·1	elevated	NR
Lyon & Veale, 1970	52	NR	6 d	NR	NR		10-20	430 mg/g creat.	absent
	53	NR	4 d	NR	NR		20	NR	absent
Cain & Holton, 1968	54	M	3y,3m	86	NR	grand mal seizures: anaemia with microcytosis, hypochromia: delayed bone age: ht. & wt. on 10th percentile	NR	NR	absent
Auerbach et al., 1967	55	F	7 mo	normal	-	normal clinically	15·6	NR	absent
	56			normal	-	normal clinically	7·6	NR	NR
	57			normal	-	normal clinically	9·1	NR	NR
	58	M	12 yr	normal	-	normal clinically	8·8	34·4mg/100ml	NR
	59			normal	-	normal clinically	5·6	NR	NR
	60			normal	-	normal clinically	4·6	23·2mg/100ml	NR
Kibel and Levy, 1970	61	F	15 yr	46-50	NR	abnormal EEG; arachnodactyly	14·2	elevated	absent
Kierland et al., 1968	62	M	6 yr	93	NR	some atopic dermatitis, ht. and wt. both below 3rd %ile.	9·46	elevated	NR
Carton et al., 1970	63	M	16 mo	NR	NR	idiopathic thrombocytopenic purpura; normal EEC (concomitant hyperalaninaemia)	6·2-9·4	8 X controls	absent

APPENDIX cont...

Kihara et al., 1968	64	M	38 yr	retarded	NR	spastic diplegia	7·1-11·8	5 X normal	very low
	65	F	4 yr	NR	NR		27·8		very low
	66	M	20 yr	NR	NR		12·8		very low
Massengill & Smith, 1967	67	M	3 yr	normal	+	generally healthy	elevated	elevated	NR
Thalhammer et al., 1971	68		newborn	NR	NR		8-21	elevated	absent
	69		newborn	NR	NR	history of mental retardation in the family	6-15	elevated	absent
	70		newborn	NR	NR	history of mental retardation in the family	6-18	elevated	absent
Neville et al., 1972 Whitfield and Shepherd, 1970	71	F	3 yr	<20	+	gross developmental retardation: frequent major seizures: signs of mixed athetoid and spastic cerebral palsy	5·1 (fasting) 15 (random)	elevated	very low or absent (<2%)
	72	F	5y,6m	114	-	sister of case 74 clinically normal	6·0 (f) 12 (r)	elevated	very low or absent (<2%)
	73	M	12 yr	35	+	severely mentally retarded with features of early-onset infantile psychosis (autism)	7·5 (f) 15 (r)	elevated	greatly reduced (15%)
	74	F	18 yr	114	-	sister of case 76 clinically normal	6·2 (f) 5·2 (r)	elevated	very low or absent (<3%)
	75	F	4y,6m	<65	+	severely subnormal with features of early infantile psychosis (autism). Development probably normal until 2 years	4·0 (f) 10·0 (r)	elevated	very low (6%)
Neville et al., 1971 Neville et al., 1972	76	F	28 yr	100	-	Infrequent major seizures from age 16 yrs. Depressive episodes. Immature personality	7·9 (f) 7-10(r)	390-720 mg/ 24 hr	very low or absent (< 3%)

APPENDIX cont...

Neville et al., 1972	77	M	23 yr	65	+	apparently normal development till aged 2 yrs. Major seizures. Severely subnormal	9·0	elevated	very low or absent (<3%)
Neville et al., 1971	78	M	16 yr	80	NR	epileptic. Brother of case 79	elevated	NR	NR
Lyon et al., 1974	79	M	27 yr	65	NR	mentally retarded - institutionalised	12·9	1040 mg/ 24 hr	NR
	80	M	52 yr	normal	-	normal	11·0	NR	NR
	81	F	37 yr	105-115	-	normal	9·6	NR	NR
Stevens et al., 1975	82	M	17 yr	normal	-	Marfan syndrome	13·1 9·2	1490 mg/ 24 hr	reduced or absent
	83	M	18 yr	normal	-	Marfan syndrome, brother of case 82	12·1 10·3	3,090 mg/ 24 hr	reduced or absent
Roberts and Ireland, 1978 Hudson et al., 1963	84	F	2 yr	NR	NR		12·6	112 mg/ 24 hr	NR
Duffner & Cohen, 1975	85	M	2 yr	36 (DQ)		EEG showed hypsarrhythmia, infantile spasms	12-14	NR	NR
Kappelman et al., 1971	86	F	7 yr	77	+	seizures, hyperactivity, learning problems	13	NR	absent
Anakura et al., 1975	87	M	6 yr	60	+	slow development, abnormal EEG	13	275 mg/ 24 hr	absent
	88	M	13 yr	90	-		6	113 mg/ 24 hr	very low

APPENDIX cont...

Brown et al., 1977	89	M	3 yr	low	+	seizures, unrelated encephalopathy, delayed bone age	8·5	increased	NR
	90	F	1 yr	low	NR	seizures, unrelated encephalopathy	11	increased	NR
	91	F	10 yr	101	-		7·5	increased	about $\frac{1}{3}$ of normal
Armstrong, 1975	92	F	NR	normal	-	gave birth to 4 normal children	10·6	1132 mg/ 24 hr	NR
Rostenberg et al., 1974	93	M	8 mo	35		abnormal EEG, moderate diffuse cortical atrophy on air encephalogram	2·1	NR	NR
Popkin et al., 1974	94	NK	7 mo	normal		normal development on low histidine diet	8·1	increased	NR
Plöchl et al., 1978	95	M	14 yr	114	+	hearing disability	6-8	1195 umol/ 24 hr	NR
Kotsopoulos and Kutty, 1979	96	M	10 yr	NR	+ (autistic)	normal development till aged 2 yrs then developed infantile autism	9·2-10·9	454 mg/ 24 hr	NR
	97	F	14 yr	N	-		8·3	NR	NR
	98	F	16 yr	N	-		9·5	NR	NR
	99	F	40 yr	N	-	mother of cases 96-98	8·7	NR	NR
Wadman et al., 1973	100	M	11 yr	101	-	abnormal EEG, seizures (perhaps related to perinatal hypoglycaemic brain damage)	13·3	2018 mg/ g. creat.	NR
Levy et al., 1974	101 to 126	12M 14F	4-12 yr	N	-	detected in neonatal screening surveys	10·1 to 17·3	4·5-27·0 mmol/ g. creat.	NR

APPENDIX cont...

Griffiths, 1973	127	F	2 yr	108	-	detected in neonatal screening survey: developing normally on low histidine diet	13·2	increased	NR
	128	F	1y,8m	N	-		9·3-12·4	increased	NR
	129	F	2 yr	N	-		18·6	increased	NR
	130	M	2 yr	N	-		18·6-21·7	increased	NR
Beauvais et al., 1971	131	F	2 yr	91	-		12·4	76	very low
Rao et al., 1974	132	M	8 yr	70	+	consanguineous parents. Delayed development. Frequent respiratory infections. Stunted growth	10	350-400 mg/24 hr	NR
Gehler and Stopfkuchen, 1977	133	M	13 yr	mildly subnormal	NR	features of Marfan's syndrome Delayed developmental milestones.	increased	increased	NR
Holmgren et al., 1974	134	F	2 yr	normal	-		7·2	6830 µmol/ g. creat.	NR
	135	F	12 yr	normal	-		11·2	6970 µmol/ g. creat.	NR
	136	M	23 yr	79	+	psychosis of unknown nature	4·8	6560 µmol/ g. creat.	NR

ATYPICAL HISTIDINAEMIA

Woody et al., 1965	1	M	11 yr	52	+	abnormal EEG. Sweat UA normal, urinary UA reduced	1·8-4·3	500-2200 mg/g. creat.	present
	2	M	10 yr	47	+	abnormal EEG, seizures. Sweat UA normal, urinary UA reduced	1·5-6·4	500-2000 mg/g. creat.	present
	3	F	6 yr	53	+	abnormal EEG, seizures. Sweat UA normal, urinary UA reduced	1·4-3·4	250-800 mg/g. creat.	present

APPENDIX cont...

Kothari et al., 1970	4	F	8 yr	83	+	behaviour problems, abnormal EEG. Negative ferric chloride test in urine. Increased sweat UA.	14·5(normal range 0·5 -5)	40·6 mg/100ml (normal range 14 - 19)	NR
	5	F	10 yr	50	+	normal EEG	17·6(n.r. 0·5-5)	NR	NR
Levy et al., 1974	6-7	NR	NR	NR	NR	skin UA about 25% of normal	0·22-0·29 mM	"mild increase"	NR

HISTIDINURIA

Sabater et al., 1976	1	M	9 yr	53		renal tubular reabsorption of histidine 40·1%. Reduced intestinal histidine absorption. Urine negative for histidine metabolites	0·94	299·9-314·3 mg/24 hr	NR
	2	M	11 yr	58		renal tubular reabsorption of histidine 52·8%. Reduced intestinal histidine absorption. Urine -ve for histidine metabolites	0·47	151·3-202·7 mg/24 hr	NR
Holmgren et al., 1974	3	M		normal		developed myoclonic seizures at age 13 years	1·4	2100 µmol/ 24 hr	NR

DECLARATION

I declare that the composition and work described in this thesis are my own except where specifically stated.

Signed,

REFERENCES

Ackroyd, H., and Hopkins, F.G. (1916) Feeding experiments with deficiencies in the amino-acid supply: arginine and histidine as possible precursors of purines. Biochem. J., 10, 551.

Agrawal, H.C., Davis, J.M., and Himwich, W.A. (1968) Developmental changes in mouse brain: weight, water content and free amino acids. J. Neurochem., 15, 917.

Agrawal, H.C., and Davison, A.N. (1973) Myelination and amino acid imbalance in the developing brain. In Biochemistry of the Developing Brain, Vol. 1, Ed. W.A. Himwich. New York: M. Dekker, Inc. p 143.

Albers, R.W., Koval, G.J., and Jakoby, W.B. (1962) Transamination reactions of rat brain. Exp. Neurol., 6, 85.

Albrecht, W. (1956) Änderung des gehalts der verschiedenen phosphatfraktionen in gehirn and muskel der maus während des wachstums. Z. Natwforsch., 118, 248.

Allan, J.D., and Brown, J.K. (1968) Maternal phenylketonuria and foetal brain damage: an attempt at prevention by dietary control. In Some Recent Advances in Inborn Errors of Metabolism. Eds. K.S. Holt and V.P. Coffey. Edinburgh: Livingstone. p 14.

Altman, J., (1969) DNA metabolism and cell proliferation. In Handbook of Neurochemistry. Ed. A. Laytha. New York and London: Plenum Press. Vol. 2, p 137.

Altman, J., Das, G.D., and Sudarshan, K. (1970) The influence of nutrition on neural and behavioural development. I. Devel. Psychobiol., 3, 281.

Anakura, M., Matsuda, I., Arashima, S., Fukushima, N., and Oka, Y. (1975) Histidinemia - classical and atypical forms. Amer. J. Dis. Child., 129, 858.

Anastasi, A., Correale, P., and Erspamer, V. (1964) Occurrence of N-acetylhistidine in the central nervous system and the eye of Rana Esculenta. J. Neurochem., 11, 63.

Andrews, B.F., Crosby, P.F., Angel, C.R. (1962) Histidinaemia: a new metabolic disorder. Southern Med. J., 55, 1326.

Andrews, T.M., McKeran, R.O., Watts, R.W.E., McPherson, K., and Lax, R. (1973) A relationship between the granulocyte phenylalanine content and the degree of disability in phenylketonuria. Quart. J. Med., 42, 805.

Arakawa, T., Fujü, M., and Ohara, K. (1966) Erythrocyte formiminotransferase activity in formiminotransferase deficiency syndrome. Tohoku J. Exp. Med., 88, 195.

Arakawa, T. (1970) Congenital defects in folate utilisation. Am. J. Med., 48, 594.

Armstrong, M.D. (1975) Maternal histidinaemia. Arch. Dis. Childh., 50, 830.

Auerbach, V.H., and Waisman , H.A. (1959) Tryptophan peroxidase - oxidase, histidase and transaminase activity in the liver of the developing rat. J. Biol. Chem., 234, 304.

Auerbach, V.H., Di George, A.M., Baldridge, R.C., Tourtellotte, C.D., and Brigham, M.P. (1962) Histidinaemia. J. Pediat., 60, 487.

Auerbach, V.H., Di George, A.M., Carpenter, G.C., and Baldridge, R.C. (1966) Histidinaemia, further observations with direct demonstration of absent histidase activity in liver. Abstr. 36th Ann. Meet., Soc. Pediat. Res.

Auerbach, V.H., Di George, A.M., and Carpenter, G.G. (1967) Histidin emia. In Amino Acid Metabolism and Genetic Variation, Ed. W.L. Nyman. New York: McGraw-Hill Book Company.

Aures, D., Håkanson, R., and Clark, W.G., (1972) Histidine decarboxylase and dopa decarboxylase. In Perspectives in Neuro-Pharmacology, Ed. S.H. Snyder. London: Oxford University Press. p 165.

Autilio, L.A., Norton, W.T., and Terry, R.D. (1964) The preparation and some properties of purified myelin from the central nervous system. J. Neurochem., 11, 17.

Bachur, N.R., and Udenfriend, S. (1966) Microsomal synthesis of fatty acid amides. J. Biol. Chem., 241, 1308.

Baddiley, J., Cantoni, G.L., and Jamieson, G.A. (1953) Structural observations on "active methionine". J. Chem. Soc. (London), 3, 2662.

Baden, H.P., Sviokla, S., Mittler, B. and Pathak, A.M. (1968) Histidase activity in hyperplastic and neoplastic rat epidermis and liver. Cancer Res., 28, 1463.

Baden, H.P., Hori, Y., Pathak, M.A., and Levy, H.L. (1969) Epidermis in histidinaemia. Arch. Dermatol., 100, 431.

Baden, H.P. (1972) Pers. Commun. quoted in Ghadimi (1974).

Baden, H.P., and Gavioli, L. (1974) Histidase activity in rat liver and epidermis. J. Invest. Dermatol., 63, 479.

Bakwin, H. (1968) Delayed speech. Ped. Clins. N. Am., 15, 627.

Balazs, R., Kovacs, S., Teichgraber, P., Cocks, W.A., and Eayrs, J.T. (1968) Biochemical effects of thyroid deficiency on the developing brain. J. Neurochem., 15, 1335.

Baldridge, R.C., and Greenberg, N. (1963) A method for the determination of histidine in blood. J. Lab. Clin. Med., 61, 700.

Baldridge, R.C., and Tourtellotte, C.D. (1958) The metabolism of histidine III urinary metabolites. J. Biol. Chem., 233, 125.

Bardin, C.W., Bullock, L.P., Sherins, R.J., Mowszowicz, I., and Blackburn, W.R. (1973) Androgen metabolism and mechanism of action in male pseudohermaphroditism: a study of testicular ferminization. Rec. Progr. Horm. Res., 29, 65.

Barnhisel, M.L., Priest, R.E., and Priest, J.H. (1970) Histidase activity in human cell culture. J. Cell. Physiol., 76, 7.

Baslow, M.H. (1967) N-Acetyl-L-histidine metabolism in the fish eye: evidence for ocular fluid-lens l-histidine recycling. Exptl., Eye Res., 6, 336.

Beauvais, P., Klein, F., Humbel, R., Dreyfus, J., Gast, M. (1971) Etude clinique et biochemique d'un cas d'histidinemie. Arch. Franç. Péd., 28, 191.

Beaven, M.A. (1975) Radiochemical assay procedures for drugs and transmitters. In Handbook of Psychopharmacology, 1. Eds. L.L. Iversen, S.H. Iversen, and S.H. Snyder. London: Plenum p 253.

Beaven, M.A. (1978) Histamine: its role in physiological and pathological processes. Monographs in Allergy, Vol 13. Basel: Karger.

Berl, S. (1973) Metabolic compartmentation in developing brain. In Biochemistry of the Developing Brain. Ed. W.A. Himwich. New York: M. Dekker Inc. p 219.

Berlow, S., Arends, R., Harries, C. (1965) Studies in histidinaemia. Lancet, 85, 241.

Beutler, E. (1972) Glucose-6-phosphate dehydrogenase deficiency. In The Metabolic Basis of Inherited Disease. Eds. J.B. Stanburg, J.B. Wyngaarden, and D.S. Fredrickson. New York: McGraw-Hill Book Company. p 1358.

Bhargava, M.M., and Feigelson, M. (1976a) Studies on the mechanisms of histidase development in rat skin and liver. I Basis for tissue specific developmental changes in catalytic activity. Develop. Biol., 48, 212.

Bhargava, M.M., and Feigelson, M. (1976b) Studies on the mechanisms of histidase development in rat skin and liver. II Alterations in enzyme levels and synthetic rates during development. Develop. Biol., 48, 226.

Blume, K.G., Hoffbauer, R.W., Busch, D., Arnold, H., and Löhr, G.W. (1971) Purification and properties of pyruvate kinase in normal and in pyruvate kinase deficient human red blood cells. Biochem. Biophys. Acta, 227, 364.

Borek, B., and Waelsch, H. (1953) The enzymatic degradation of histidine. J. Biol. Chem., 205, 459.

Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G., and Lowy, P.H. (1950) Metabolism of ^{14}C - labelled glycine, L-histidine, L-leucine and L-lysine. J. Biol. Chem., 187, 839.

Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G., and Lowy, P.H. (1952) Incorporation *in vitro* of labelled amino acids into proteins of rabbit reticulocytes. J. Biol. Chem., 196, 669.

Boyer, S.H., Siggers, D.C., and Krueger, L.J. (1973) Caveat to protein replacement therapy for genetic disease. Lancet, 2, 654.

Bowden, J.A., McArthur, C.L., and Fried, M. (1970) The inhibition of pyruvate decarboxylation in rat brain by α-ketoisocaproic acid. Biochem. Med., 5, 101.

Brand, L.M., and Harper, A.E. (1975) Characterization of histidine: ammonia lyase from rat liver and its inhibition *in vitro* and *in vivo*. Fed. Proc., 34, 509.

Brand, L.M., and Harper, A.E. (1976) Histidine ammonia-lyase from rat liver. Purification, properties and inhibition by substrate analogues. Biochemistry, 15, 1814.

Brock, D.J.H. (1972) Inborn errors of metabolism. In the Biochemical Genetics of Man, 1st edition. Eds. D.J.H. Brock, and O. Mayo. London and New York: Academic Press.

Brock, D.J.H. (1978) Inborn errors of metabolism. In the Biochemical Genetics of Man, 2nd edition. Eds. D.J.H. Brock, and O. Mayo. London and New York: Academic Press.

Brown, D.D., and Kies, M.W. (1959) The mammalian metabolism of L-histidine. J. Biol. Chem., 234, 3182.

Brown, D.D., Tomchick, R., and Axelrod, J. (1959) The distribution and properties of a histamine-methylating enzyme. J. Biol. Chem., 234, 2948.

Brown, D.D., Silva, O.L., McDonald, P.B., Snyder, S.H., and Kies, M.W. (1960) The mammalian metabolism of L-histidine. III The urinary metabolites of L-histidine-14C in the monkey, human and rat. J. Biol. Chem., 235, 154.

Brown, E.S., Waisman, H.A., Geison, R.L., and Gerritsen, T. (1977) Sibship with histidinemia and an unrelated encephalopathy. Helv. Paediat. Acta, 32, 401.

Brownstein, M.J., Saavedra, J.M., Palkovits, M., and Axelrod, J. (1974) Histamine content of hypothalamic nuclei of the rat. Brain Res., 77, 151.

Bruckman, C., Berry, H.K., Dasenbrock, R.J. (1970) Histidinaemia in two successive generations. Am. J. Dis. Child., 119, 221.

Brusilow, S.W., and Ikai, K. (1968) Urocanic acid in sweat: an artifact of elution from the epidermis. Science, 160, 1257.

Budillon, G., Del Vecchio-Blanco, C., and Coltorti, M. (1971) Enzymatic aspects of histidine metabolism in liver tissue. Enzyme, 12, 329.

Buffoni, F. (1966) Histaminase and related amine oxidases. Pharmacol. Rev., 18, 1163.

Bulfield, G., and Kacser, H. (1974) Histidinaemia in mouse and man. Arch. Dis. Childh., 49, 545.

Bulfield, G., and Kacser, H. (1975) Histamine and histidine levels in the brain of the histidinaemic mutant mouse. J. Neurochem., 24, 403.

Bulfield, G. (1978) Genetic variation in the activity of the histidine catabolic enzymes between inbred strains of mice: a structural locus for a cytosol histidine aminotransferase isozyme (Hat-1). Biochem. Genet., 16, 1233.

Burstein, S., Bhavnani, B.R., and Bauer, C.W. (1967) Genetic aspects of cortisol hydroxylation in guinea pigs: kinetics of enzymatic 2-α- and 6-β- hydroxylation by adrenal homogenates. Endocrinology, 80, 663.

Burton, K. (1956) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J., 62, 315.

Butterfield, L.J., and O'Brien, D. (1963) The effect of maternal toxaemia and diabetes on transplacental gradients of free amino acids. Arch. Dis. Childh., 38, 326.

Cain, A.R.R., and Holton, J.B. (1968) Histidinaemia: a child and his family. Arch. Dis. Childh., 43, 62.

Cantarow, A., and Schepatz, B. (1967) Biochemistry. 4th edition. Philadelphia and London: W.B. Saunders Co.

Carton, D., Dhondt, F., De Schrijver, F., Samyn, W., Kint, J., Delbeke, M.J., and Hooft, C. (1970) Histidinaemia. Helv. Paediat. Acta, 25, 127.

Celada, F., and Strom, R. (1972) Antibody-induced conformational changes in proteins. Quart. Rev. Biophys., 5, 395.

Ceriotti, G. (1955) Determination of nucleic acids in animal tissues. J. Biol. Chem., 214, 59.

Chase, H.P., Lindsley, W.F.B., and O'Brien, D. (1969) Undernutrition and cerebellar development. Nature, Lond., 221, 554.

Chase, H.P., and O'Brien, D. (1970) Effect of excess phenylalanine and of other amino acids on brain development in the infant rat. Pediatr. Res., 4, 96.

Childs, B., and Der Kaloustian, V.M. (1968) Genetic heterogeneity. N. Engl. J. Med., 279, 1205, and 1267.

Chovnick, A., Gelbart, W.M., McCarron, M., Osmond, B., Candido, E.P.M., and Baillie, D.L. (1976) Organization of the rosy locus in Drosophila melanogaster: evidence for a control element adjacent to the xanthine dehydrogenase structural element. Genetics, 84, 233.

Clarance, G.A., and Bowman, J.K. (1966) Further case of histidinaemia. Brit. Med. J., 1, 1019.

Clarke, A.D.B., and Clarke, A.M. (1974) Mental retardation and behavioural change. Brit. Med. Bull., 30, 179.

Coltorti, M., Di Simone, A., and Budillon, G. (1966) Histidase and urocanase activities of liver and plasma. Correlations between tissue enzyme levels and plasmatic increases during human and mouse viral hepatitis. Clin. Chim. Acta, 13, 568.

Cook, J.G.H. (1975) Factors influencing the assay of creatinine. Ann. Clin. Biochem., 12, 219.

Cornell, N.W., and Villie, C.A. (1968) The purification and properties of rat liver histidase. Biochim. Biophys. Acta, 167, 172.

Corner, B.D., Holton, J.B., Norman, R.M., and Williams, P.M. (1968) A case of histidinaemia controlled with a low histidine diet. Pediatrics, 41, 1074.

Crome, L.C., and Stern, J. (1967) Pathology of Mental Retardation. London: Churchill.

Crowley, G.M. (1964) The enzymatic synthesis of 5-phosphobosyl-imidazoleacetic acid. J. Biol. Chem., 239, 2593.

Daniel, P.M., Moorhouse, S.R., and Pratt, O.E. (1976) Amino acid precursors of monoamine neurotransmitters and some factors influencing their supply to the brain. Psychol. Med., 6, 277.

Davies, H.E., and Robinson, M.J. (1963) A case of histidinaemia. Arch. Dis. Childh. 38, 80.

Davis, B.J. (1964) Disc electrophoresis - II method and application to human serum proteins. Ann. N.Y. Acad. Sci., 121, 404.

Davis, J.M., and Himwich, W.A. (1973) Amino acids and proteins of developing mammalian brain. In Biochemistry of the Developing Brain, Vol. 1. Ed. W.A. Himwich. New York: M. Dekker Inc. p 55.

Davison, A.N. (1968) Lipid metabolism of nervous tissue. In Applied Neurochemistry. Eds. A.N. Davison and J. Dobbing. Oxford and Edinburgh: Blackwell Scientific Publications. p 178.

Davison, A.N., and Dobbing, J. (1968) The developing brain. In Applied Neurochemistry. Eds. A.N. Davison and J. Dobbing. Oxford and Edinburgh: Blackwell Scientific Publications. p 253.

Davison, A.N. (1977) The biochemistry of brain development and mental retardation. Brit. J. Psychiat., 131, 565.

Deol, M.S. (1964a) The abnormalities of the inner ear in Kreister mice. J. Embryol. exp. Morph., 12, 475.

Deol, M.S. (1964b) The origin of the abnormalities of the inner ear in dreher mice. J. Embryol. exp. Morph., 12, 727.

Dickerson, J.W.T., and Dobbing, J. (1967) Prenatal and postnatal growth and development of the central nervous system of the pig. Proc. Roy. Soc. B., 166, 384.

Dizik, M., and Elliott, R.W. (1978) A second gene affecting the sialylation of lysosomal α-mannosidase in mouse liver. Biochem. Genet., 16, 247.

Dobbing, J. (1968) Vulnerable periods in developing brain. In Applied Neurochemistry. Eds. A.N. Davison and J. Dobbing. Oxford and Edinburgh: Blackwell Scientific Publications. p 287.

Dobbing, J. (1970) Undernutrition and the developing brain. The relevance of animal models to the human problem. Amer. J. Dis. Childh., 120, 411.

Dobbing. J. (1974) The later development of the brain and its vulnerability. In Scientific Foundations of Paediatrics. Eds. J.A. Davis and J. Dobbing. London: Heinemann Medical. p 565.

Dobbing, J., and Smart, J.L. (1974) Vulnerability of developing brain and behaviour. Brit. Med. Bull., 30, 164.

Duffner, P.K., Cohen, M.E. (1975) Infantile spasms associated with histidinaemia. Neurology (Minneapolis), 25, 195.

Eayrs, J.T., and Goodhead, B. (1959) Postnatal development of the cerebral cortex in the rat. J. Anat. (Lond.), 93, 385.

Edlbacher, S. (1926) Zur Kenntris des intermediaren stoffwechsels des histidins. Z. physiol. Chem., 157, 106.

Edwards, D.J., and Blau, K. (1972) Aromatic acids derived from phenylalanine in the tissues of rats with experimentally induced phenylketonuria-like characteristics. Biochem. J., 130, 495.

Eldjarn, L., Jellum, E., and Stokke, O. (1975) Inborn errors of metabolism - a challenge to the clinical chemist. Clin. Chem., 21, 63.

Endo, T., and Schwartz, D. (1966) Tissue specific variations in the urea sensitivity of the E_1 esterase in maize. Genetics, 54, 233.

Everett, M.A., Anglin, J.H., and Bever, A.T. (1961) Ultraviolet induces biochemical alterations in skin. Arch. Dermatol., 84, 59.

Faulkner, W.R., and King, J.W. (1970) Renal Function Tests. In Fundamentals of Clinical Chemistry. Ed. N.W. Tietz. New York: W.B. Saunders Co. p 698.

Feigelson, M. (1968) Estrogenic regulation of hepatic histidase during postnatal development and adulthood. J. Biol. Chem., 243, 5088.

Feigelson, M. (1971a) Hypophyseal regulation of hepatic histidase during postnatal development and adulthood. I Pituitary suppression of histidase activity. Biochim. Biophys. Acta, 230, 296.

Feigelson, M. (1971b) Hypophyseal regulation of hepatic histidase during postnatal development and adulthood. II Pituitary-estrogen interrelationship. Biochim. Biophys. Acta, 230, 309.

Feigelson, M. (1971c) Androgenic regulation of rat liver histidase. Endocrinology, 89, 625.

Feigelson, M. (1972) Differential hormonal control of histidase in liver and epidermis during postnatal development. Abstract 21, Fourth International Congress on Endocrinology, Washington D.C. p 8.

Feigelson, M. (1973a) Studies on the postnatal development of responses of hepatic histidase to endocrine control. Biochim. Biophys. Acta, 304, 669.

Feigelson, M. (1973b) Multihormonal regulation of hepatic histidase during postnatal development. Enzyme, 15, 169.

Feigelson, M. (1973c) Hormonal control of hepatic histidase during postnatal development. Abstract 85-11, Ninth International Congress on Biochemistry, Stockholm. p 357.

Feigelson, M. (1974) Relationship between tissue specific responses to hormones and differential postnatal developmental patterns of histidase in rat liver and epidermis. Abstract 248, Fourth International Congress on Hormonal Steroids, Mexico City. J. Steroid Biochem., 5, 355.

Figge, F.H.J., and Strong, L.C. (1941) Xanthine oxidase (dehydrogenase) activity in livers of mice of cancer-susceptible and cancer-resistant strains. Canc. Res., 1, 779.

Fisher, R.A., and Harris, H. (1971a) Studies on the separate isozymes of red cell acid phosphatase phenotypes A. and B. I Ann. Hum. Genet. Lond., 34, 431.

Fisher, R.A., and Harris, H. (1971b) Studies on the separate isozymes of red cell acid phosphatase phenotypes A. and B. II Ann. Hum. Genet. Lond., 34, 439.

Fisher, R.A., and Harris, H. (1972) 'Secondary' isozymes derived from the three PGM loci. Ann Hum. Genet. Lond., 34, 69.

Fleck, A., and Begg, D. (1965) The estimation of ribonucleic acid using ultraviolet absorption measurements. Biochim. Biophys. Acta, 108, 333.

Flexner, L.V. (1955) In Biochemistry of the Developing Nervous System. Ed. H. Waelsch. New York: Academic Press. p 281.

Folch-Pi, J. (1955) Composition of the brain in relation to maturation. In Biochemistry of the Developing Nervous System. Ed. H. Waelsch. New York: Academic Press. p 121.

Fox, M.W. (1965) Reflex-ontogeny and behavioural development of the mouse. Anim. Behav., 13, 234.

Frankfater, A., and Fridovich, I. (1970) The purification and properties of oxidised derivatives of L-histidine ammonia-lyase. Biochim. Biophys. Acta, 206, 457.

Frew, I.J.C., and Menon, G.N. (1976) Betahistine hydrochloride in Ménière's disease. Postgrad. Med. J., 52, 501.

Funakoshi, S., and Deutsch, H.F. (1969) Human carbonic anhydrases. II Some physico-chemical properties of native isozymes and of similar isozymes generated in vitro. J. Biol. Chem., 244, 3438.

Gaitonde, M.K., and Richter, D. (1966) Changes with age in the utilisation of glucose carbon in liver and brain. J. Neurochem., 13, 1309.

Galamon, T., and Szulc-Kuberska, J. (1966) Hereditary abnormality of histidine in stuttering. Abstr. 3rd Ann. Meet., Fed. Eur. Biochem. Soc. (Free Papers). Warsaw.

Ganrot, P.O., Rosengren, A.M., and Rosengren, E. (1961) On the presence of different histidine decarboxylating enzymes in mammalian tissues. Experientia, 17, 263.

Ganschow, R. (1971) Pers. Commun. quoted in Paigen (1971) p 12.

Garrod, A.E. (1902) The incidence of alkaptonuria: a study in chemical individuality.
Lancet, 2, 1616.

Garrod, A.E., (1908) Croonian lectures on inborn errors of metabolism. Lancet, 2, 1, 73, 142 and 214.

Gatfield, P.D., Knights R.M., Devereuz, M., and Pozsonyi J.P. (1969) Histidinaemia: report of four new cases in one family and the effect of low-histidine diets. Can. Med. Assoc. J., 101, 465.

Gaull, G.E., Tallan, H.H., Lajtha, A., and Rassin, D.K. (1975) Pathogenesis of brain dysfunction in inborn errors of amino acid metabolism. In Biology of Brain Dysfunction. Ed. G.E. Gaull. New York: Plenum Press. p 47.

Gayet, J., and Bonichon, A. (1961) Morphological differentiation and metabolism in the optic lobes of the chick embryo. In Regional Neurochemistry. Eds. S.S. Kety and J. Elkes. London: Pergamon Press. p 135.

Gelehrter, T., Motulsky, A.G., and Omenn, G.S. (1970) Genetic control mechanisms in man and other animals. Science, 169, 791.

Gehler, J., and Stopfkuchen, H. (1977) Histidinamie mit klinischen zeichen des Marfan-syndrom. Mschr. Kinderheilk., 125, 481.

Gerritsen, T. (1964) Histidinaemia and mental retardation. In Proceedings International Copenhagen Congress on the Scientific Study of Mental Retardation, 1, 94.

Gerritsen, T., and Siegel, F.L. (1972) The use of animal models for the study of amino acidopathies. In Monogr. Human Genet., Vol. 6, Karger, Basel. p 22.

Ghadimi, H., Partington, M.W., Hunter, A. (1961a) A familial disturbance of histidine metabolism. New Eng. J. Med., 265, 221.

Ghadimi, H., Partington, M.W., Hunter, A., and Bailey, I.D. (1961b) An inborn error of histidine metabolism mimicking phenylketonuria. Am. J. Dis. Childh., 102, 454.

Ghadimi, H. Partington, M.W., Hunter, A. (1962) Inborn error of histidine metabolism. Pediatrics, N.Y., 29, 714.

Ghadimi, H., Partington, M.W., (1967) Salient features of histidinaemia. Amer. J. Dis. Childh., 113, 83.

Ghadimi, H., and Zischka, R. (1967) In Amino Acid Metabolism and Genetic Variation, Chapt. 6. New York: McGraw-hill.

Ghadimi, H., (1974) Histidinaemia: emerging clinical picture. In Heritable Disorders of Amino Acid Metabolism. Ed. W.L. Nyhan. London: John Wiley and Sons.

Giles, K.W., and Myers, A. (1965) An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature, Lond., 206, 93.

Gilman, P.A., and Howell, R.R. (1969) The simultaneous occurrence of histidinemia and congenital hypoplastic anemia. J. Paediat., 75, 878.

Gitlow, S., Mendlowitz, M., Wilk, E.K., Wolf, R., and Glick, J. (1965) Excretion of catecholamine metabolites by normal children and those with familial dysautonemia. J. Clin. Invest., 44, 1049.

Givot, I.L., Smith, T.A., and Abeles, R.H. (1969) Studies on the mechanism of action and the structure of the electrophilic centre of histidine ammonia-lyase. J. Biol. Chem., 244, 6341.

Givot, I.L., and Abeles, R.H. (1970) Mammalian histidine ammonia lyase. In vivo inactivation and presence of an electrophilic center at the active site. J. Biol. Chem., 245, 3271.

Givot, I.L., Mildvan, A.S., and Abeles, R.H. (1970) Magnetic resonance studies of an enzyme-metal-substrate bridge complex in histidine ammonia lyase. Fed. Proc., 29, 1590.

Glowinski, J., and Iversen, L.L. (1966) Regional studies of catecholamines in the rat brain - I. J. Neurochem., 13, 655.

Gordon, N. (1970) Delayed speech and histidinaemia. Develop. Med. and Child Neurol., 12, 104.

Gray, G.M., Conklin, K.A., and Townley, R.R.W. (1976) Sucrose-isomaltase deficiency. New Eng. J. Med., 294, 750.

Green, J.P. (1970) Histamine. In Handbook of Neurochemistry. Ed. A. Lajtha. New York: Plenum Press. Ch. 4, p 221.

Griffiths, M. (1973) Clinical aspects of the dietary treatment of histidinaemia. A report of four cases. In Treatment of Inborn Errors of Metabolism. Eds. J.W.T. Seakins, R.A. Saunders and C. Toothill. Edinburgh: Churchill Livingstone. p 87.

Guthrie, R. (1968) Screening for "inborn errors of metabolism" in the newborn infant - a multiple test program. Birth Defects, Original Article Series, 6, 92.

Guthrie, R., and Murphy, W.H. (1971) Microbiologic screening procedures for detection of inborn errors of metabolism in the newborn infant. In Phenylketonuria and Some Other Inborn Errors of Amino Acid Metabolism: Biochemistry, Genetics, Diagnosis and Therapy. Eds. H. Bickel, R.P. Hudson and L. Wolf. Stuttgart Thieme. p 132.

Haavaldsen, R. (1962) Transamination of aromatic amino-acids in nervous tissue. Nature, Lond., 196, 577.

Haddara, M. (1956) A quantitative study of the postnatal changes in the packing density of the neurons in the visual cortex of the mouse. J. Anat., 90, 494.

Hague, R.V., and Holton, J.B. (1971) An intravenous load test for the detection of heterozygotes for histidinaemia. Clin. Chim. Acta, 33, 462.

Hais, I.M., and Zenisek, A. (1959) Urocanic acid, a physiological sunscreen. Amer. Perfum. and Aromatics, 73, 26.

Hall, D.A. (1952) Histidine -deaminase and the production of urocanic acid in the mammal. Biochem. J., 51, 499.

Hanford, W.C., Nep, R.L., and Arfin, S.M. (1974) Genetic variation in histidine ammonia-lyase activity in the mouse. Biochem. Biophys. Res. Commun., 61, 1434.

Hanford, W.C., and Arfin, S.M. (1977) Genetic differences in the rate of histidase synthesis in inbred mice. J. Biol. Chem., 252, 6695.

Hanson, A. (1966) La N-acétylhistidine comme donneur d'acétyle dans la biosynthèse de l'acétylcholine due cerveau de poisson. Acta Chem. Scand., 20, 159.

Hanson, K.R. and Havir, E.A. (1972) In The Enzymes, 3rd edition. Ed. P.D. Boyer. New York: Academic Press. Ch. I, p 75.

Harris, H. (1975) The Principles of Human Biochemical Genetics, 2nd edition. Publishen North-Holland/American Elsevier.

Harris, H. (1979) Multilocus enzymes in man. In Human Genetics: Possibilities and Realities, Ciba Foundation Symp., 66 (new series). Excerpta Medica, p 187.

Hassall, H., and Greenberg, D.M. (1963) The oxidation of 4(5)-imidazolone-5(4)-propionic acid to hydanton-5-propionic acid by xanthine oxidase. Biochim. Biophys. Acta, 67, 507.

Hassall, H., Lunn, P.J., and Ryall-Wilson, J. (1970) Detection of histidase and urocanase after disc electrophoresis on polyacrylamide gels. Anal. Biochem., 35, 326.

Hassall, H., and Soutar, A.K. (1974) Amino acid sequence of peptide containing the active cysteine residue of histidase. Biochem. J., 137, 559.

Heller, I.H., and Elliott, K.A.C. (1954) Desoxyribonucleic acid content and cell density in brain and human brain tumors. Canad. J. Biochem., 32, 584.

Herbert, J.D., Coulson, R.A., and Hernandez, T. (1966) Free amino acids in the caiman and rat. Comp. Biochem. Physiol., 17, 583.

Hess, A. (1955) Blood-brain barrier and ground substance of central nervous system. Arch. Neurol. Psychiat., 73, 380.

Hess, H.H. and Thalheimer, C. (1965) Microassay of biochemical structural components in nervous tissues. J. Neurochem., 12, 193.

Himwich, W.A. (1962) Biochemical and neurophysiological development of the brain in the neonatal period. Int. Rev. Neurobiol., 4, 117.

Holmgren, G., Hambraeus, L., de Chateau, P. (1974) Histidinaemia and normohistidinemic histidinuria: report of three cases and the effect of different protein intakes on urinary excretion of histidine. Acta Paediatr. Scand., 63, 220.

Holmquist, W.R., and Schroeder, W.A. (1966) A new N-terminal blocking group involving a schiff base in hemoglobin. Biochemistry, 5, 2489.

Holton, J.B. (1964) Histidinaemia. F.E.B.S. Abstracts of Communications Presented at the First Meeting of the Federation. New York: Academic Press. p 117.

Holton, J.B., Lewis, F.J.W., and Moore, G.R. (1964) Biochemical investigation of histidinaemia. J. Clin. Path., 17, 671.

Holton, J.B. (1965) Skin L-histidine ammonia-lyase activity in the family of a child with histidinaemia. Clin. Chim. Acta, 11, 193.

Holton, J.B., Jones, S., and Small, N.A. (1968) Amino acid changes associated with febrile illness in a case of histidinaemia and in other children. Clin. Sci., 35, 425.

Hopkinson, D.A., and Harris, H. (1969) The investigation of reactive sulphydryls in enzymes and their variants by starch gel electrophoresis. Studies on red cell deaminase. Ann. Hum. Genet., Lond., 33, 81.

Hopkinson, D.A. (1970) The investigation of reactive sulphydryls in enzymes and their variants by starch gel electrophoresis: studies on the human phosphohexose isomerase variant, PH1 5-1. Ann. Hum. Genet., Lond., 34, 79.

Hosein, E.A., and Smart, M. (1960) The presence of anserine and carnosine in brain tissue. Can. J. Biochem. Physiol., 38, 569.

Howard, E. (1968) Reductions in size and total DNA of cerebrum and cerebellum in adult mice after corticosteril treatment in infancy. Exp. Neurol., 22, 191.

Howell, R.R. (1970) Inborn errors of metabolism: some thoughts about their basic mechanisms. Pediat., 45, 901.

Hudson, F.P., Dickinson, R.A., and Ireland, J.T. (1963) Experiences in the detection and treatment of phenylketonuria. Pediatrics, 31, 47.

Hunt, R.K., and Jacobson, M. (1971) Neurogenesis in frogs and early larval treatment with somatotropin or prolactin. Develop. Biol., 26, 100.

Hutchison, W.C., and Munro, H.N. (1961) The determination of nucleic acids in biological materials. The Analyst, 86, 768.

Jacob, F., and Monod, J. (1961) Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 3, 318.

Jacobson, M. (1970) In Developmental Neurobiology. Holt, Rinehart and Winston, Inc.

Kacser, H., Bulfield, G., and Wallace, M. (1973) Histidinaemic mutant in the mouse. Nature, Lond., 244, 5411, 77.

Kacser, H., Mya Mya, K., Duncker, M., Wright, A.F., and Bulfield, G. (1977) Maternal histidine metabolism and its effect on foetal development. Nature, Lond., 265, 5591, 262.

Kacser, H., Bulfield, G., and Wright, A. (1979a) The biochemistry and genetics of histidinaemia in the mouse. In Models for the Study of Inborn Errors of Metabolism. Ed. F.A. Hommes. Elsevier/North-Holland. p 33.

Kacser, H., Mya Mya, K., and Bulfield, G. (1979b) Endogenous teratogenesis in maternal histidinaemia. In Models for the Study of Inborn Errors of Metabolism. Ed. F.A. Hommes. Elsevier/North-Holland. p 43.

Kahlson, G., Rosengren, E., Westling, H., and White, T. (1958) The site of increased formation of histamine in the pregnant rat. J. Physiol., Lond., 144, 337.

Kahlson, G.E., Rosengren, E., and Thunberg, R. (1963) Observations on the inhibition of histamine formation. J. Physiol., Lond., 169, 467.

Kahlson, G., and Rosengren, E. (1968) New approaches to the physiology of histamine. Physiol. Revs., 48, 155.

Kakimoto, Y., and Armstrong, M.D. (1961) β-L-Aspartyl-L-histidine, a normal constituent of human urine. J. Biol. Chem., 236, 3280.

Kapeller-Adler, R., and Iggo, B., (1957) Histamine and its derivatives in human urine. Biochim. Biophys. Acta, 25, 394.

Kapeller-Adler, R. (1970) Amine oxidases and methods for their study. Wiley - Interscience.

Kapellman, M., Thomas, G.H., and Howell, R.R. (1971) Histidinemia in a negro child. Amer. J. Dis. Childh., 122, 212.

Karjala, S.A., Turnquest, B.W., and Schayer, R.W. (1956) Urinary metabolites of radioactive histamine. J. Biol. Chem., 219, 9.

Karjala, S.A., and Turnquest, B.W. (1955) The characterization of two methylimidazoleacetic acids as radioactive histamine metabolites. J. Amer. Chem. Soc., 77, 6358.

Karn, R.C., Shulkin, J.D., Merritt, A.D., and Newell, R.C. (1973) Evidence for post-transcriptional modification of human salivary amylase (Amy,) isozymes. Biochem. Genet., 10, 341.

Karrer, R., and Cahilly, G. (1965) Experimental attempts to produce phenylketonuria in animals: a critical review. Psychol. Bull., 64, 52.

Kato, A., Yoshioka, Y., Watanabe, M., and Suda, M. (1955) Metabolism of histidine. VI. J. Biochem. (Tokyo), 42, 305.

Kerr, G.R., and Waisman, H.A. (1966) Phenylalanine: transplacental concentrations in rhesus monkeys. Science, 151, 824.

Khanolkar, V.R., and Chitre, R.G. (1942) Studies in esterase (butyric) activity. I. Esterase content of serum of mice from certain cancer-resistant and cancer-susceptible strains. Canc. Res., 2, 567.

Kibel, M.A., and Levy, H.L. (1970) A further case of histidinaemia: clinical and biochemical aspects. South African Med. J., 44, 242.

Kierland, R.R., Perry, H.O., Winkelmann, R.K., Muller, S.A., and Sams, W.M. (1968) Society transactions. Arch. Dermatol., 98, 316.

Kilhara, H., Boggs, D.E., Lassila, E.L., and Wright, S.W. (1968) Histidinaemia: studies on histidase activity in stratum corneum. Biochem. Med., 2, 243.

Kirkman, H.N. (1972) Enzyme defects. In Progr. Med. Genet., 8, 125.

Klee, C.B. (1970) Reversible polymerization of histidine ammonia-lyase. The Role of Sulfhydryl groups in the activity and polymeric state of the enzyme. J. Biol. Chem., 245, 3143.

Klee, C.B. (1972) Metal activation of histidine ammonia-lyase. J. Biol. Chem., 247, 1398.

Klee, C.B., Kirk, K.L., Cohen, L.A., and McPhie, P. (1975) Histidine ammonia-lyase. The use of 4-fluorohistidine in identification of the rate-determining step. J. Biol. Chem., 250, 5033.

Klee, C.B., Kirk, K.L., and Cohen, L.A. (1979) 4-Nitro-L-histidine as a substrate for histidine ammonia-lyase: the role of -hydrogen acidity in the rate-limiting step. Biochem. Biophys. Res. Commun., 87, 343.

Knox, W.E. (1972) Phenylketonuria. In The Metabolic Basis of Inherited Disease, 3rd edition. Eds. J.B. Stanbury, J.B. Wyngaarden, and D.S. Fredrickson. New York: McGraw-Hill. p 262.

Kobayashi, T. (1963) Brain-to-body ratios and time of maturation of the mouse brain. Am. J. Physiol., 204, 343.

Kobayashi, Y., and Maudsley, D.V. (1972) A single-isotope enzyme assay for histamine. Anal. Biochem., 46, 85.

Koch, R., Dobson, J.C., Blaskovics, M., Williamson, Y.L., Ernest, A.E., Friedman, E.G., and Parker, C.E. (1973) Collaborative study of children treated for phenylketonuria. In Treatment of Inborn Errors of Metabolism. Eds. J.W.T. Seakins, R.A. Saunders, and C. Toothill. Edinburgh: Churchill Livingstone. p 3.

Konishi, H., and Kakimoto, Y. (1976) Formation of -glutamylhistamine from histamine in rat brain. J. Neurochem., 27, 1461.

Kothari, P.D., Desai, A.D., Banerjee, A.P., and Karandikar, P.V. (1970) Histidinemia. Neurol. (India), 22, 141.

Kotsopoulos, S., and Kutty, K.M. (1979) Histidinemia and infantile autism. J. Aut. Dev. Dis., 9, 55.

Krebs, E.G., Kent, A.B., and Fischer, E.H. (1958) The muscle phosphorylase kinase reaction. J. Biol. Chem., 231, 73.

Kwogochi, V., Fukui, Y., Nakagawa, T., and Yamamoto, I. (1957) A note on as-urocanic acid. Jap. J. Pharmacol., 6, 147.

La Du, B.N., Howell, R.R., Jacoby, G.A., Seegmiller, J.E., Zannoni, V.G. (1962) The enzymatic defect in histidinaemia. Biochem. Biophys. Res. Commun., 7, 398.

La Du, B.N., Howell, R.R., Jacoby, G.A., Seegmiller J.E., Sober, E.K., Zannoni, V.G., Canby, J.R., and Ziegler, L.K. (1963) Clinical and biochemical studies on two cases of histidinaemia. Pediatrics, 32, 216.

La Du, B.N. (1972) Histidinaemia. In The Metabolic Basis of Inherited Disease, 3rd edition. Eds. J.B. Wyngaarden and D.S. Frederickson. New York: McGraw-Hill.

La Du, B.N. (1978) Histidinaemia. In The Metabolic Basis of Inherited Disease, 4th edition. Eds. J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. New York: McGraw-Hill.

Lajtha, A., and Toth, J. (1973) Perinatal changes in the free amino acid pool of the brain in mice. Brain Res., 55, 238.

Lalley, D.A., and Shows, T.B. (1977) Lysosomal acid phosphatase deficiency: liver specfic variant in the mouse. Genetics, 87, 305.

Lamartiniere, C.A., and Feigelson, M. (1977) Effects of estrogen, glucocorticoid, glucagen, and adenosine 3 : 5-monophosphate on catalytic activity, amount, and rate of de novo synthesis of hepatic histidase. J. Biol. Chem., 252, 3234.

Lamartiniere, C.A., and Lucier, G.W. (1978) Programming of hepatic histidase following prenatal administration of diethylstilbestrol. J. Steroid Biochem., 9, 595.

Lancet (1974) The duration of treatment of phenylketonuria. Editorial, 1, 971.

Langridge, J. (1968a) Genetic and enzymatic experiments relating to the tertiary structure of β-galactosidase. J. Bacteriol., 96, 1711.

Langridge, J. (1968b) Genetic evidence of the disposition of the substrate binding site of β-galactosidase. Proc. Nat. Acad. Sci., U.S., 60, 1260.

Lapham, L.W. (1968) Tetraploid DNA content of Purkinje neurons of human cerebellar cortex. Science, 159, 310.

Lee, S.C., and Harper, A.E. (1971) Responses of mammalian histidine catabolic enzymes to dietary and hormonal treatments. Biochim. Biophys. Acta, 244, 135.

Lehmann, H., and Carrell, R.W. (1969) Variations in the structure of human hemoglobin: with particular reference to the unstable hemoglobins. Brit. Med. Bull., 25, 14.

Lehrer, G.M., Bornstein, M.B., Weiss, C., and Silides, D.J. (1970) Enzymatic maturation of mouse cerebral neocortex in vitro and in situ. Exp. Neurol., 26, 595.

Levenson, J., Lindahl-Kiessling, K., and Rayner, S. (1964) Carnosine excretion in juvenile amaurotic idiocy. Lancet, 2, 756.

Levy, H.L. (1973) Genetic screening. Adv. Hum. Genet., 4, 1.

Levy, H.L., Shih, V.E., and Madigan, P.M. (1974) Routine newborn screening for histidinemia. New Engl. J. Med., 291, 1214.

Levy, H.L., and Shih, V.E. (1974) Maternal phenylketonuria and hyperphenylalaninemia. A prospective study. Pediatr. Res., 8, 391.

Lin, E.C.C., Pitt, B.M., Civen, M., Knox, W.E. (1958) Assay of aromatic amino acid transaminations and keto acid oxidation in the enol-borate-tautomerase method. J. Biol. Chem., 233, 668.

Litwack, G., and Nemeth, A.M. (1965) Development of liver tyrosine aminotransferase activity in the rabbit, guinea pig and chicken. Arch. Biochem., 109, 316.

Lott, I.T., Wheelden, J.A., Levy, H.L. (1970) Speech and histidinemia: methodology and evaluation of four cases. Devel. Med. Child Neurol., 12, 596.

Lovenberg, W., Weissbach, H., and Udenfriend, S. (1962) Aromatic L-amino acid decarboxylase. J. Biol. Chem., 237, 89.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265.

Lumsden, J., and Coggins, J.R. (1977) The subunit structure of the arom multienzyme complex of neurospora crassa: a possible pentafunctional polypeptide chain. Biochem. J., 161, 599.

Lyon, I.C.T., and Veale, A.M.O. (1970) Histidinemia. Proc. Univ. of Otago Med. School, 48, 68.

Lyon, I.C.T., Gardner, R.J.M., and Veale, A.M.O. (1974) Maternal histidinaemia. Arch. Dis. Childh., 49, 581.

Lyon, M.F., Glenister, P.H., and Lamoreux, M.L. (1975) Normal spermatozoa from androgen-resistant germ cells of chimaeric mice and the role of androgen in spermatogenesis. Nature, Lond., 258, 620.

Mabry, C.C., Denniston, J.C., Nelson, T.L., and Son, C.D. (1963) Maternal phenylketonuria. New Eng. J. Med., 269, 1404.

Mabry, C.C., Denniston, J.C., and Coldwell, J.G. (1966) Mental retardation in children of phenylketonuric mothers. New Eng. J. Med., 275, 1331.

MacCready, R.A., and Levy, H.L. (1972) The problem of maternal phenylketonuria. Amer. J. Obs. Gynecol., 113, 121.

McKusick, V.A. (1975) Mendelian Inheritance in Man, 4th edition. Baltimore and London: The Johns Hopkins University Press.

McManus, I.R. (1956) Biological mehtylation of anserine. Fed. Proc., 15, 312.

MacMillan, A. (1970) Pers. Commun. quoted in Ghadimi (1974).

MacPherson, H.T. (1942) Modified procedures for the colorimetric estimation of arginine and histidine. Biochem. J., 36, 59.

Makoff, R., and Baldridge, R.C. (1969) The metabolism of histidine. Liver enzyme changes during development. Biochim. Biophys. Acta, 90, 282.

Mandel, P., and Bieth, R. (1952) Comparative study of the biochemical development of the brain in various mammals. C.R. Acad. Sci., 235, 485.

Margolis, F.L. (1974) Carnosine in the primary olfactory pathway. Science, 184, 909.

Marti, H.R., Beale, D., and Lehmann, H. (1967) Hemoglobin Koelliker: the new hemoglobin appearing after severe hemolysis: α-2 minus 141 Arg β-2. Acta haemat., Basel, 37, 174.

Martignoni, P., and Winick, T. (1954) Biosynthesis of carnosine and anserine in the chick. J. Biol. Chem., 208, 251.

Martin, J.J., and Schlote, W. (1972a) Central nervous system lesions in disorders of amino-acid metabolism: a neuropathological study. J. Neurol. Sci., 15, 49.

Martin, J.J., and Schlote, W. (1972b) Neuropathological study of aminoacidurias. In Aminoacidopathies, Immunoglobinopathies, Neuro-genetics and Neuro-Ophthalmology. Ed. J. François. Basel: S. Karger. p 64.

Martinez, D.M. (1972) The effect of Serc (betahistine hydrochloride) on the circulation of the inner ear in experimental animals. Acta Otolaryngol. (Suppl.), 305, 29.

Massengill, R., Jr., and Smith, D.D. (1967) Histidinemia and language development. A case report. J. Comm. Dis., 1, 51.

Maudsley, D.V., and West, G.B. (1964) The site of increased formation of histamine in the pregnant mouse. J. Physiol., Lond., 174, 473.

Mehler, A.H., and Tabor, H. (1953) Deamination of histidine to form urocanic acid in liver. Biol. Chem., 201, 775.

Meister, A., and Fraser, P.E. (1954) Enzymatic formation of L-asparagine by transamination. J. Biol. Chem., 210, 37.

Meister, A. (1965) Biochemistry of the Amino Acids. New York and London: Academic Press.

Melancon, S.B., Lee, S.Y., and Nadler, H.L. (1971) Histidase activity in cultivated amniotic fluid cells. Science, 173, 627.

Midelfort, C.F., and Mehler, A.H. (1972) Deamidation in-vivo of an asparagine residue of rabbit muscle aldolase. Proc. Natl. Acad. Sci., U.S., 69, 1816.

Minatogawa, Y., Noguchi, T., and Kido, R. (1977) Species distribution and properties of hepatic phenylalanine (histidine): pyruvate aminotransferase. Hoppe-Seyler's Z. physiol. Chem., 358, 59.

Mirsky, A.E., and Ris, H. (1951) The desoxyribonucleic acid content of animal cells and its evolutionary significance. J. Gen. Physiol., 34, 451.

Moog, F. (1971) The control of enzyme activity in mammals in early development and in old age. In Enzyme Synthesis and Degradation in Mammalian Systems. Ed. M. Rechcigl. Basel: Karger. p 47.

Morris, M.L., Lee, S.C., and Harper, A.E. (1972) Influence of differential induction of histidine catabolic enzymes on histidine degradation in vivo. J. Biol. Chem., 247, 5793.

Morris, M.L., Lee, S.C., and Harper, A.E. (1973) A comparison of the responses of mitochondrial and cytosol histidine - pyruvate aminotransferase to nutritional and hormonal treatments. J. Biol. Chem., 248, 1459.

Morrow, A.G., Greenspan, E.M., and Carroll, D.M. (1949) Liver - glucuronidase activity of inbred mouse strains. J. Nat. Cancer Inst., 10, 657.

Morrow, A.G., Greenspan, E.M., and Carroll, D.M. (1950) Comparative studies of liver - glucuronidase activity in inbred mice. J. Nat. Cancer Inst., 10, 1199.

Mudd, S.H., Edwards, W.A., Loeb, P.M., Brown, M.S. and Laster, L. (1970) Homocystinuria due to cystathionine synthase deficiency: the effect of pyridoxine. J. Clin. Invest., 49, 1762.

Muller, C.J. (1961) A comparative study on the structure of mammalian and avian hemoglobins. In Molecular Evolution. Netherlands: Van Gorcum, Assen.

Munro, H.N., and Fleck, A. (1966) Recent developments in the measurement of nucleic acids in biological materials. The Analyst, 91, 78.

Munro, H.N. (1974) Control of plasma amino acid concentrations. In Aromatic Amino Acids in the Brain. CIBA Foundation Symposium, 22. Amsterdam: Elsevier. p 5.

Mya Mya, K. (1978) Inner ear development in maternal histidinaemia in the mouse. PhD. Thesis, University of Edinburgh.

Nakagawa, I., Takahashi, T., Suzuki, T., and Kobayashi, K. (1963) Amino acid requirements of children: minimal needs of tryptophan, arginine and histidine based on nitrogen balance method. J. Nutr., 80, 305.

Nakajima, T., Wolfgram, F., and Clark, W.G. (1967) The isolation of homoanserine from bovine brain. J. Neurochem., 14, 1107.

Neame, K.D. (1968) Transport, metabolism and pharmacology of amino acids in brain. In Applied Neurochemistry. Eds. A.N. Davison and J. Dobbing. Oxford and Edinburgh: Blackwell Scientific Publications. p 119.

Neville, B.G.R., Harris, R.F., Stern, D.J., Stern, J. (1971) Maternal histidinaemia. Arch. Dis. Childh., 46, 119.

Neville, B.G.R., Bentovim, A., Clayton, B.E., and Shepherd, J. (1972) Histidinaemia. Study of relation between clinical and biological findings in 7 subjects. Arch. Dis. Childh., 47, 190.

Neville, B.G.R., and Lilley, P.M. (1973) Histidinaemia: its significance in neonatal screening. Arch. Dis. Childh., 48, 325.

Noda, K., and Nakagawa, H. (1972) Study on the sex difference in rat liver histidase. Endocrinol., 90, 207.

Noguchi, T., Okuno, E., Minatogawa, Y., and Kido, R. (1976a) Purification, characterization and identification of rat liver histidine - pyruvate aminotransferase isoenzymes. Biochem. J., 155, 107.

Noguchi, T., Minatogawa, Y., Okuno, E., and Kido, R. (1976b) Organ distribution of rat histidine - pyruvate aminotransferase isoenzymes. Biochem. J., 157, 635.

O'Gorman, L.P. (1968) The estimation of vanilmandelic acid (VMA) in serum and urine by a radioactive ^{14}C labelling technique. Clin. Chim. Acta, 19, 485.

Ohno, S., Payne, H.W., Morrison, M., and Beutler, E. (1966) Hexose-6-phosphate dehydrogenase found in human liver. Science, 153, 1015.

Okamura, H., Nishida, T., and Nakagawa, H. (1974) L-histidine ammonia-lyase in rat liver. I purification and general characteristics. J. Biochem., 75, 139.

Ono, S., and Hagen, P. (1959) Pyridoxal phosphate: a coenzyme for histidine decarboxylase. Nature, Lond., 184, 1143.

Orr, E.L., and Quay, W.B. (1975) Hypothalamic 24-hour rhythms in histamine, histidine decarboxylase and histamine-N-methyltransferase. Endocrinol., 96, 941.

Padua, R.A., Bulfield, G., and Peters, J. (1978) Biochemical genetics of a new glucosephosphate isomerase allele ($Gpi-1^c$) from wild mice. Biochem. Genet., 16, 127.

Paigen, K.M. (1971) The genetics of enzyme realization. In Enzyme Synthesis and Degradation in Mammalian Systems. Ed. M. Rechcigl. Basel: Karger. p 1.

Paigen, K., Swank, R.T., Tomino, S., and Ganschow, R.E. (1975) The molecullar genetics of mammalian glucuronidase. J. Cell. Physiol., 85, 379.

Patel, A.J., and Balazs, R. (1975) Effect of X-irradiation on the biochemical maturation of rat cerebellum: metabolism of [^{14}C]-glucose and [^{14}C]-acetate. Radiat. Res., 62, 456.

Penrose, L.S. (1963) The Biology of Mental Defect, 3rd edition. London: Sidgwick and Jackson Ltd.

Perry, T.L., Hansen, S., Tischler, B., Bunting, R., and Berry, K. (1967) Carnosinemia: a new metabolic disorder associated with neurologic disease and mental defect. New Engl. J. Med., 277, 1219.

Perry, T.L., Hansen, S., and Love, D.L. (1968) Serum-carnosinase deficiency in carnosinaemia. Lancet, 1, 1229.

Perutz, M.F., and Lehmann, H. (1968) Molecular pathology of human haemoglobin. Nature, Lond., 19, 902.

Peterkofsky, A. (1962) The mechanism of action of histidase: amino-enzyme formation and partial reactions. J. Biol. Chem., 237, 787.

Peterkofsky, A., and Mehler, L.N. (1963) Inhibition of pseudomonas histidase. Evidence for a metal cofactor. Biochim. Biophys. Acta, 73, 159.

Phillis, J.W., Tebecis, A.K., and York, D.H. (1968) Histamine and some antihistamines: their actions on cerebral cortical neurones. B.J. Pharmacol., 33, 426.

Piomelli, S., Corash, L.M., Davenport, D.D., Miraglia, J., and Ambrose, E.L. (1968) Glucose-6-phosphate dehydrogenase deficiency (Types Gd^{A-} and Gd ^{Mediterranean}): the result of in vivo instability of the mutant enzymes. J. Clin. Invest., 47, 940.

Pisano, J.P., Wilson, J.D., Cohen, L., Abraham, D., and Udenfriend, S. (1961) Isolation of γ- aminobutyrylhistidine (homocarnoside) from brain.
J. Biol. Chem., 236, 499.

Plöchl, E., Tasser, C., and Jarosch, E. (1978) Histidinämie bei einem normal intelligenten, jedoch hörbehinderten knaben. Padiatr. Padologie, 13, 303.

Polar, E., and Metcoff, J. (1965) "True" creatinine chromogen determination in serum and urine by semi-auto-mated analysis. Clin. Chem., 11, 763.

Popkin, J.S., Clow, C.L., Scriver, C.R., and Grove, J. (1974) Is hereditary histidinaemia harmful? Lancet, 1, 721.

Pratt, O.E. (1976) The transport of metabolizable substances into the living brain. In Transport Phenomena in the Nervous System; Physiological and Pathological Aspects. Eds. G. Levi, L. Battistin and A. Lajtha. New York: Plenum Press. p 55.

Quinn, M.R., and Fisher, H. (1977) Effect of dietary histidine on olfaction, and rat brain and muscle concentration of histidine-containing dipeptides. J. Neurochem., 29, 717.

Ramón y Cajal, S. (1960) In Studies on Vertebrate Neurogenesis. Illinois: Charles C. Thomas, Springfield. p 327.

Rao, S.R., Subhash, M.N., and Narayanan, H.S. (1974) Histidinemia. Indian J. Pediat., 41, 312.

Rechcigl, M., Jr., and Heston, W.E. (1967) Genetic regulation of enzyme activity in mammalian system by the alteration of the rate of enzyme degradation. Biochem. Biophys. Res. Commun., 27, 119.

Rechler, M.M. (1969) The purification and characterization of L-histidine ammonia-lyase (pseudomonas). J. Biol. Chem., 244, 551.

Reichelt, K.L., Edminson, P.D., and Kvamme, E. (1975) Amine-dependent synthesis of peptides and peptido-amines in nervous tissue. Biochem. Soc. Trans., 3, 112.

Reichlin, M. (1972) Localizing antigenic determinants in human haemoglobin with mutants: molecular correlations of immunological tolerance. J. Mol. Biol., 64, 485.

Reichlin, M. (1975) Amino acid substitution and the antigenicity of globular proteins. Adv. Immunol., 20, 71.

Renfree, M.B., and McLaren, A. (1974) Foetal origin of transferrin in mouse amniotic fluid. Nature, Lond., 252, 159.

Renfree, M.B., Hensleigh, H.C., and McLaren, A. (1975) Developmental changes in the composition and amount of mouse foetal fluids. J. Embryol. exp. Morph., 33, 435.

Retey, J., Fierz , H., and Zeylemaker, W.P. (1970) Steric course of the histidase reaction. Febs Lett., 6, 203.

Richards, G.M. (1974) Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. Anal. Biochem., 57, 369.

Roberts, E.E.G., and Ireland, J.T. (1978) Pers. Commun. quoted in La Du (1978).

Roberts, E., Harman, P.J., and Frankel, S. (1951) γ-Aminobutyric acid content and glutamic decarboxylase activity in developing mouse brain. Proc. Soc. Exp. Biol. Med., 78, 799.

Robinson, D., and Stirling, J.L. (1968) N-acetyl- -glucose auminidascs in human spleen. Biochem. J., 107, 321.

Rose, W.C., Haines, W.J., Warner, D.T., and Johnson, J.E. (1951) The amino acid requirements of man, II The role of threonine and histidine. J. Biol. Chem., 188, 49.

Rosenberg, L.E., and Scriver, C.R. (1974) Disorders of amino acid metabolism. In Duncan's Diseases of Metabolism, 7th edition. Eds. P.K. Bandy and L.E. Rosenberg. New York: W.B. Saunders Co. p 465.

Rosenblatt, D., Mohyuddin, F., and Scriver, C.R. (1970) Histidinemia discovered by urine screening after renal transplantation. Pediatrics, 46, 47.

Rosengren, E. (1963) Histamine metabolism in the pregnant mouse. J. Physiol., 169, 499.

Rosengren, E. (1965) Histamine metabolism in the pregnant and non-pregnant hamster. Proc. Soc. Exptl. Biol. Med., 118, 884.

Rostenberg, I., Guizar, J., Alejandre, I., and Armendares, S. (1974) Histidinaemia in a consanguineous marriage. J. Med. Genet., 11.

Roth, E.D., and Hug, D.H. (1971) Activity stain for urocanase and histidase on polyacrylamide gel. J. Chromatogr., 54, 286.

Rothschild, Z., and Schayer, R.W. (1958) Synthesis and metabolism of a histamine metabolite, 1-methyl-4-(β-aminoethyl)-imidazole. Biochim. Biophys. Acta, 30, 23.

Rubenstein, A.H., and Steiner, D.F. (1970) Proinsulin: the single chain precursor of insulin. Med. Clin. N. Amer., 54, 191.

Sabater, J., Ferrẽ, C., Puliol, M., Maya, A. (1976) Histidinuria: a renal and intestinal histidine transport deficiency found in two mentally retarded children. Clin. Genet., 9, 117.

Sahib, M.K., and Krishna Murti, C.R. (1969) Induction of histidine degrading enzymes in protein starved rats and regulation of histidine metabolism. J. Biol. Chem., 244, 4730.

Sakaguchi, M., Sugiyama, M., Sugiyama, T., and Kawai, A. (1970) Histidine metabolism in fish. IV Comparative study on histidine deaminases from muscle and liver of mackerel and bacteria. Bull. Jap. Soc. Scientific Fisheries, 36, 200.

Sawin, P.B., and Glick, D. (1943) Atropinesterase, a genetically determined enzyme in the rabbit. Proc. Natl. Acad. Sci., U.S., 29, 55.

Schayer, R.W. (1956) The metabolism of histamine in various species. B.J. Pharmacol., 11, 472.

Schayer, R.W., and Karjala, S.A. (1956) Ring N methylation; a major route of histamine metabolism. J. Biol. Chem., 221, 307.

Schayer, R.W. (1959) Catabolism of physiological quantities of histamine in vivo. Physiol. Rev., 39, 116.

Schayer, R.W. (1966) Enzymatic formation of histamine from histidine. In Handbook of Experimental Pharmacology. Eds. O. Eichler and A. Farah. Berlin: Springer. Vol. 18, p 688.

Schayer, R.W., and Reilly, M.A. (1974a) Effect of histidine decarboxylase inhibitors and other drugs on histamine formation in vivo. Agents and Actions, 4, 133.

Schayer, R.W., and Reilly, M.A. (1974b) Histamine formation in guinea pig brain and other tissues: effect of α-methyl-dopa. Agents and Actions, 4, 139.

Schayer, R.W., and Reilly, M.A. (1975) Activity changes in mouse brain histidine decarboxylase. Agents and Actions, 5, 115.

Schirmer, M.D., and Harper, A.E. (1970) Adaptive responses of mammalian histidine-degrading enzymes. J. Biol. Chem., 245, 1204.

Schmid-Ruter, E. (1972) Screening results in eight countries of Western Europe. Abstr. European Soc. Paediat. Res., Germany, Sept. 11-14.

Schmidt, G., and Thannhauser, S.J. (1945) A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. Biol. Chem., 161, 83.

Schnieder, E.A. (1975) A contribution to the physiology of the perilymph. Part IV: effect of histamine on the cochlear microcirculation. Ann. Otol. Rhinol. Laryngol., 84, 228.

Schroeder, W.A., Cua, J.T., Matsuda, G., and Fenninger, W.D. (1962) Hemoglobin F1, an acetyl-containing hemoglobin. Biochim. Biophys. Acta, 63, 532.

Schwartz, E., (1961) Abbau von histidin zu urocaninsaure in der epidermis. Biochem. Ztschr., 344, 415.

Schwartz, D. (1964) Genetic studies on mutant enzymes in maize. V. In vitro interconversion of allelic isozymes. Proc. Nat. Acad. Sci., U.S., 52, 222.

Schwartz, J.C. Lampart, C., and Rose, C.(1970). Properties and regional distribution of histidine decarboxylase in rat brain. J. Neurochem., 17, 1527.

Schwartz, J.C., Lampart, C., Rose, C., Rehault, M.C., Bischoff, S., and Pollard, H. (1971) Histamine formation in rat brain during development. J. Neurochem., 18, 1787.

Schwartz, J.C., Lampart, C., and Rose, C. (1972) Histamine formation in rat brain in vivo: effects of histidine loads. J. Neurochem., 19, 801.

Schwartz, J.C. (1977) Histaminergic mechanisms in brain. Ann. Rev. Pharmacol. Toxicol., 17, 325.

Scriver, C.R. (1973) Vitamin-responsive inborn errors of metabolism. Metabolism, 22, 1319.

Seakins, J.W.T., and Holton, J.B. (1969) Histidinaemia. Biochem. J., 111, 4.

Sereni, F., and Kenney, F.T., and Kretchmer, N. (1959) Factors influencing the development of tyrosine aminotransferase activity in rat liver. J. Biol. Chem., 234, 609.

Shapiro, B.M., Kingdom, H.S., and Stadtman, E.R. (1967) Regulation of glutamine synthetase. VII: adenylyl glutamine synthetase, a new form of the enzyme with altered regulatory and kinetic properties. Proc. Natl. Acad. Sci., U.S., 58, 642.

Shapiro, B.M., and Stadtman, E.R. (1967) Regulation of glutamine synthetase. IX. J. Biol. Chem., 242, 5069.

Shaw, K.N.F., Boder, E., Gutenstein, M., and Jacobs, E.E. (1963) Histidinemia (abstract). J. Pediat., 63, 720.

Shire, J.G.M. (1976) The forms, uses and significance of genetic variation in endocrine systems. Biol. Rev., 51, 105.

Sick, K., Beale, D., Irvine, D. Lehmann, H., Goodall, P.T., and MacDougall, S. (1967) Hemoglobin G. Copenhagen and Hemoglobin J Cambridge. Two new β-chain variants of hemoglobin A. Biochim. Biophys. Acta, 140, 231.

Small, N.A., Holton, J.B., and Ancill, R.J. (1970) In vitro inhibition of serotonin and γ-aminobutyric acid synthesis in rat brain by histidine metabolites. Brain Res., 21, 55.

Smart, I. (1961) The subependymal buyer of the mouse brain and its cell production as shown by radioautography after thymidine-H^3 injection. J. Comp. Neurol., 116, 325.

Smart, I., and Leblond, C.P. (1961) Evidence for division and transformations of neurologlia cells in the mouse brain, as derived from radioautography after injection of thimidine H^3. J. Comp. Neurol., 116, 349.

Smith, M., Hopkinson, D.A., and Harris, H. (1971) Developmental changes and polymorphisms in human alcohol dehydrogenase. Ann. Hum. Genet., Lond., 34, 251.

Smith, T.A., Cordelle, F.H., and Abeles, R.H. (1967) Inactivation of histidine deaminase by carbonyl reagents. Arch. Biochem. Biophys., 120, 724.

Smithies, O., and Cornell, G.E. (1959) Biochemical aspects of the inherited variations in human serum haptoglobins and transferrins. In Ciba Foundation Symp. on Biochemistry of Human Genetics. Eds. G.E. Wolstenholme and C.H. O'Connor. London: Churchill.

Snedecor, G.W., and Cochran, W.G. (1967) Statistical Methods, 6th edition. Iowa: State University Press.

Snyder, S.H., Silva, O.L., and Kies, M.W. (1961) The mammalian metabolism of L-histidine. J. Biol. Chem., 236, 2996.

Snyder, S.H. (1971) Determination of tissue histamine by an enzymatic isotopic assay. In Methods in Enzymology. Eds. S.P. Colowick and N.O. Kaplan. New York and London: Academic Press. Vol. 17, p 838.

Snyderman, S. (1965) An eczemoid dermatitis in histidine deficiency. J. Pediatr., 66, 212.

Snyderman, S.E., Boyder, A., Roitman, E., Holt, L.E., Jr., and Prose, P.H. (1963) Pediatrics, 31, 786.

Soutar, A.K., and Hassall, H. (1969) Multiple polymers of histidine ammonia-lyase. Biochem. J., 114, 79.

Spolter, P.D., and Baldridge, R.C. (1963) The metabolism of histidine V. on the assay of enzymes in rat liver. J. Biol. Chem., 238, 2071.

Srivastava, S.K. and Beutler, E. (1969) Auxiliary pathways of galactose metabolism. J. Biol. Chem., 244, 6377.

Stamatoyannopoulos, G. (1972) The molecullar basis of haemoglobin disease. Ann. Rev. Genet., 6, 47.

Stanbury, J.B., Wyngaarden, J.B., and Fredrickson, D.S. (1978) Inherited variation and metabolic abnormality. In The Metabolic Basis of Inherited Disease, 4th edition. Eds. J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. New York: McGraw-Hill.

Steele, W.J., Okamura, N., Busch, H. (1964) Prevention of loss of RNA, DNA and protein into lipid solvents. Biochim. Biophys. Acta, 87, 490.

Stefanowska, M. (1898) Évolution des cellules nerveuses corticales chez la souris après la naissance. Trav. Lab. Inst. Solvay (Brux.), 2, 1-44.

Stein, W.H., and Moore, S. (1954) The free amino acids of human plasma. J. Biol. Chem., 211, 915.

Stevenson, R.E., and Huntley, C.C. (1967) Congenital malformations in the offspring of phenylketonuric mothers. Pediatrics, 40, 33.

Stevens, R., Cross, H.E., and Morrow, G. (1975) Histidinemia with features of the Marfan syndrome. J. Pediat., 86, 907.

Stifel, F.B., and Herman, R.H. (1972) Is histidine an essential amino acid in man? Amer. J. Clin. Nutr., 25, 182.

Strecker, H.J. (1970) Biochemistry of selected amino acids.

In Handbook of Neurochemistry. Ed. A. Lajtha. New York: Plenum Press. Vol. 3, p 173.

Sugita, N.J. (1918) Comparative studies on the growth of the cerebral cortex. J. Comp. Neurol., 29, 1.

Sutton, H.E., and Wagner, R.P. (1975) Mutation and enzyme function in humans. Ann. Rev. Genet., 9, 187.

Swank, R.T., Paigen, K., and Ganschow, R.E. (1973) Genetic control of glucuronidase induction in mice. J. Mol. Biol., 81, 225.

Swift, H.H. (1950) The deboxyribose nucleic acid content of animal nuclei. Physiol. Zool., 23, 169.

Tabor, H., (1951) Diamine oxidase. J. Biol., Chem., 188, 125.

Tabor, H. (1954) Metabolic studies on histidine, histamine and related imidazoles. Pharmacol. Rev., 6, 299.

Tabor, H., and Mehler, A.H. (1955) Histidase and urocanase. In Methods in Enzymology. Eds. Colowick, S.P., and Kaplan, N.O. New York and London: Academic Press. Vol. 2, p 228.

Tanret, M.C. (1909) Sur une base nouvelle retirée du seigée ergoté, l'ergothioneine. C.R. Acad. Sci., 149, 222.

Taylor, K.M., and Snyder, S.H. (1972) Dynamics of the regulation of histamine levels in mouse brain. J. Neurochem., 19, 341.

Taylor, K.M. (1975) Brain histamine. In Handbook of Psychopharmacology. Eds. L.L. Iversen, S.H. Iversen and S.H. Snyder. New York and London: Plenum Press. Vol. 3, p 327.

Tesar, C., and Rittenberg, D. (1947) The metabolism of L-histidine. J. Biol. Chem., 170, 35.

Thalhammer, O., Scheibenreiter, S., and Pantlitschko, M. (1971) Histidinaemia: detection by routine newborn screening and biochemical observations on three unrelated cases. Zeitschr. Kinderheilk., 109, 279.

Thomas, G.H., Parmley, T.H., Stevenson, R.E., and Howell, R.R. (1971) Developmental changes in amino acid concentrations in human amniotic fluid. Abnormal findings in maternal phenylketonuria. Amer. J. Obs. Gynecol., 111, 38.

Tocci, P.M., and Bessman, S.P. (1967) Histidine peptiduria. In Amino Acid Metabolism and Genetic Variation. Ed. W.L. Nyhan. New York: McGraw-Hill. p 161.

Tourian, A.Y., and Sidbury, J.B. (1978) Phenylketonuria. In The Metabolic Basis of Inherited Disease, 4th edition. Eds. J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. New York: McGraw-Hill.

Tunnicliff, G., Wein, J., and Roberts, E. (1972) Effects of imidazoleacetic acid on brain amino acids and body temperature in mice. J. Neurochem., 19, 2017.

Tuomisto, L. (1977) Ontogenesis and regional distribution of histamine and histamine-N-methyltransferase in the guinea pig brain. J. Neurochem., 28, 271.

Urbach, K.F. (1949) Nature and probable origin of conjugated histamine excreted after ingestion of histamine. Proc. Soc. Exptl. Biol. Med., 70, 146.

Uzman, L.L., and Rumley, M.K. (1958) Changes in the composition of the developing mouse brain during early myelination. J. Neurochem., 3, 170.

Van Arsdel, P.P., and Beall, G.N. (1960) The metabolism and functions of histamine. Arch. Int. Med., 106, 714.

Van Balgooy, J.N.A., Marshall, F.D., and Roberts, E. (1972) Metabolism of intracerebrally administered histidine, histamine and imidazoleacetic acid in mice and frogs. J. Neurochem., 19, 2341.

Van den Berg, C.J. (1970) Compartmentation of glutamate metabolism in the developing brain: experiments with labelled glucose, acetate, phenylalanine, tyrosine and proline. J. Neurochm., 17, 973.

Van Sprang, F.J., and Wadman, S.K. (1967) Treatment of a patient with histidinemia. Acta Paediat. Scand., 56, 493.

Vendrely, R., and Vendrely, C. (1949) La teneur du moyau cellulaire en acide desoxyribonucleique à travers les organse, les individus et les espèces animals. Experientia, 5, 327.

Wadman, S.K., Van Sprang, F.J., Van Stekelenburg, G.J., and Debree, P.K. (1967) Three new cases of histidinemia. Acta Paediat., Scand., 56, 485.

Wadman, S.K., De Bree, P.K., Van der Heiden, C., and Van Sprang, F.J. (1971) Automatic column chromatographic analysis of urinary and serum imidazoles in patients with histidinemia and normals. Clin. Chim. Acta, 31, 215.

Wadman, S.K., De Bree, P.K., Van Biervhiet, J.P.G.M., and Van Sprang, F.J. (1973) Dietary correction of histidinemia in older children possible. Clin. Chim. Acta, 49, 377.

Waisman, H.A. (1967) Variations in clinical and laboratory findings in histidinemia. Am. J. Dis. Childh., 113, 93.

Waldmeier, P.C., Feldtrauer, J.J., and Maitre, L. (1977) Methylhistamine: evidence for selective deamination by MAO in the rat brain in vivo. J. Neurochem., 29, 785.

Wallace, M. (1970) Balance defect. Mouse Newsletter, 42, 20.

Wallace, M.E. (1971) Environ. Pollut., 1, 175. An unprecedented number of mutants in a colony of wild mice.

Ward, L.C., and Buttery, P.J. (1978) N -methylhistidine - an index of the true rate of myofibrillar degradation ? An appraisal. Life Sci., 23, 1103.

Weissbach, H., Lovenberg, W., and Udenfriend, S. (1961) Characteristics of mammalian histidine decarboxylating enzymes. Biochim. Biophys. Acta, 50, 177.

White, J.M. (1978) Haemoglobin variation. In The Biochemical Genetics of Man, 2nd edition. Eds. D.J.H. Brock and O. Mayo. London and New York: Academic Press. p 561.

Whitfield, A.E., and Shepherd, J. (1970) Measurement of L-histidine ammonia-lyase activity and urocanic acid content of human skin. Clin. Chim. Acta, 29, 181.

Wickner, R.B. (1969) Dehydroalanine in histidine ammonia lyase. J. Biol. Chem., 244, 6550.

Williams, V.R., and Hiroms, J.M. (1967) Reversibility of the "irreversible" histidine ammonia-lyase reaction. Biochim. Biophys. Acta, 139, 214.

Wilmot, T.J., and Menon, G.N. (1976) Betahistine in Ménière's disease. J. Laryngol. Otol., 90, 833.

Winnick, M., and Rosso, P. (1973) Effects of malnutrition on brain development. In Biology of Brain Dysfunction. Ed. G.E. Gaull. New York: Plenum. Ch. 8.

Witkop, C.J., Jr., and Henry, F.V. (1963) Sjogren-Larsson Syndrome and histidinaemia: hereditary biochemical diseases. J. Speech Hearing Dis., 28, 2.

Wixon, R.L., Andersen, H.L., Terry, B.E., and Sheng, Y. B. (1977) Total parenteral nutrition with selective histidine depletion in man. I Responses in nitrogen metabolism and related areas. Amer. J. Clin. Nutr., 30, 887.

Woody, N.C., Snyder, C.H., and Harris, J.A. (1965) Histidinaemia. Amer. J. Dis. Childh., 110, 606.

Woolf, L.I., Griffiths, R., Moncrieff, A., Coates, S., and Dillistone, F. (1958) The dietary treatment of phenylketonuria. Arch. Dis. Childh., 33, 31.

Woolf, L.I. (1970) Myelin deficiencies related to inborn errors of human metabolism. In Myelination. Eds. A.N. Davison and A. Peters. American Lecture Series No. 782. Illinois: Charles Thomas. p 183.

World Health Organisation (1969) Biochemistry of Mental Disorders, Technical Report Series No. 427, 6.

Yates, C.M., Dewar, A.J., Wilson, H., Winterburn, A.K., and Reading, H.W. (1974) Histological and biochemical studies on the retina of a new strain of dystrophic rat. Exp. Eye Res., 18, 119.

Zak, B. (1957) Simple rapid microtechnic for serum total cholesterol. Amer. J. Clin. Path., 27, 583-588.

Zannoni, V.G. and La Du, B.N. (1963) Determination of histidine α-deaminase in human stratum corneum and its absence in histidinaemia. Biocehm. J., 88, 160.

Zenisek, A., Kral, J.A., and Hais, I.M. (1955) "Sun-screening" effect of urocanic acid. Biochim. Biophys. Acta, 18, 589.

Zenisek, A., and Kral, J.A., (1953) The occurrence of urocanic acid in human sweat. Biochim. Biophys. Acta, 12, 479.