

MUTANTS OF AROMATIC AMINO ACID METABOLISM
IN THE MOUSE

by

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SUMMARY

The effects of alleles at the b-, c- and d-loci on the melanin content of the coat and the tyrosine hydroxylase and phenylalanine hydroxylase activity of mouse skin and liver were measured in an attempt at an investigation of the quantitative effects on the components of a metabolic pathway of in vivo variation of enzyme activity.

The d-locus alleles were found to have no effect on phenylalanine hydroxylase activity or melanin content: it was suggested that the grey dilution associated with the alleles 'dilute' and 'dilute-lethal' is an optical effect due to clumping of the pigment granules.

The c-locus alleles 'chinchilla' and 'extreme dilution' were found to affect the K_M for tyrosine and the heat stability of skin, but not liver, tyrosine hydroxylase. In all heterozygotes examined tyrosine hydroxylase activity was found to be close to the parental mean. It was therefore concluded that the c-locus is the structural locus for skin tyrosine hydroxylase, although the pattern of development of tyrosine hydroxylase was also affected by the c-locus alleles. This was suggested to be due to altered responses of the mutant enzyme molecules to activating agents and/or different K_I 's for melanin. A non-competitive inhibition relationship between skin melanin and tyrosine hydroxylase in aaBBCC was demonstrated. The difficulty of expressing tyrosine hydroxylase activity and melanin content in terms consistently proportional to follicle number was also discussed.

The b-locus alleles 'cordovan' and 'brown' were found to increase tyrosine hydroxylase activity by about $1\frac{1}{2}$ and 2 times respectively but K_M and heat stability were not affected and dominance of the

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INTRODUCTION.

This thesis describes an investigation into the effects of mutation and gene dose on the kinetic properties of an enzyme and the consequent quantitative changes in the end-product of the affected metabolic pathway, in this case the phenotype.

There have been few quantitative studies of gene-enzyme-phenotype relationships in higher organisms for two main reasons: first, that the biochemical complexity of organisms with a high degree of cellular differentiation makes instances of suitable systems for study very rare, and second, that in the absence of a coherent theory of metabolic control from which predictions of quantitative relationships might be made, there is little motivation for such an approach.

Most early investigations of gene-phenotype relationships, carried out in attempts to explain the nature of gene action, were on morphogenic systems whose components were impossible to isolate or define biochemically and the phenotypes not accessible to quantitative measurement. From work on intersexuality in Lymantria dispar, however, Goldschmidt (1917a,b; 1920) postulated that the physiological basis of gene action was the control of rates of developmental processes. A similar theory was simultaneously advanced by Wright (1916) to explain the effects of multiple alleles on the coat colour of the guinea-pig. At this time the biochemistry of melanin formation was known in outline and Wright suggested that the action of the alleles was to affect the rate of production of an enzyme involved in melanogenesis. He presented this idea later in a more general form (1929, 1934) in which the physiological processes underlying the phenotype were visualised as a chain of reactions starting from the primary gene product, suggested to be an enzyme. By considering at one step the rate of formation of product from substrate supplied at a given rate by an

enzyme produced at different rates depending on genotype, he derived a hyperbolic relationship between genotype and phenotype. Though quantitative estimates of pigmentation intensity were available for the various coat-colour mutants, corresponding data for enzyme activity were not available and so comparison of experimentally and theoretically derived relationships was not possible.

It was not generally accepted at the time that the universal action of genes could be through enzymes, probably because the importance of enzymes themselves in metabolism was not appreciated, and Wright's explanation was considered a special case by Goldschmidt (Goldschmidt 1938). As the connection between gene and enzyme was recognised, however, biochemical genetics concentrated on microorganisms where the more simple relationships between gene and phenotype gave meaningful data on the mechanism of gene action and the large numbers of organisms able to be used in single experiments afforded a high degree of genetic resolution. Its return to the investigation of higher organisms has been a slow one, but evidence increases to show that essentially the same molecular mechanisms of gene action operate here. The extensive work on the primary, tertiary and quaternary structures of variant haemoglobins, for instance, (listed by Harris, 1970), give a very clear picture of the effects of mutation in a structural gene on the polypeptide it produces. General mechanisms of genetic regulation, however, still remain to be elucidated.

In the light of present knowledge it is possible to re-draw Wright's model as a metabolic pathway with an enzyme involved at each step and to describe changes in enzyme activity by measurements of kinetic parameters already well-defined by enzymology. Given the rate equation for every enzyme it is possible also to write down a

set of equations to describe the rates of change of all the metabolites in the pathway, but unless the starting conditions are specified, there will be no unique solution for it. By considering a steady-state system, however, Kacser & Burns (1973, 1968) formulated theoretical predictions of the changes expected in the flux of metabolites through a pathway following changes in enzyme activity. In the steady-state system the sum of the rates at which any metabolite is being produced is exactly equal to the sum of the rates at which it is being removed by the next step in the pathway or pathways it is involved in and the backward reaction. There is thus a net flux through the pathway although the concentrations of the intermediate metabolites remain constant, fixed at levels which are a function of in vivo enzyme activity, which in turn may be affected by substrate and product concentrations. The predicted relationship between flux and in vitro enzyme activity at any one step is non-linear - i.e. the flux becomes increasingly insensitive to changes in enzyme activity as this increases. The derivation of this relationship will be discussed in another section of this chapter.

Tateson (1972) investigated the applicability of this model to the arginine biosynthetic pathway in Neurospora crassa. Variation in the extractable activity of four enzymes at successive steps in the pathway was produced by constructing heterokaryons with different proportions of nuclei with wild-type and mutant structural genes. The mutant enzyme forms showed so little activity that the observed activity could be considered to be due to a homogeneous population of wild-type molecules. From growth-rate measurements it was possible to correct for flux to growth (protein incorporation), and after allowance had been made for some enzyme derepression seen at

low levels of activity, a general agreement with the predicted relationship was obtained. Not only might information on mechanisms of metabolic control be obtained from such investigations (Kacser & Burns, 1973), but the existence of a non-linear relationship between flux and enzyme activity also gives an insight into the metabolic basis of phenotypic dominance which will be discussed in detail later.

There are no other examples in the literature where the direct effect of gene dose on enzyme activity has been measured for a wide range of dosage. Data from human biochemical genetics indicates, however, that where the effect of a mutation is to reduce the activity of an enzyme to a very low level compared with normal the heterozygote has half the activity of the normal homozygote. (Harris, 1970). Intermediacy of the heterozygote is now used as one criterion for deciding whether or not a mutation is at the structural locus for an affected enzyme (Paigen, 1971). There is no further indication that the gene-dose/enzyme activity relationship continues to be linear over a wider range of gene dosage, but assuming this to be the probable case, two instances where gene dose has been able to be varied and its effect on quantitative measurements of phenotype will be discussed.

Mangelsdorf & Fraps (1931) studied the effect of dosage of the gene Y and its allele y on the carotenoid content of maize endosperm. In this triploid tissue four genotypes were measured: yyy (white kernel), yyY (pale yellow), yYY (yellow) and YYY (deep yellow), which had respective carotenoid contents of less than 1%, 30%, 65% and 100% of wild-type - a linear relationship. Randolph and Hand (1940) made similar measurements on endosperm from diploid and tetraploid strains, thus obtaining gene dosages of 3Y and 6Y. The carotenoid content of the tetraploid was found to have increased by only 40%, which is

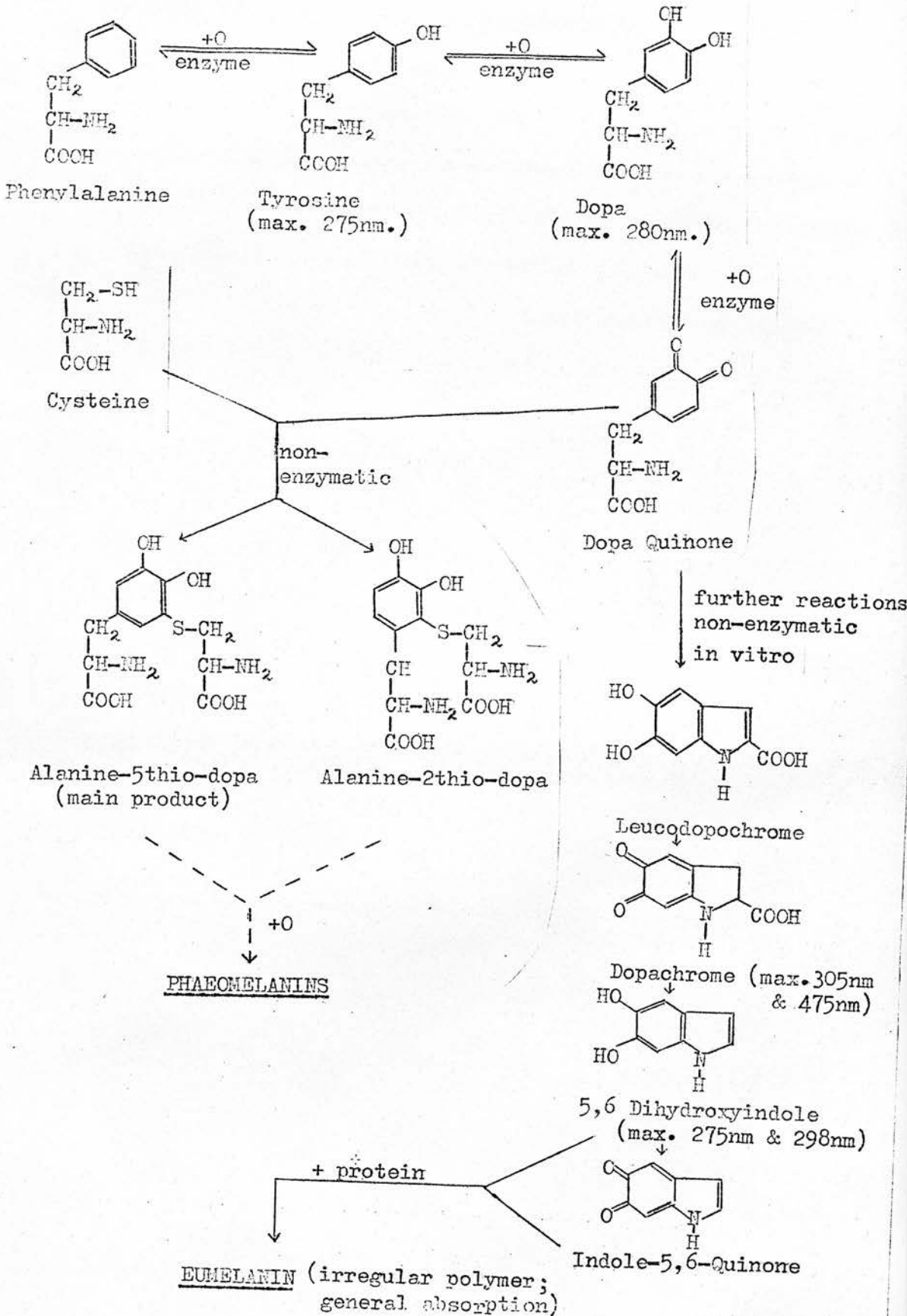
indicative of a non-linear relationship of the type predicted but in this case the duplication of a whole genome was being considered rather than selective duplication of a single gene.

A second example of a non-linear gene-dose/phenotype relationship is found in the Bar-eye series in Drosophila melanogaster. Here the Bar phenotype is associated with the duplication of a certain chromosomal region (Bridges, 1936). Triplication and further reiteration of this region can be produced by unequal crossing-over during meiosis and Rapoport (1940) produced 5- to 9- fold tandem repetitions by X-irradiation. A non-linear relationship between number of Bar regions on the chromosome and facet number was obtained, the proportional effect on facet number decreasing with increasing Bar dose. Rapoport suggested that the action of Bar was to prevent one or more divisions of the presumptive facet cells in an early period of exponential growth of the eye anlage. The relationship between Bar dose and facet number was investigated quantitatively by Andrews (1970) and several possible mechanisms for the action of Bar were considered. A simple inhibition of cell division did not account for the relationship obtained, nor did the consideration of the Bar gene product as a competitive or non-competitive inhibitor of a facet-differentiating stimulus. It was concluded that the Bar substance probably acted at the level of differentiation to reduce a facet-producing stimulus and the response of the presumptive facet cells to it. Until the biochemical basis of facet differentiation is known, therefore, further quantitative biochemical studies cannot be carried out on this system.

In the study to be described in this thesis the effects of mutations at two loci on the enzymes involved in melanogenesis in the mouse and on consequent coat colour are investigated. This system was

chosen because of its biochemical accessibility and because of the large amount of genetic variation associated with it. The aim of the project was to find mutations which had a consistent and measurable effect on melanogenic enzymes, to investigate the effect in each case and, by constructing as many different genotypes as possible, to investigate interactions and the relationships between enzyme activity and melanin content of skin and hair and hence possible mechanisms of metabolic regulation in a higher organism.

Fig. 1. The Melanin Pathway.



The Melanin Pathway.

The main stages in the oxidation of tyrosine and its derivatives to eumelanin (black or brown pigment) and phaeomelanin (yellow or red pigment) precursors are shown in fig. 1. The eumelanin pathway was largely worked out by Raper (1928) who investigated the oxidation of tyrosine by a tyrosinase from the meal-worm Tenebrio molitor. A spectrophotometric investigation by Mason (1948) confirmed Raper's scheme for the pathway intermediates and led to the suggestion that melanin itself was a polyindolequinone of the general formula $(C_8H_3O_2N)_n$, which in vivo is conjugated with protein. The incorporation of different tyrosine derivatives into eumelanin has since been demonstrated, however, by the use of radioactively labelled compounds (Kirby & Ogunkoya 1965; Chen & Chavin 1966), structural studies (Nicolaus 1962; Nicolaus & Piatelli 1965) and tracer studies (Swan, 1964; Robson & Swan 1966) suggesting that the Raper-Mason scheme is a greatly oversimplified representation of the polymerisation process. Eumelanin appears to be a very irregular polymer whose composition varies with differences in biological or chemical conditions. The phaeomelanin pathway shown here is taken from a scheme recently proposed by Prota (1972) from biosynthetic work and analytical studies of feather pigments.

The existence of tyrosinase in mammalian tissue was first demonstrated by Hogeboom & Adams (1942) using Harding-Passey mouse melanoma. These workers reported a partial separation of tyrosine hydroxylase and dopa oxidase activity: studies such as Raper's (1928) had shown tyrosinase from plant and invertebrate sources to possess both activities. Lerner et al (1949) were unable to repeat this but demonstrated that, as was already known to be the case in plants, dopa

could act as a catalyst for the tyrosine hydroxylase reaction. When tyrosine is used as substrate for tyrosinase there is usually a long lag period but this can be shortened or eliminated if dopa is added in catalytic amounts. All tyrosinases so far isolated have been shown to contain copper and Mason (1955) suggested a mechanism by which tyrosinase could be active towards both tyrosine and dopa by using the oscillation of the copper between the cuprous and cupric states. In the presence of dopa (or other reducing system), cupric ions are reduced to the cuprous state and the hydroxylation is able to take place. Thus, depending on the oxidation potential of the system, it may be necessary for tyrosine to catalyse the second step before the first. Dopa was widely held to be the in vivo melanogenic substrate in mammals, however, until Fitzpatrick et al (1950) demonstrated that tyrosine was a melanogenic substrate in human skin. In vitro the oxidation of dopa to melanin proceeds non-enzymatically and as yet no enzymes have been isolated which catalyse further oxidation and polymerisation reactions. The conversion of dopa-quinone to melanin is therefore generally considered to be non-enzymatic in vivo also.

Tyrosine is obtained in mammals from the diet and by the hydroxylation of phenylalanine, which is an essential amino acid. The absence of phenylalanine hydroxylase in man causes phenylketonuria, a condition which is characterised by high phenylalanine blood levels, excretion of degradation products of phenylalanine, neurological abnormalities and reduced pigmentation. In tyrosine-feeding experiments on phenylketonurics by Snyderman et al (1955) tyrosine was shown to cure hypo-melanosis of the hair. Miyamoto & Fitzpatrick (1957) found that this hypo-pigmentation was due to tyrosinase inhibition which could be removed by addition of tyrosine. These

results together suggest that hypopigmentation associated with phenylketonuria is due not mainly to the decrease in the tyrosine available for conversion to melanin because of the block in the pathway but to the competitive inhibition of tyrosinase by phenylalanine or its derivatives. Nevertheless, for present purposes it was decided to consider phenylalanine the first step in the melanin pathway.

In mammals phenylalanine and tyrosine can be deaminated to phenylpyruvic acid and p-hydroxyphenylpyruvic acid respectively. Under normal physiological conditions the bulk of the phenylalanine is hydroxylated to tyrosine in the liver and its degradation products are found in quantity only in phenylketonurics. Tyrosine aminotransferase is known to be induced by its substrate in rat liver (Rosen et al 1963) but its activity in skin has not been recorded. Dopa is also decarboxylated to dopamine, a precursor of catecholamines, in liver, kidneys and parts of the peripheral and central nervous system. Again, the extent to which this reaction occurs in the skin or whether there are differences in this respect between the different pigment genotypes in the skin and other parts of the body is not known.

The Mammalian Pigmentary System.

Melanin synthesis in mammals takes place in specialised cells, melanocytes. In 1948 Masson concluded from histological studies of skin sections using dopa as a substrate for tyrosinase that the melanocyte is the only melanin-producing cell in the skin. These observations were confirmed by Billingham (1948, 1949) who showed in addition that on transplantation melanocytes maintained a type-specific lineage and secreted pigment granules into epidermal cells. More recent studies suggest that some melanin synthesis may also occur in mast cells (Okun et al 1972). 1 to 3% of mast cells was reported by these workers to be dopa-positive and this percentage was increased to 90% in cells which had suffered mechanical damage. In normal tissue, however, melanin synthesis in these cells would be insignificant compared with that in melanocytes. Rawles (1948) showed that melanocytes arise during embryogenesis from the neural crest and migrate to the skin where they proliferate, maintaining the characteristic dendritic shape of the neurone. In the mouse melanocytes are not normally found in trunk-skin epidermis except in association with the hair bulb (Markert & Silvers 1956).

In a detailed histological study Russell (1946) showed that the hair colour of different mouse mutants were due to the deposition of pigment as qualitatively distinct granules. Lerner et al (1949) found tyrosinase activity in fractions of mouse melanoma obtained by differential centrifugation to be associated with the microsomes. Electron microscope studies by Birbeck, Mercer & Barnicot (1956) showed that melanocytes contained a system of membranes similar to those seen in secretory cells, and distinguished several stages of pigment granule development. The granules appeared to start as hollow

vacuoles which then developed internal lamellae on which an electron-dense material was deposited. These observations were confirmed by Moyer (1961) who was able to distinguish four stages of pigment granule development in the mouse eye. Moyer's observations also led him to suggest that the pigment granule, now termed the 'melanosome', originated as an intracisternal dilation of the endoplasmic reticulum, as occurs with the products of other secretory cells. Seiji et al (1963) examined mouse melanoma fractions for tyrosinase activity and also located this activity in the cell by means of autoradiography. Their combined data suggested that tyrosinase is synthesised on ribosomes and subsequently transferred through the endoplasmic reticulum to the melanosomes where melanogenesis takes place. The fully melanised granule is then eventually secreted into other cells.

The process of melanogenesis is thus a complicated one involving specialised subcellular structures within specialised cells and so affording many possible targets for the action of mutation.

The Genetics of Pigmentation in the Mouse.

Mutations affecting pigmentation are known at over thirty loci in the mouse. Wolfe & Coleman (1966) have produced a classification of most of these according to probable site of action - e.g. hair follicle environment, melanoblast differentiation or migration, and sites within the melanocyte. These criteria are based largely on the results of histological studies and transplantation experiments: in very few cases is there any suggestion of the biochemical basis of the effect. The main exceptions are the a, b, c and d loci which will now be briefly discussed.

The wild-type 'agouti' colouration in the mouse is produced by the insertion of a sub-apical yellow band in an otherwise black hair. The alleles at the a (agouti) locus determine the amount of this yellow pigment (phaeomelanin) present. The dominant allele A^y suppresses eumelanin completely, resulting in uniformly yellow hairs; A is the wild-type allele, which is recessive to A^y but dominant to a (non-agouti) which when homozygous suppresses the agouti band to form totally black hairs, although yellow hairs remain round the ears, mammae and perineum. Pigment produced at sites other than hair follicles is always eumelanic regardless of the genotype at the a locus and transplantation of melanocytes has indicated that the alleles of this locus act only in the hair follicles (Silvers & Russell 1955; Markert & Silvers 1956). All skin explants in tissue culture produce only eumelanin on addition of tyrosine, but addition of sulphhydryl compounds to the medium cause yellow pigment to be produced. Cleffmann (1963, 1964) showed that the amount of sulphhydryl required for this switch is related to the genotype at the a locus, A^ya skin requiring less than aa skin. These observations support the scheme

for the biosynthesis of phaeomelanins recently proposed by Prota (1972). Tyrosinase activity appears to be depressed during phaeomelanin synthesis: Coleman (1962) found that incorporation of ^{14}C -tyrosine by yellow (A^y-) skin slices was only one third of that of black skin. Moyer (1966) observed from electron micrographs that the melanosomes synthesising phaeomelanin consist of randomly arranged protein fibrils, whereas those synthesising eumelanin consist of parallel fibrils held together in a matrix by regular cross-linking. Holstein et al (1967, 1970) found three electrophoretic variants of tyrosinase in skin of mouse during eumelanin synthesis, and only one from skin producing phaeomelanin. The two missing forms might represent activity closely associated with the regular matrix and their absence in yellow skin could be due to the presence of high concentrations of sulphhydryl groups inhibiting the formation of disulphide bridges between protein molecules and hence limiting attachment sites for the tyrosinase molecules.

The b (black/brown) locus determines the type of eumelanin produced. The dominant allele, B, is responsible for the wild-type black pigment and alleles b^c and b for cordovan (dark brown) and brown pigment respectively. The effect of the b alleles seems to be on both melanosome morphology and tyrosinase activity. Russell (1946) found that pigment granules in the hair of homozygous bb mice were rounded instead of oval and had half the diameter of wild-type black granules. Several investigations of the effect on tyrosinase activity have been made. (Russell & Russell 1948; Foster 1951, 1959; Fitzpatrick & Kukita 1959; Coleman 1962). All of these established that brown mice had as much tyrosinase activity as black mice except for Foster's study which suggested the presence of an inhibitor in brown and by some assays

brown appeared to have twice the activity of black.

Alleles at the c (albino) locus determine pigment intensity. The homozygous cc mouse is a pink-eyed albino whatever its genetic constitution at the a and b loci. Russell & Russell (1948) used a histochemical dopa oxidase assay on four homozygotes in this series; wild-type (CC), chinchilla (c^{ch}c^{ch}), extreme dilution (c^ec^e) and albino (cc). The estimated activities roughly corresponded to what might be expected from visual examination of the phenotypes. No activity was detected in the albino. Similar results were obtained by Coleman (1962) for the incorporation of ¹⁴C-tyrosine into skin slices, with one additional significant observation: the genotypes CC and Cc, though not visually distinguishable, were biochemically so, Cc having half the activity of CC. At the same time it was also shown that the tyrosinase produced by the himalayan (c^hc^h) mutant was more heat-labile than that produced by the wild-type. These latter results suggest that the c locus could be the structural locus for tyrosinase.

Alleles at the d (dilute) locus cause a coat colour dilution which has a greying effect on the fur of genotypes which would otherwise be dark. The effect is less noticeable in lighter phenotypes. Russell (1948) examined the distribution of pigment granules in the hair of homozygous dilute (dd) mice and found that the granules were clumped together in large irregular masses. An estimation of pigment volume from measurements of pigment clumps suggested that there was no great reduction of pigment. Markert & Silvers (1956) reported that dd melanocytes showed abnormal dendrite morphology. The dilute-lethal (d¹) allele is recessive to dilute but has no additional effect on colour dilution, dd and d¹d¹ animals being identical in colour. The dilute-lethal mouse develops severe muscular and neurological abnormalities

characterised by opisthotonic convulsions and usually dies at weaning (Searle 1952). The CNS shows myelin degeneration (Kelton & Rauch 1962). In a study of phenylalanine metabolism in dilute genotypes Coleman (1960) pointed out several similarities between these mutants and the inborn error of metabolism phenylketonuria in man. Coleman reported the tyrosine hydroxylase activity in liver slices and homogenates in Dd, dd¹ and d¹d¹ animals to be 69%, 51% and 14% respectively of that of DD at three weeks of age. This reduction in activity was found to be caused by a particulate inhibitor. A corresponding increase in blood phenylacetic acid was also observed and dilute animals cleared phenylalanine less efficiently in loading tests. A ten-fold increase in blood phenylalanine levels was also observed in dilute-lethal animals by Rauch and Yost (1963), who confirmed that phenylalanine hydroxylase activity was low but did not observe intermediacy at the enzyme level, Dd¹ animals having normal activity.

Mice homozygous for pink-eyed dilution (pp) which reduces eumelanin in the hair and eyes have also been reported to have low tyrosinase activities, as have homozygous ruby-eyed (ruru) animals (Fitzpatrick & Kukita 1959; Coleman 1962; Russell & Russell 1948).

In the present study non-agouti (aa) animals were used in order to avoid changes in tyrosinase activity during phaeomelanin synthesis. Genotype at the b, c, and d and possibly other loci would then be varied.

Theoretical Background.

The flux (v_1) through a single reversible reaction in which a quantity of substrate S_0 is converted to a quantity of product S_1 by an enzyme e_1 is given by the expression:

$$v_1 = \frac{V_1/M_1 (S_0 - S_1/K_1)}{1 + (S_0/M_1 + S_1/M_{-1})} \dots \dots \dots (i)$$

where V_1 is the maximal velocity (V_{max}) of the enzyme, M_1 and M_{-1} are the Michaelis constants (K_M) for the forward and backward reactions respectively and K_1 the equilibrium constant for the pools S_0 and S_1 . This equation can be used to describe the flux at each step in a pathway consisting of a chain of unimolecular reactions in which product S_1 becomes substrate for enzyme e_2 which converts it to product S_2 and so on. In the steady state the rate at which each intermediate is produced is exactly equal to the rate at which it is removed so that all the intermediate fluxes are equal and equal to the overall flux (F) through the pathway. Using this relationship, Burns (1972) showed that for a pathway of n steps summation of all the individual flux equations for each step gave an expression for the pathway flux from which intermediate pools were eliminated, i.e.

$$F = \frac{S_0 - S_n / (K_1 K_2 K_3 \dots K_n)}{\frac{1}{E_1} + \frac{+1}{E_2 \cdot K_1} + \frac{+1}{E_3 \cdot K_1 K_2} \dots + \frac{+1}{E_n \cdot (K_1 K_2 \dots K_{n-1})}} \dots \dots (ii)$$

where E is the ratio V/M for a particular enzyme.

This is the simplest possible expression which can be derived by such an approach. For this particular case the pathway enzymes were

assumed to be unsaturated so that the ratios S_0/M_1 and S_1/M_{-1} are small and the denominator of equation (i) approaches unity. Modifications arising from enzyme saturation, feedback inhibition and other factors are discussed by Kacser & Burns (1973).

The effects on the pathway flux of altering the activity of one enzyme in the pathway can be examined by considering equation (ii). It can be seen that the plot of F vs. E (i.e. V_{\max}/K_M) for any one enzyme for which V_{\max} is changing will be an asymptotic curve passing through the origin and the larger the value of E the smaller its relative contribution to the sum of the reciprocal terms involving $1/E$ and hence the smaller its proportional effect on the flux. The curvature of the curve depends on the number of enzymes in the pathway since changes in one term will be more noticeable amongst a small number of terms than a large number. If this basic relationship holds for a particular system, measurements of intermediate metabolite concentrations for various levels of enzyme activity could yield information on the mechanisms of regulation operating in a pathway when departures from the predicted values are seen.

The application of this model to theories of the biochemical basis of dominance in diploid organisms is important. If on the graph of flux (which in some cases will be a direct measurement of phenotype, as in pigmentation) vs. enzyme activity 50% activity corresponds to an only slightly reduced value for flux, very little or no change in phenotype will be observed in a heterozygote with effectively only one dose of enzyme. If codominance at the enzyme level is seen for a series of alleles it will be the allele which in the homozygous state produces the highest enzyme activity which will be dominant at the phenotypic level. Hence dominance is seen as an inherent property of

a metabolic system and 'dominance modifiers' as factors which change the relative magnitudes and thereby the relative significance of the reciprocal terms of the denominator of equation (ii) so that the shape of the flux vs. enzyme activity curve is altered and further alterations in the activities of other enzymes in a pathway may have phenotypic effects greater or smaller than previously.

In this context the melanin biosynthetic pathway appeared to present a valuable opportunity to investigate the biochemical basis of dominance. By utilising some of the genetic variation available the effects of allelic substitution on the enzymes involved in the pathway and on a phenotype which in this case is the direct end-product of the pathway could be measured and an attempt made to compare the experimental results with the theoretical predictions. If genetic variation at the structural locus of an enzyme is to be used, however, the change in the value of E may be due either to an altered V_{\max} or K_M , or both, so that the ratio V_{\max}/K_M must be calculated in each instance.

There are difficulties, however, in applying this model to higher organisms. First, it is not clear how near to a 'steady state' an individual might be. Strictly considered, this term should refer to an organism where growth and differentiation have ceased and metabolic activity is geared to maintenance and a constant level of physical activity, or where growth is merely a uniform expansion and proliferation of every component, although it might be that the time taken by a system to reach a steady state is very small compared with the time taken for the growth process and an organism could be considered to be in a series of instantaneous steady states during differentiation. Certainly, differential growth when considered

over a long period in a higher organism would require a more complicated correction than that used for Neurospora (Tateson, 1972). Secondly, the model assumes no intracellular compartmentation of enzymes and metabolite pools - all are assumed to be equally accessible to each other. In a system as specialised as the mouse pigmentary system this might not be so.

There is some evidence, however, to suggest that the model does have application to this system. Coleman (1962) observed intermediacy at the enzyme level for the effects of the *c*-locus alleles on tyrosinase activity in skin for all genotypes investigated including heterozygotes with the wild-type 'dominant' allele which were visually indistinguishable from the homozygous wild-type. In brown mice (bb) enzyme activity decreased as alleles causing decreasing pigmentation were substituted at the *c*-locus, although in each case the level of tyrosinase activity was higher than the corresponding black (BB) genotype. No measurements of pigment intensity are available to make detailed quantitative comparisons but visual estimations of pigment intensity were produced by Dunn(1936) for genotypes at the *c*-locus on black and brown backgrounds. The genotypes aabb^{ch}c^{ch} and aabbCC are given the same grading: brown chinchilla mice are distinguishable from brown wild-type only by the presence of white instead of yellow hairs in the ears, whereas black chinchilla mice appear dark brown beside wild-type black and are given a different grading on the visual scale. If, as has been suggested, the b allele causes an increase in tyrosinase activity, the apparent ineffectiveness of the chinchilla allele in combination with brown could be explained in the light of the preceding discussion. Coleman (1962) also reported that tyrosinase activity was lower than normal in yellow (A^y-) genotypes: here the

effect of the chinchilla and lower alleles in almost suppressing pigment formation in the absence of the wild-type allele could be similarly explained.

It was therefore planned to investigate the enzyme-flux relationships in the melanin pathway by the following experiments.

Plan of Experiments.

Where coat-colour mutations were found to affect tyrosinase or phenylalanine hydroxylase activity in a consistent and measurable way it was intended to use them to vary enzyme activity in vivo at the three adjacent steps in the melanin pathway mediated by these enzymes and to measure the effects on intermediate metabolite pools and the consequent effects on the end-product of the pathway, melanin.

A tyrosinase assay was required which would give reliable estimates for K_M and V_{max} . Previous attempts to assay tyrosinase in mouse skin have either made use of histological assays which depend ultimately on visual assessment of the degree of darkening of an already pigmented skin (Russell & Russell 1948) or indirect assays such as oxygen uptake (Foster 1951) or incorporation of radioactive tyrosine into melanin (Fitzpatrick & Kukita 1959; Coleman 1962), producing conflicting results in several instances. None of these assays is specific for a single step in the pathway: oxygen is required for several melanogenic reactions and also for other metabolic processes in the skin and tyrosine incorporation is effectively another means of measuring flux. It was therefore decided to use the tyrosine hydroxylase assay developed by Pomerantz (1966) in which the amount of tritiated water produced from 3,5 3H -tyrosine on its oxidation to dopa is measured. This assay is specific for the hydroxylation step and is potentially more quantitative than those described above. Possible tyrosinase mutants could thereby be investigated in more detail in order to select suitable activity variants and after producing as many different levels of tyrosinase activity as possible by constructing different genotypes, quantitative measurements would then be made. Measurements of the associated dopa oxidase activity would

then be attempted by the spectrophotometric estimation of dopachrome which is not the immediate product of the oxidation (see fig. 1) but the nearest intermediate able to be measured in this way. A specific radioactive assay for dopa oxidase is not available.

It was decided to measure melanin content spectrophotometrically as by this method melanins from different genotypes could be compared by their spectra as well as by estimates of intensity at a single wavelength.

The relationship of enzyme activity and melanin content would then be compared for different genotypes of the same age for as many different ages as possible, and for single genotypes at different ages. In addition, an attempt would be made to study the effects of dosage of the C allele by using Cattanach's translocation (Cattanach 1961). This is an autosome-X translocation in which a segment of the chromosome carrying the c-locus is inserted into the X chromosome. In males, where the single X is not Lyonised it should therefore be possible to get three doses of the C allele - two on the autosomes and one on the X (X^CY;aaBBCC). The effects of a possible increase in V_{max} without an associated change in K_M of tyrosinase on the pathway could then be investigated.

MATERIALS and METHODS.I. Source of Animals.

aaBBCC animals were obtained from the C57BL/6J stock maintained in Edinburgh University Genetics Department and aaBBcc animals came from a JU strain also maintained here.

aab^cb^cCC and aabbCC animals were obtained from the Jackson Laboratories, Bar Harbor, on C57 background. Since difficulties were encountered in breeding the latter genotype, brown males were mated to black females and the laboratory stock established from the F2 generation.

All other alleles at the c-locus (c^{ch}, c^h, c^e, c^p) were also obtained from Bar Harbor on C57 background in aaBB animals and the genotypes used for this investigation bred from these.

Animals carrying Cattanach's translocation were kindly given by Dr. Bruce Cattanach, M.R.C. Radiobiology Unit, Harwell. This stock had to be maintained on an outbred background as the translocation causes reduced viability which is increased on inbreeding.

aaBBCCdd and d¹d¹ animals were obtained from Edinburgh University Genetics Department on non-inbred background.

The sources of other mutants will be mentioned as they occur in the text.

Animals were maintained at 22°C in a daily cycle of 12hrs. light and 12hrs. dark. Two females were used per male in the same cage for breeding. Food was given ad. lib..

II. Production of aaBBCCC Animals.

Males carrying Cattanach's translocation show considerably reduced viability and fertility especially on inbred backgrounds and are produced at a much lower frequency than expected. Two fertile translocation males of genotype $X^C Y; aaBBcc$ were obtained, however, and used to produce $X^C Y; aaBBCC$ animals by first mating with C57 females to give $X^C X; aaBBCC$ female offspring. These were then mated with C57 males to give four classes of male offspring: $XY; Cc$, $XY; CC$, $X^C Y; Cc$ and $X^C Y; CC$ - i.e. 1, 2, 2 and 3 doses of the C allele respectively. It was originally hoped to use females with an X-linked marker in the first mating so that in the final crosses males not carrying the translocation could be distinguished from those which did carry it. The effects of this marker should be distinguishable before 5 days of age when the enzyme assays were to be performed and should not affect hair colour or structure so that the two classes of males with two doses of C could be compared. Crosses were started using X-linked anaemia (sla) from an outbred stock in the Genetics Department of Edinburgh University which had been selected for increased expression. Attempts to combine this mutation with $aaBBCC$ by crossing into C57 were unsuccessful, however, as the mutant phenotype became increasingly difficult to distinguish and there was insufficient time to start a fresh selection process. The final crosses therefore produced four classes of males which were not phenotypically distinguishable and each animal was assayed individually.

III. Extraction and Measurement of Pigment.

Skin was removed from the back of the mouse, scraped free of fat and chopped into small pieces. 50mgm. was then incubated with 0.1N NaOH for 30mins. in a boiling water bath. This treatment was sufficient to break down skin and hair and free the melanin granules but did not dissolve any pigment as acidification of the supernatant obtained after centrifuging produced no black precipitate. (Melanin is insoluble in acid and is precipitated as a black flocculent mass on acidification of alkaline 'solution'). After centrifuging the pigment granules were resuspended in distilled water and recentrifuged. This process was repeated once more and the washed precipitate then suspended in 2ml. 1.0N NaOH and heated in a boiling water bath for 3-4hrs. A clear, tea-coloured solution was obtained which turned slightly cloudy on cooling. This cloudiness was removed by filtering and did not return. Optical density was measured at 360nm., this being the wavelength of maximum absorption determined from absorption spectra, using quartz cuvettes and 0.5N NaOH as a blank.

IV. Determination of Phenylalanine Hydroxylase. (E.C. 1.99.1.2.).

Phenylalanine hydroxylase was assayed by the method of Woolf et al (1970). Liver or skin was removed immediately after animals had been killed by severing the spinal cord just behind the head, weighed and homogenised at 0°C with 0.15M KCl. 3ml. KCl was taken per 1gm. of tissue. The homogenate was centrifuged for 30mins. at 30,000g. 0.2ml. supernatant was added to a reaction mixture containing 0.2ml. 0.2M phosphate buffer (pH 7.4), 0.5µmoles cofactor (2-amino-4-hydroxy-6,7-dimethyltetrahydropterine) in 0.1ml., 0.8µmoles NADH in 0.1ml., 0.5µmoles nicotinamide in 0.1ml., and 2µmoles phenylalanine in 0.1ml. 0.2ml. KCl was used instead of supernatant in controls. The mixture was incubated for 30mins. in air without shaking at 25°C. 0.2ml. 30% w/v trichloroacetic acid was used to stop the reaction and the mixture allowed to stand for 30mins. at 0°C, then centrifuged and the tyrosine produced assayed by the method of Udenfriend & Cooper (1952). The supernatant was incubated in a water bath for 30mins. at 55°C with 0.5ml. 20% nitric acid and 0.5ml. solution of 1.0% nitroscaphthol in 95% ethanol. After cooling 5ml. dichloroethylene was added and the mixture shaken thoroughly and centrifuged. The absorption of the aqueous layer was read at 450nm. and O.D. values transformed to µmoles tyrosine by using standard curves.

V. Determination of Tyrosine Hydroxylase. (o-diphenol: O₂ oxidoreductase
E.C. 1.10.3.1.)

Tyrosine hydroxylase activity was assayed by a modification of the method of Menon & Haberman (1968), based on that developed by Pomerantz (1966). Dorsal skin was removed immediately after death, scraped free of fat, washed, and excess moisture blotted away. It was then chopped into small pieces, weighed, and homogenised in 0.1M phosphate buffer pH 7.4 at 0°C. 3ml. buffer was used per 1gm. tissue. 0.4ml. homogenate was added to a reaction mixture containing 0.15ml. 0.1M phosphate buffer (pH 7.4), 2.0µmoles tyrosine dissolved in 0.01N HCl added in 0.25ml., 0.2µmoles dopa in 0.15ml. (fresh solution) and 1.0µCi 3,5 ³H-tyrosine added in 0.1ml. The mixture was incubated for 1hr. in a shaking water bath at 37°C and the reaction stopped with 0.2ml. 30% w/v TCA. Controls were treated with TCA before incubation. After centrifuging in a bench centrifuge the supernatant was vortexed with 1.0ml. Dowex 50X12 400 resin (suspension containing 10gm. resin to 7.5ml. Na/Li buffer pH 2.0). The mixture was centrifuged and the supernatant vortexed with a fresh 1ml. resin suspension. This process was repeated once more and the supernatant taken for determination of ³HOH, by liquid scintillation counting. The supernatant was divided into two equal halves as the volume was too large for a single scintillation vial, and each half added to a vial with 2ml. solubiliser (Nuclear Enterprises NE50) and 10ml. of a solution of 0.5% PPO and 0.03% POPOP in scintillation grade toluene as scintillation fluid. The efficiency of the counter was checked each time by standards. The linearity of the assay over 2hrs. was checked using aaBBCC, aaBBc^{ch}c^{ch}, and aabbCC skin homogenates.

Note: Tyrosine hydroxylase activity is expressed as µmoles dopa formed/min./ml. homogenate' (tables 6a,b,c pp.41-43). This transformation of scintillation counts per minute to µmoles was made by doing a simple proportion sum, knowing the initial number of counts introduced into 2.0 µmoles unlabelled tyrosine at the start of the assay. The possibility of quenching during counting was thus not allowed for.

VI. Determination of Dopa Oxidase. (o-diphenol: O₂ oxidoreductase
E.C. 1.10.3.1.)

The following assay was found to be successful for solutions in phosphate buffer of mushroom tyrosinase which gave similar levels of tyrosine hydroxylase activity to those shown by skin homogenates. 0.8ml. enzyme solution was added to 2.0µmoles dopa in 1.2ml. 0.1M phosphate buffer. Increase in absorption was measured at 475nm. Several temperatures from 20°C to 40°C were tried and several pH's, from 6.0 to 8.0. Other modifications made in attempts to adapt the assay for skin extracts are described in the results.

VII. Determination of Tyrosine Aminotransferase. (L-tyrosine: 2 oxo-
glutarate aminotransferase E.C. 2.6.1.5.)

Tyrosine aminotransferase was assayed by a modification of the radioactive method of Miller & Thompson (1972). Skin and liver homogenates were prepared as described for the tyrosine hydroxylase assay and 0.4ml. homogenate added to a reaction mixture containing 10.0µmoles α-ketoglutarate in 0.25ml. 0.1M phosphate buffer (pH 7.4), 2.0µmoles tyrosine in 0.25ml. 0.01N HCl, 0.075µmoles pyridoxal phosphate in 0.05ml. and 1.0µCi ¹⁴C-tyrosine in 0.05ml. The mixture was incubated in a shaking water bath at 37°C for 1hr. and the reaction terminated with 0.2ml. 30% TCA. After centrifuging the supernatant was acidified with 0.8ml. 5N HCl and the p-hydroxyphenylpyruvate formed by transamination extracted with 5ml. 4:1 ethyl acetate/toluene mixture. The organic layer was taken for counting in a liquid scintillation counter by the procedure described in the tyrosine hydroxylase assay (solubiliser was omitted as the solution to be counted was miscible with toluene).

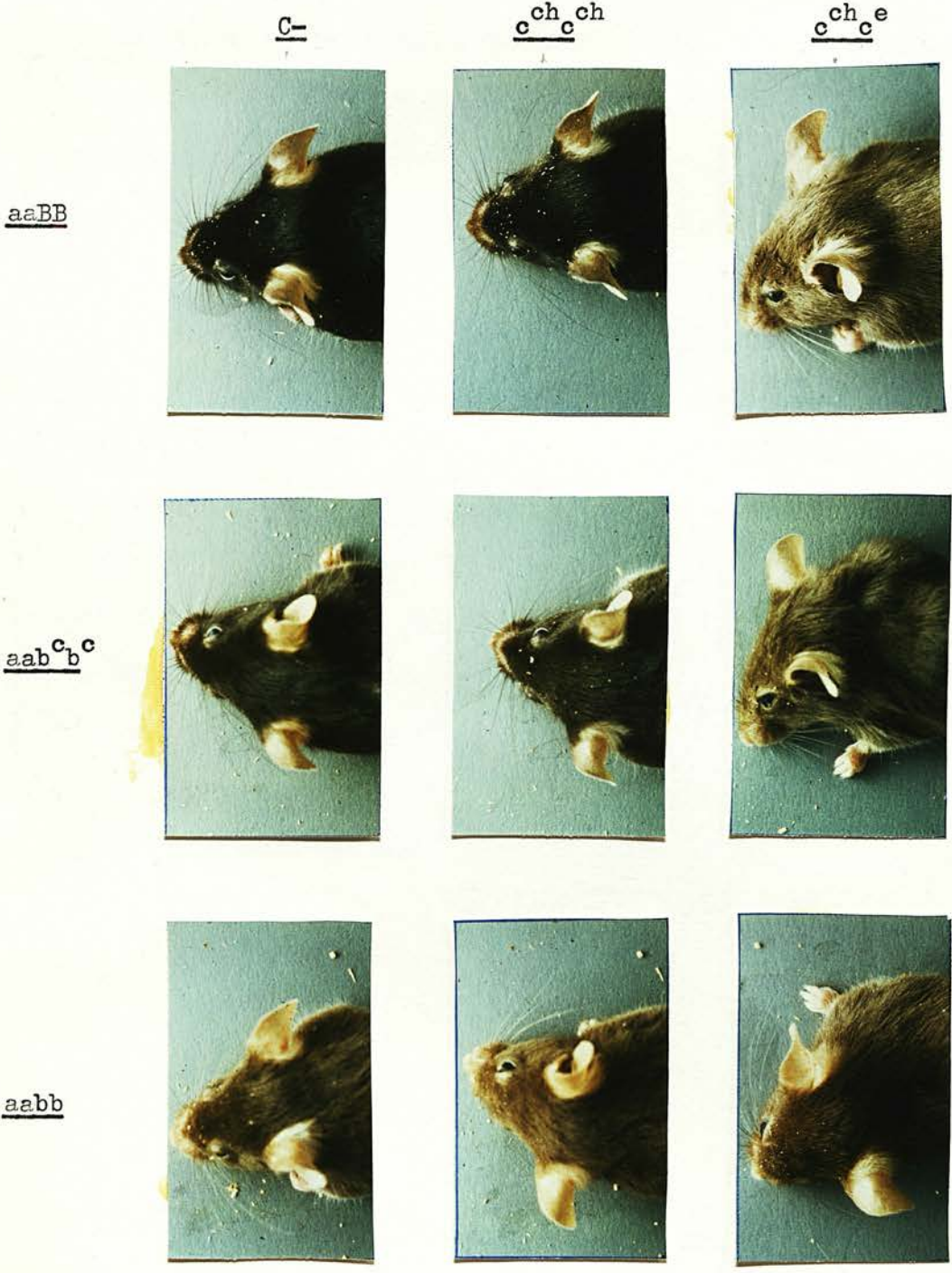
VIII. Extraction and Measurement of Free Amino Acid Pools.

Skin homogenates were prepared as described for the tyrosine hydroxylase assay. 0.2ml. standard AGPA solution was added for each 1ml. homogenate such that the final AGPA concentration was 0.2 μ moles/ml. The mixture was vortexed and protein precipitated with 0.2ml. 30% w/v sulphosalicylic acid at 0°C. After centrifuging the supernatant was applied to the column of a Technicon Auto Amino Acid Analyser. The quantities of the amino acids eluted were calculated as μ moles/ml homogenate using the AGPA peak as a standard.

IX. Determination of Protein.

Protein was determined by a modification of the method of Lowry. 0.4ml. solution under assay was incubated with 2ml. reagent solution at room temperature for 20mins. The reagent solution was made fresh daily by addition of 0.5ml. 0.5% CuSO_4 to 24.5ml. of a solution containing 2.0% Na_2CO_3 , 0.4% NaOH and 0.02% sodium potassium tartrate. After incubation 0.2ml. Folin & Ciocalteu's phenol reagent was added, the mixture shaken and then left for a further 30mins. The colour developed was read at 750nm. and protein weight estimated from a standard curve constructed by using BSA solutions of different concentrations.

Fig.2. The Visual Effects of Allelic Substitution at the c-locus on aaBB, aab^c_b^c, and aabb Backgrounds.



$\frac{c^{ch}}{c}$



$\frac{c^e}{c}$



$\frac{c^e}{c}$



$\frac{cc}{cc}$

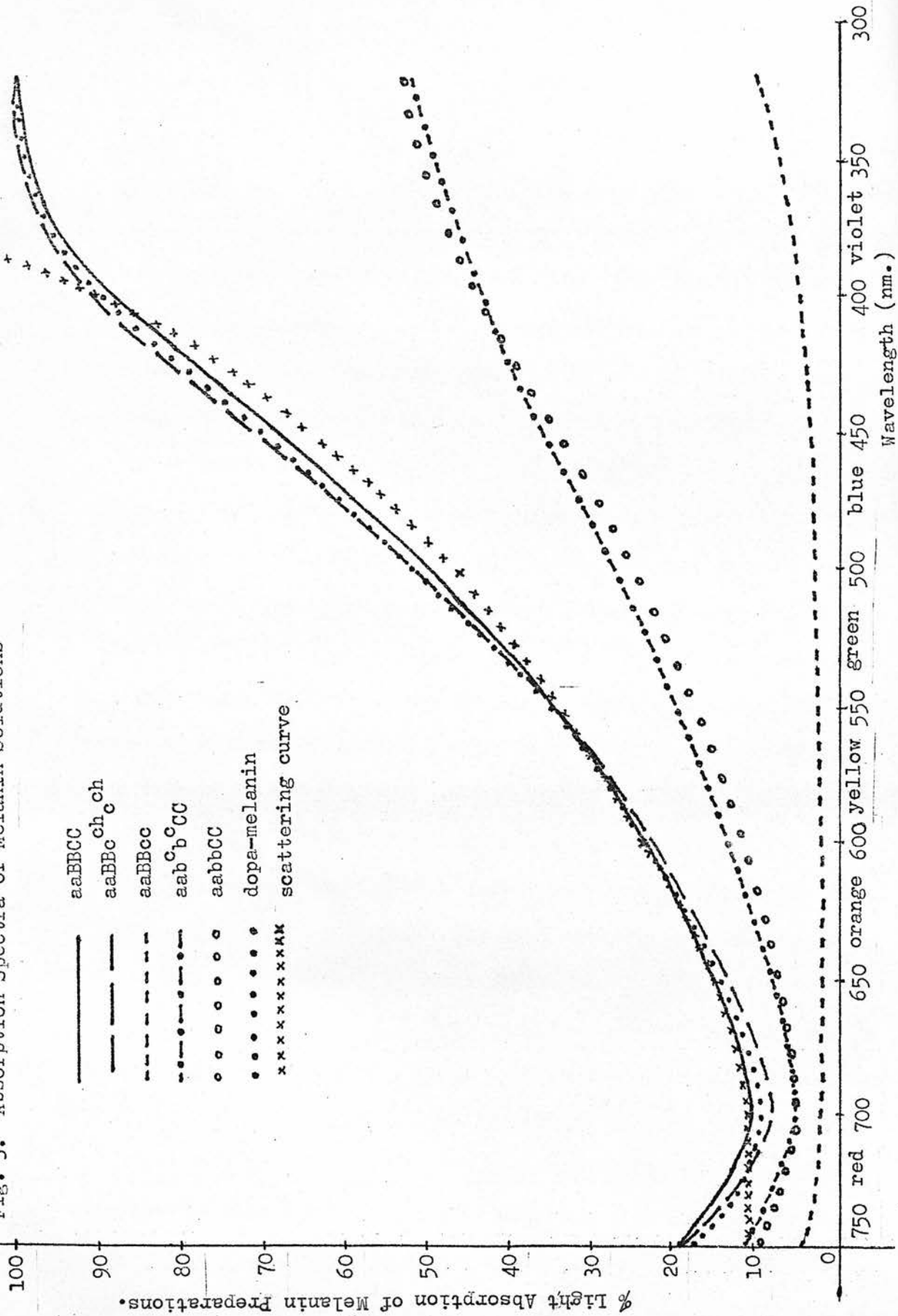


RESULTSI. Melanin Determinations.

(i). The visual effects of allelic substitution at the c-locus in non-agouti black, cordovan and brown animals.

Photographs of the range of genotypes on which detailed investigations were made are shown in fig. 2. The c-locus alleles produce distinct effects on a non-agouti black (aaBB) background when the wild-type dominant (C) allele is not present. The black-chinchilla homozygote (aaBBc^{ch}c^{ch}) is dark brown in colour and, in direct light, easily distinguishable from wild-type although this difference does not show clearly in the photographs. A gradation of coat colour from dark brown to white can be produced by various combinations of the c^{ch}, c^e, and c alleles. c^ec animals are only just distinguishable from albinos by coat colour but have black eyes unlike true albinos, which have pink eyes. The effects of the same combinations of c-locus alleles with cordovan are less marked: although wild-type pigmentation is reduced to a dark brown similar to that of black chinchilla by cordovan, other allelic combinations produce a darker colour with cordovan than with black except for c^ec which again is almost white, with black eyes. c^{ch} appears to have no effect on eumelanin in cordovan animals, aab^cb^cc^{ch}c^{ch} animals being distinguished from wild-type only by the presence of white, instead of yellow hairs, in and around the ears. The trend is continued with brown animals, in which wild-type, c^{ch}c^{ch}, c^{ch}c^e, and c^{ch}c are phenotypically indistinguishable in body colour, though white hairs are present in the ears of the latter three genotypes. bbc^ec^e animals are slightly darker than b^cb^cc^ec^e animals, which in turn are darker than BBc^ec^e.

Fig. 3. Absorption Spectra of Melanin Solutions



(ii). Absorption spectra of melanin extractions.

Pigment granules were extracted from aaBBCC, aaBBc^{ch}c^{ch}, aaBBcc, aab^cb^cCC and aabbCC animals and the absorption spectra of the extractions obtained compared with each other and with a solution of 'dopa-melanin' prepared by the action of mushroom tyrosinase on a dopa solution and then solubilised in the same way as extracted pigment. The spectra obtained are shown in fig. 3 opposite, together with the curve expected from the scattering of light by small particles (less than 90 μ) in suspension. Rayleigh's Law of Scattering states that scattering varies inversely as the fourth power of the wavelength. During the preparation of the extracts the process of solution was monitored by measuring optical density at 360nm. at regular intervals. O.D. decreased for about 2 $\frac{1}{2}$ hrs. and then remained steady for over three hours. All the solutions obtained obeyed Beer-Lambert's Law. No sediment was seen after centrifuging for 1hr. at 100,000g. Subsequent measurements of optical density were made at 360nm. where absorption values are high and differences in intensity between solutions at their maximum.

Daniel (1938) obtained similar absorption curves over the range 425-675nm. for melanin extracts from AABBCC and AABBc^{ch}c animals and pointed out that the slope of the curves over this range of wavelength could indicate light scattering by suspended particles. The curves in fig. 3, however, show a marked departure from that expected on this hypothesis at 700-750 and 320-400nm. As the solutions obtained obey Beer-Lambert's Law it may be concluded that the spectrophotometric measurement is quantitative; if true absorption rather than scattering is being measured it may also be concluded from the similarity of the spectra obtained that the difference in colour between the genotypes

investigated are due to quantitative rather than qualitative differences in the melanin present. If the curves are due mainly to light scattering by undissolved suspended particles, however, no conclusions can be drawn as to the qualitative nature of the melanin present but measurements of absorption at a particular wavelength will give an indication of the number of particles present and to this extent will be a quantitative estimate of the amount of pigment present. Acidification of the extracts caused a black, flocculent precipitate to appear which could then be 'redissolved' on heating with alkali. This apparent precipitation and solvation cannot be distinguished from an aggregation and dispersal of small particles, however. Nicolaus (1968) reported that alkali treatment degraded eumelanin to its constituent indoles: if this is the case in the preparations used here, the O.D. values of the extracts may not reflect differences in coat colour due to different polymeric structures of the melanin from different genotypes so that, although the extracts are solutions rather than suspensions, O.D. measurements and absorption spectra may give no information on the qualitative nature of the pigment present.

Fig. 4a. Melanin Content vs. Age, (1).

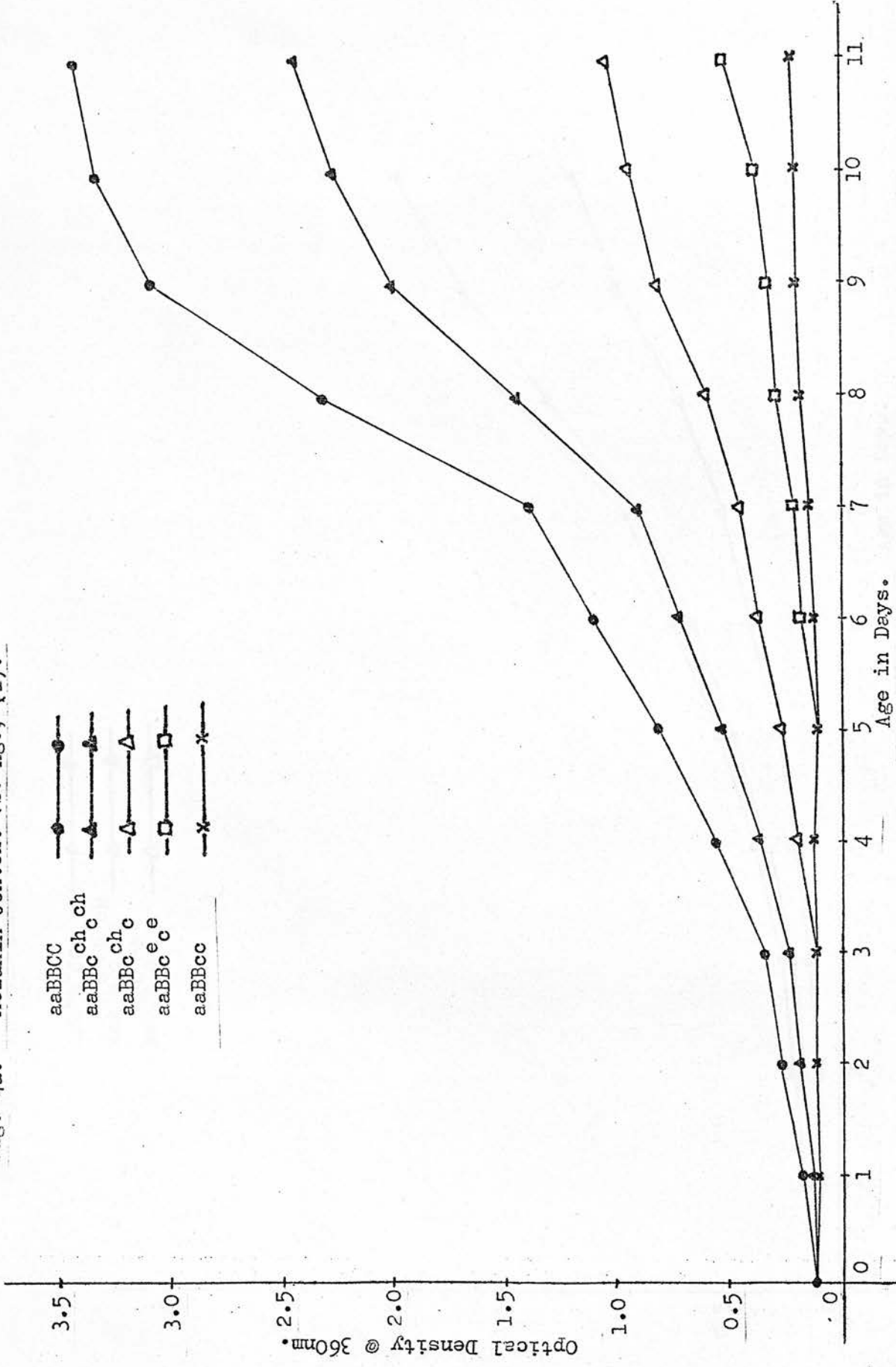


Fig. 4b. Melanin Content vs. Age, (2).

$ab^c_b^c c c$ —●—
 $ab^c_c^c ch_c ch$ —▲—
 $ab^c_c^c ch_c c$ —△—

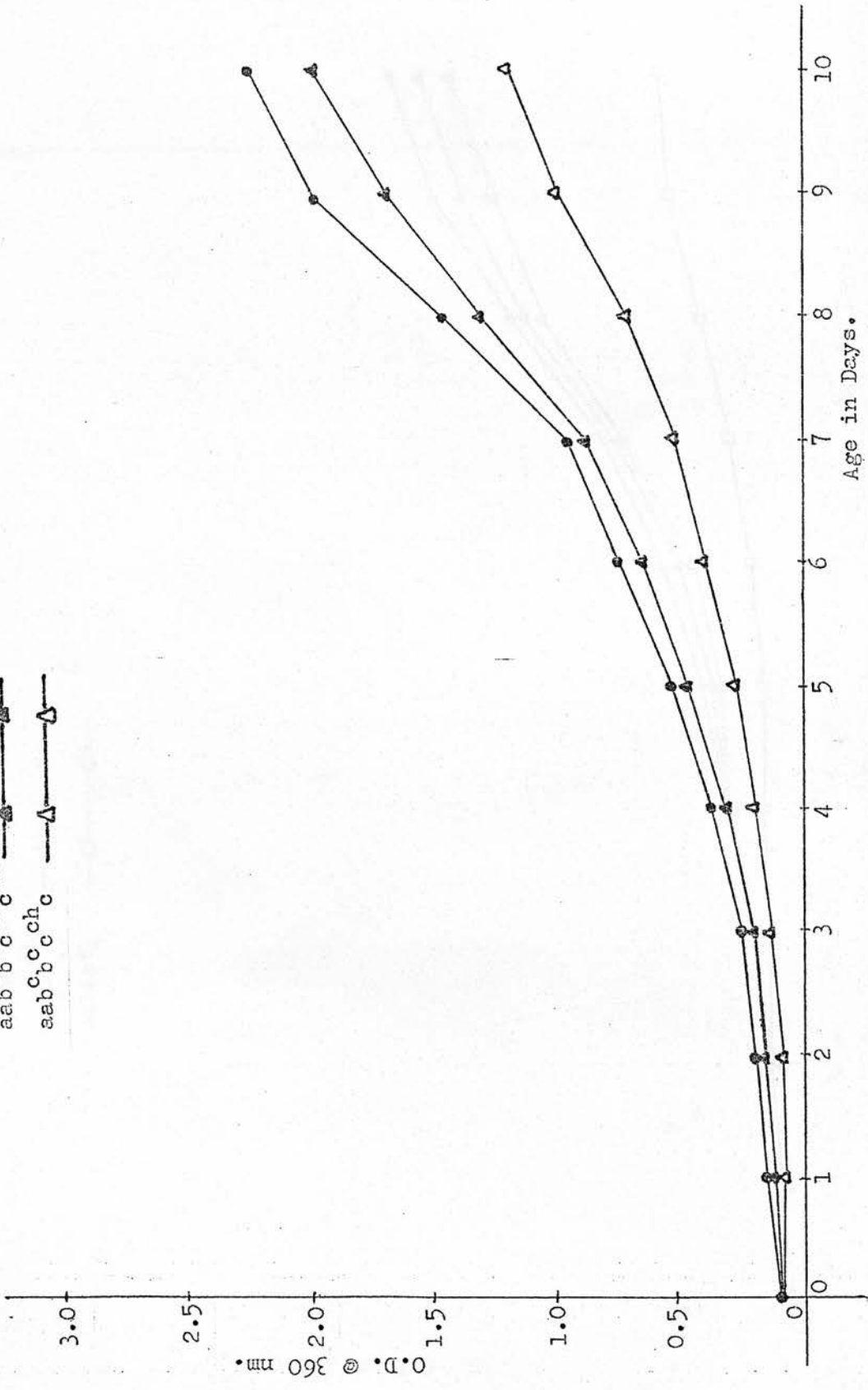
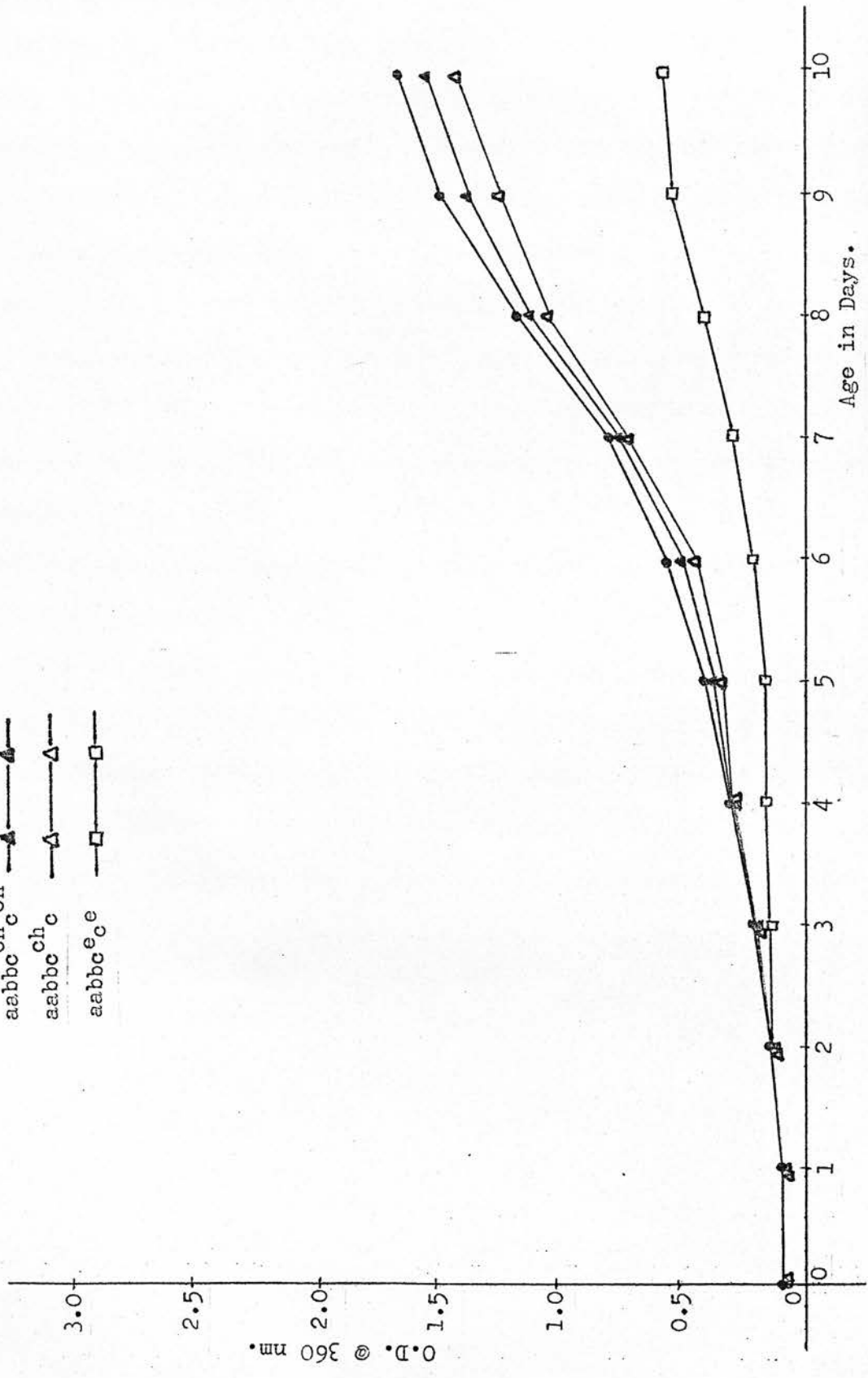


Fig. 4c. Melanin Content vs. Age, (3).

- aabbCC —●—
- aabbcc^{ch} —▲—
- aabbcc^{ch} —△—
- aabbcc^e —□—



(iii). O.D. values of black, cordovan and brown genotypes from 0-11days.

The optical densities of pigment extractions prepared from young mice from birth to 10 or 11 days of age are shown in tables la, b and c below. 50mgm. dorsal skin was used in each case for the preparation of 1ml. solution. At least 3 animals were used per litter for each set of determinations and the values given below are means of determinations from at least 5 litters.

Table la. O.D. @ 360nm. of melanin extractions of aaBB genotypes.

Age.	O.D./ml. melanin extractions from 50mgm. skin.						
	<u>CC</u>	<u>Cc</u> ^{ch}	<u>Cc</u>	<u>c</u> ^{ch} <u>c</u> ^{ch}	<u>c</u> ^{ch} <u>c</u>	<u>c</u> ^e <u>c</u> ^e	<u>cc</u>
0	0.09 ±0.01	0.08 ±0.02	0.09 ±0.01	0.08 ±0.01	0.08 ±0.02	0.08 ±0.01	0.09 ±0.00
1	0.15 ±0.02	0.15 ±0.01	0.13 ±0.02	0.12 ±0.02	0.09 ±0.01	0.09 ±0.02	0.10 ±0.01
2	0.25 ±0.04	0.24 ±0.03	0.22 ±0.02	0.17 ±0.03	0.10 ±0.03	0.09 ±0.02	0.09 ±0.02
3	0.33 ±0.04	0.29 ±0.04	0.27 ±0.04	0.23 ±0.02	0.10 ±0.02	0.11 ±0.02	0.09 ±0.01
4	0.56 ±0.03	0.51 ±0.05	0.50 ±0.06	0.36 ±0.04	0.16 ±0.03	0.11 ±0.02	0.11 ±0.01
5	0.81 ±0.06	0.75 ±0.05	0.72 ±0.04	0.53 ±0.05	0.26 ±0.03	0.12 ±0.03	0.11 ±0.02
6	1.12 ±0.07	0.98 ±0.08	0.95 ±0.07	0.70 ±0.06	0.37 ±0.06	0.16 ±0.02	0.12 ±0.02
7	1.37 ±0.08	1.19 ±0.07	1.01 ±0.07	0.90 ±0.05	0.48 ±0.05	0.21 ±0.04	0.16 ±0.03
8	2.32 ±0.11	2.10 ±0.12	1.99 ±0.09	1.43 ±0.08	0.61 ±0.08	0.27 ±0.03	0.18 ±0.03
9	3.09 ±0.17	2.91 ±0.10	2.86 ±0.11	2.09 ±0.09	0.82 ±0.07	0.33 ±0.04	0.19 ±0.02
10	3.35 ±0.19	3.35 ±0.16	3.08 ±0.15	2.28 ±0.09	0.95 ±0.09	0.38 ±0.04	0.19 ±0.03
11	3.45 ±0.22	3.45 ±0.19	3.20 ±0.16	2.45 ±0.14	1.04 ±0.08	0.53 ±0.04	0.21 ±0.03

Table 1b. O.D. @ 360nm. of melanin extractions of aab^cb^c genotypes.

Age.	O.D./ml. melanin extractions from 50mgm. skin.				
	<u>CC</u>	<u>Cc</u> ^{ch}	<u>Cc</u>	<u>c</u> ^{ch} <u>c</u> ^{ch}	<u>c</u> ^{ch} <u>c</u>
0	0.10 ±0.02	0.09 ±0.01	0.08 ±0.02	0.10 ±0.02	0.08 ±0.01
1	0.13 ±0.03	0.14 ±0.03	0.12 ±0.02	0.14 ±0.01	0.10 ±0.02
2	0.18 ±0.03	0.19 ±0.03	0.17 ±0.01	0.18 ±0.02	0.11 ±0.01
3	0.25 ±0.06	0.23 ±0.04	0.24 ±0.02	0.22 ±0.02	0.15 ±0.03
4	0.35 ±0.05	0.37 ±0.04	0.34 ±0.04	0.34 ±0.04	0.21 ±0.03
5	0.52 ±0.07	0.54 ±0.05	0.50 ±0.02	0.49 ±0.04	0.30 ±0.02
6	0.75 ±0.09	0.77 ±0.08	0.76 ±0.03	0.65 ±0.06	0.39 ±0.04
7	0.96 ±0.11	0.96 ±0.08	0.93 ±0.04	0.89 ±0.09	0.53 ±0.03
8	1.48 ±0.13	1.44 ±0.16	1.41 ±0.06	1.32 ±0.09	0.72 ±0.05
9	2.01 ±0.31	2.03 ±0.21	1.99 ±0.09	1.70 ±0.08	1.05 ±0.05
10	2.27 ±0.33	2.25 ±0.27	2.20 ±0.18	2.13 ±0.21	1.25 ±0.09

Table 1c. O.D. @ 360nm. of melanin extractions of aabb genotypes.

Age.	O.D./ml. melanin extractions from 50mgm. skin.					
	<u>CC</u>	<u>Cc</u> ^{ch}	<u>Cc</u>	<u>c</u> ^{ch} <u>c</u> ^{ch}	<u>c</u> ^{ch} <u>c</u>	<u>c</u> ^e <u>c</u> ^e
0	0.08 ±0.01	0.08 ±0.01	0.10 ±0.01	0.09 ±0.02	0.10 ±0.01	0.09 ±0.02
1	0.10 ±0.02	0.09 ±0.01	0.10 ±0.01	0.10 ±0.02	0.09 ±0.02	0.09 ±0.01
2	0.16 ±0.02	0.14 ±0.03	0.15 ±0.02	0.16 ±0.03	0.14 ±0.03	0.11 ±0.01
3	0.20 ±0.04	0.23 ±0.03	0.22 ±0.03	0.21 ±0.04	0.22 ±0.03	0.10 ±0.02
4	0.29 ±0.06	0.30 ±0.05	0.31 ±0.05	0.28 ±0.05	0.29 ±0.04	0.11 ±0.01
5	0.41 ±0.05	0.43 ±0.06	0.43 ±0.04	0.39 ±0.05	0.35 ±0.06	0.13 ±0.03
6	0.54 ±0.05	0.54 ±0.04	0.53 ±0.08	0.48 ±0.07	0.44 ±0.07	0.16 ±0.02
7	0.75 ±0.07	0.74 ±0.06	0.76 ±0.09	0.72 ±0.08	0.69 ±0.05	0.19 ±0.03
8	1.18 ±0.07	1.19 ±0.08	1.21 ±0.06	1.11 ±0.06	1.06 ±0.08	0.26 ±0.03
9	1.50 ±0.10	1.48 ±0.09	1.49 ±0.09	1.39 ±0.09	1.29 ±0.08	0.35 ±0.04
10	1.68 ±0.09	1.65 ±0.13	1.65 ±0.09	1.59 ±0.10	1.46 ±0.09	0.40 ±0.03

These results are presented graphically in figs. 4a, b and c.

Table 1d below gives O.D. values obtained for 50mgm. shaved skin for aaBBCC and aaBBc^{ch}c^{ch} from 7 days of age when the hair is first visible. Although the homogenisation process used for the tyrosine hydroxylase assay left hairs intact, hair alone could be homogenised to some extent and tyrosine hydroxylase activity demonstrated. If this activity is producing melanin in vivo within the hair, the

O.D. values presented above will reflect this, but tyrosine hydroxylase activities will not, since the normal homogenisation procedure does not liberate the hair activity. As the extent of homogenisation of hair without skin and its associated enzyme activity varied a precise comparison of total skin and hair enzyme with O.D. value was not possible and it was therefore decided to extract pigment from shaved skin for comparison with tyrosine hydroxylase activity.

Table 1d. O.D. values for shaved aaBBCC and aaBBc^{ch}c^{ch} skin.

Age	<u>CC</u>	<u>c^{ch}c^{ch}</u>
7	1.39 \pm 0.06	0.95 \pm 0.07
8	1.63 \pm 0.09	1.20 \pm 0.06
9	1.78 \pm 0.10	1.44 \pm 0.07
10	1.90 \pm 0.09	1.66 \pm 0.09
11	2.01 \pm 0.11	1.94 \pm 0.08

(iv). O.D. values for aaBBCCC animals.

The O.D. values obtained for 27 offspring from matings from which aaBBCCC males were expected together with aaBBcc and aaBBCC are shown with the simultaneously-determined tyrosine hydroxylase activities in table 7 further on in this section.

(v). O.D. values for 'dilute' and 'dilute-lethal'.

The O.D. values shown in table 3 below are from 5day animals. At this age dilute animals already appear grey. The results are compared with those for normal (DD) animals and also expressed as a percentage of DD.

Table 3. O.D. values for 5day aaBBCCDD, aaBBCCdd and aaBBCCd¹d¹.

Genotype	O.D.	% of normal
<u>DD</u>	0.81 \pm 0.06	100.0
<u>dd</u>	0.79 \pm 0.07	97.5
<u>d¹d¹</u>	0.76 \pm 0.04	94.0

II. Phenylalanine Hydroxylase Activity in wild-type, dilute and dilute-lethal.

Phenylalanine hydroxylase activity was first determined in liver supernatants of 3week aaBBCCDD, aaBBCCdd and aaBBCCd¹d¹ animals. Coleman (1960) reported the presence of an inhibitor of the particulate fraction of the liver homogenate which caused dd and d¹d¹ to have 50% and 14% of wild-type activity. In a second set of experiments assays were performed on whole liver homogenates and in a third set the sediment formed after centrifugation was resuspended in the supernatant and assays performed on this mixture. The results are shown in table 4 below. Each value shown is a mean of five determinations.

Table 4. Phenylalanine hydroxylase in DD, dd and d¹d¹.

Genotype	Supernatant Activity (μ moles/min/mgm.protein)	Activity of whole homog.	Activity of reconstituted homogenate
<u>DD</u>	5.05 \pm 0.06	3.93 \pm 0.09	3.78 \pm 0.13
<u>dd</u>	5.26 \pm 0.09	4.03 \pm 0.10	4.12 \pm 0.13
<u>d¹d¹</u>	5.31 \pm 0.07	4.11 \pm 0.08	3.91 \pm 0.09

The raw data showed d¹d¹ to have higher phenylalanine hydroxylase activity than DD and dd, but as d¹d¹ liver was found to have a 50% higher protein content the difference was very much reduced when activities were expressed per mgm. protein. Attempts to perform the assay on whole skin homogenates were unsuccessful.

Phenylalanine hydroxylase assays were also carried out on 'jittery' (jiji) and 'wabblers-lethal' (wlwl) which show abnormal behaviour similar to that of dilute-lethal, and their normal heterozygous litter-mates. No significant differences in phenylalanine hydroxylase activity were found between these genotypes. ji and wl are not associated with abnormal pigmentation.

III. Tyrosine Hydroxylase.

(i). Tyrosine hydroxylase activity in skin and liver homogenates.

Tyrosine hydroxylase activity was assayed in skin and liver homogenates subjected to a number of different treatments. Week-old C57 animals were used. Menon & Haberman (1970) reported that tyrosine hydroxylase activity in microsomes and melanosomes prepared from melanomas using isotonic sucrose could be increased by several membrane-disrupting treatments including freezing and thawing and addition of Triton-X 100. Homogenates prepared in water showed increased activity which was not further increased by the above treatments. In the following experiments skin and liver homogenates were prepared in 0.1M phosphate buffer at pH 7.4 with or without 0.25M sucrose and 0.1% Triton-X 100. Homogenates were then assayed immediately or after being frozen and thawed twice. Homogenates subjected to the above treatments were also centrifuged at 60,000g for 30mins. and the supernatants taken for assay. The results are given in tables 5a and b below as counts per minute obtained for equal volumes of homogenate or supernatant.

Table 5a. Effects of different homogenising media and freezing and thawing on tyrosine hydroxylase activity of skin homogenates and supernatants.

Homogenising medium	Homogenate activity		Supernatant activity	
	fresh	frozen	fresh	frozen
0.1M phosphate	2699 \pm 136	1354 \pm 281	432 \pm 69	834 \pm 58
0.1M phosphate + 0.25M sucrose	2371 \pm 193	2016 \pm 255	327 \pm 51	857 \pm 37
0.1M phosphate + 0.1% Triton-X 100	2836 \pm 308	1073 \pm 362	504 \pm 73	691 \pm 66
0.1M phos. +0.25M sucrose +0.1% Triton-X 100	2745 \pm 239	1665 \pm 206	612 \pm 32	478 \pm 46

Table 5b. Effects of different homogenising media and freezing on the tyrosine hydroxylase activity of liver homogenates and supernatants.

homogenising medium	homogenate activity		supernatant activity	
	fresh	frozen	SN from fresh homogenate	SN fr. frozen homogenate
0.1M phosphate	1634 \pm 89	1400 \pm 56	943 \pm 46	1792 \pm 62
0.1M phos. + 0.25M sucrose	1671 \pm 52	1525 \pm 64	1009 \pm 69	1648 \pm 37
0.1M phos. + 0.1% triton-X100	1406 \pm 76	1268 \pm 68	1135 \pm 52	953 \pm 75
0.1M phos. + 0.25 M sucr. + 0.1% Triton-X 100	1522 \pm 61	1460 \pm 72	1267 \pm 60	1139 \pm 43

These experiments were originally carried out using 0.05M Tris buffer pH 7.4 but it was discovered that activity was increased almost 5-fold in the presence of phosphate buffer. Phosphate buffer was then used in all subsequent assays.

In skin highest activity was found in fresh whole homogenate prepared in 0.1M phosphate and 0.1% Triton-X 100. The results obtained, however, were very variable as can be seen from the high standard error attached to the activity value. Freezing appeared to reduce activity except in the presence of sucrose and high supernatant activities were not produced by any of the treatments. It was suggested by Menon & Haberman (1970) that the increases in extractable tyrosine hydroxylase activity seen after treatment with membrane-disruptive agents was due to the disruption of melanosomes, which would allow easier access of substrate to enzyme and enzyme to substrate by dissociating the tyrosine hydroxylase molecules from the melanosome. As the tyrosine hydroxylase activities seen in crude skin homogenates in this study were so low, it was decided to investigate possible methods of increasing the activity with the reservation that a meaningful comparison of in vitro enzyme activity with melanin produced in vivo depends on the extractable

enzyme activity being proportional to the in vivo activity in all cases for all mutant forms of enzyme. The results presented above, however, show that no activation similar to that found in melanoma by Menon & Haberman occurred in skin, nor could the tyrosine hydroxylase activity be released into the supernatant. Only the use of phosphate buffer instead of Tris had a large and repeatable effect on tyrosine hydroxylase activity. As use of Tris introduces a molecule into the assay mixture which is not found in vivo, however, it was decided to use phosphate buffer. Tyrosine hydroxylase was therefore assayed in fresh, whole homogenate with phosphate buffer as homogenising medium. Addition of the chelating agent EDTA reduces activity slightly, probably by its action on the copper moiety of the tyrosinase molecule. Addition of cupric ions as CuSO_4 had no effect on activity.

The tyrosine hydroxylase from liver shows different behaviour towards Triton-X and centrifugation. Triton-X reduces activity, which appears to be largely in the supernatant. As there are no melanosomes in the liver, tyrosine hydroxylase would be expected to be differently located, and from these results does not appear to be attached to any microsomal fraction.

Fig. 5a. Tyrosine Hydroxylase Activity vs. Age.

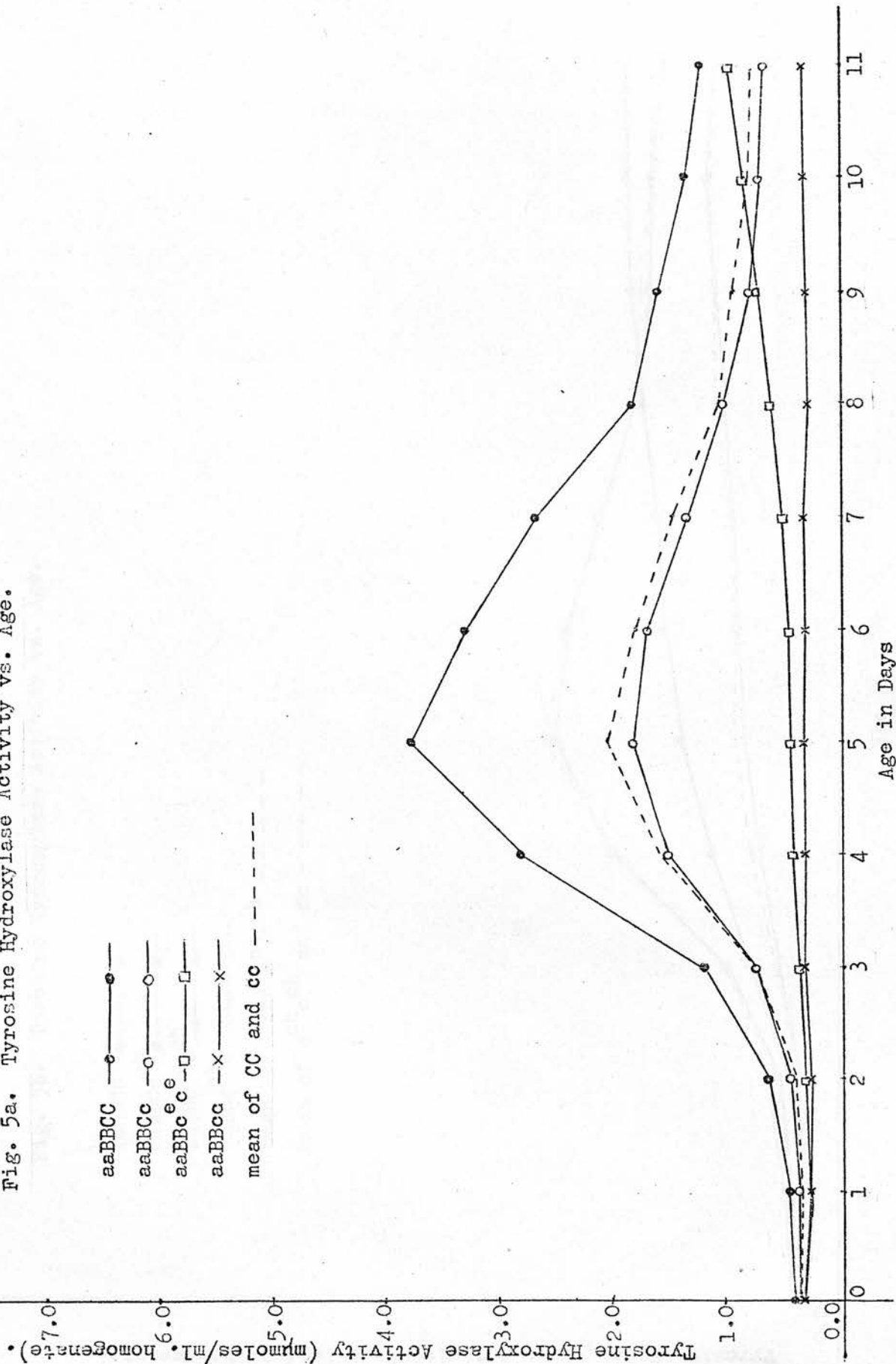


Fig. 5b. Tyrosine Hydroxylase Activity vs. Age.

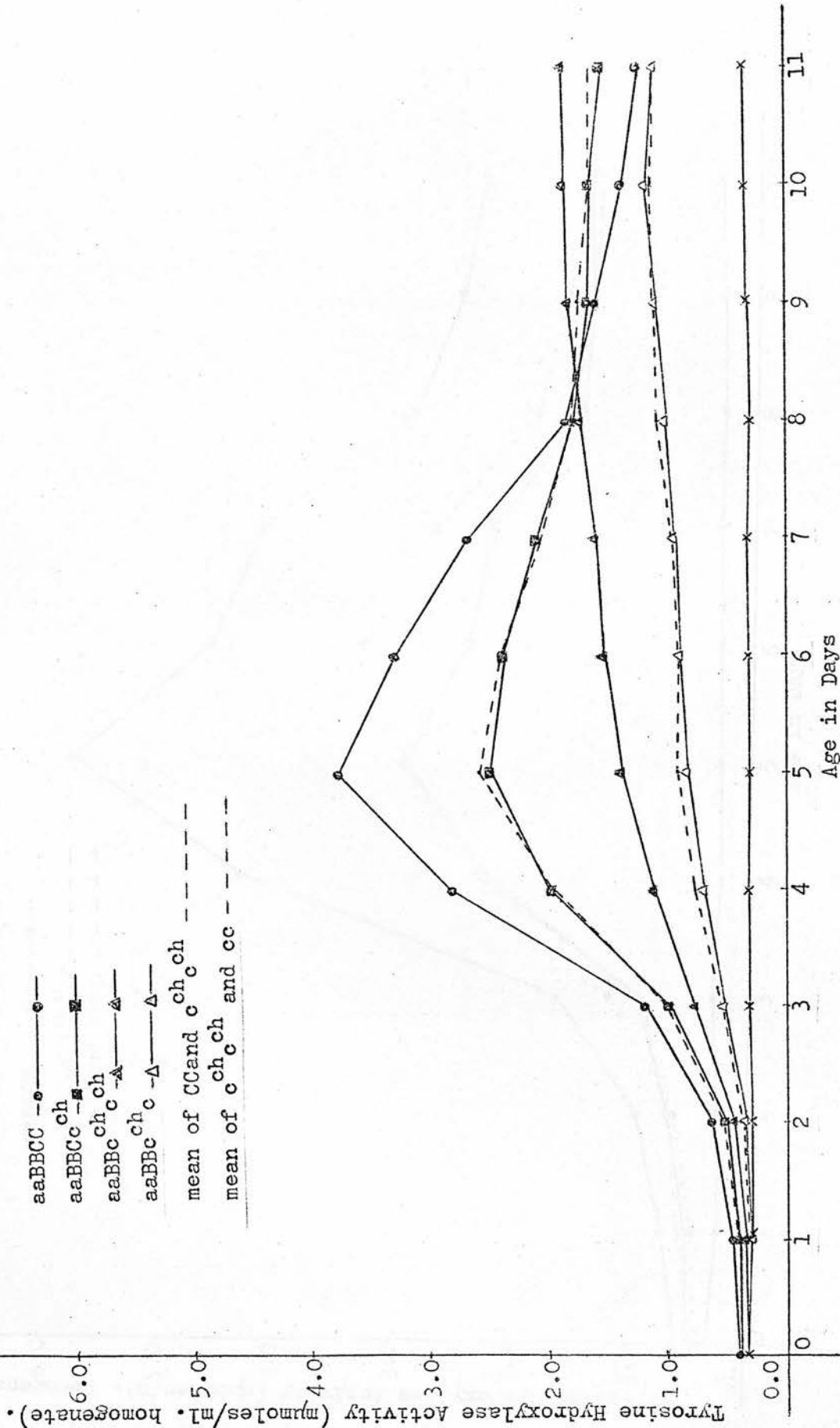


Fig. 5c. Tyrosine Hydroxylase Activity vs. Age.

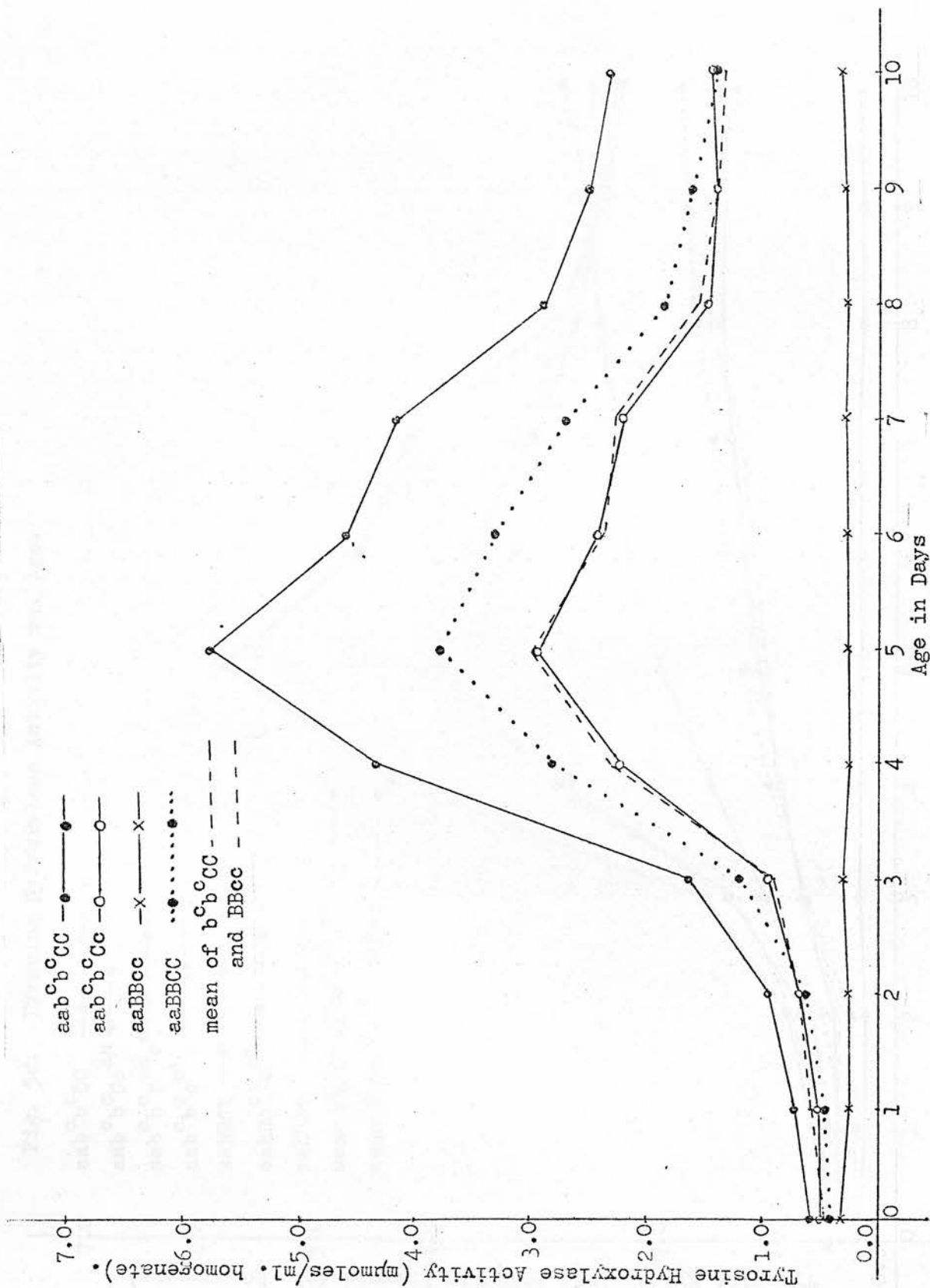
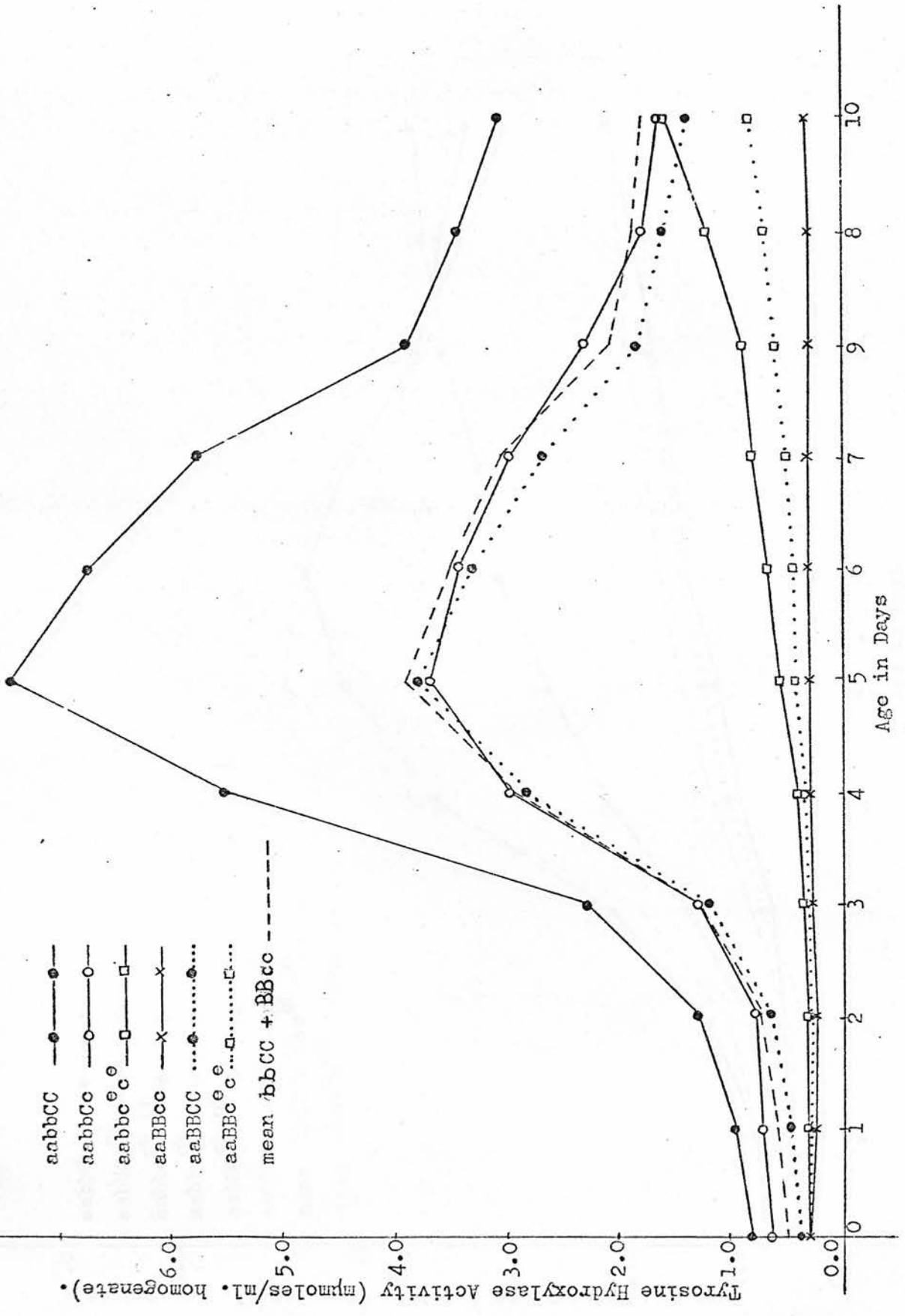


Fig. 5e. Tyrosine Hydroxylase Activity vs. Age.



(ii). Tyrosine hydroxylase activity in black, brown and cordovan genotypes.

The tyrosine hydroxylase activities of whole skin homogenates from animals from birth to 11 days are shown in tables 6a, b and c below. As far as possible the values given below are means of at least 12 determinations: 3 sets of assays on 4 different matings from which 3 or 4 littermates were taken for replicate assays. Activity is expressed as $\mu\text{moles dopa formed/min/ml. homogenate}$. Figs. 5a, b, c, d, e, f compare the development of tyrosine hydroxylase activity with age for the various genotypes.

Table 6a. Tyrosine hydroxylase activity for aaBB genotypes.

Age	$\mu\text{moles dopa formed/min/ml. homogenate}$						
	<u>CC</u>	<u>Cc</u> ^{ch}	<u>Cc</u>	<u>c</u> ^{ch} <u>c</u> ^{ch}	<u>c</u> ^{ch} <u>c</u>	<u>c</u> ^e <u>c</u> ^e	<u>cc</u>
0	0.40 ± 0.02	0.37 ± 0.03	0.36 ± 0.04	0.36 ± 0.03	0.30 ± 0.01	0.28 ± 0.03	0.30 ± 0.02
1	0.46 ± 0.02	0.39 ± 0.04	0.38 ± 0.03	0.38 ± 0.02	0.33 ± 0.04	0.31 ± 0.02	0.26 ± 0.01
2	0.62 ± 0.03	0.51 ± 0.04	0.44 ± 0.03	0.46 ± 0.05	0.36 ± 0.02	0.31 ± 0.04	0.24 ± 0.03
3	1.19 ± 0.05	0.98 ± 0.06	0.75 ± 0.06	0.82 ± 0.03	0.54 ± 0.06	0.34 ± 0.06	0.30 ± 0.03
4	2.82 ± 0.09	1.94 ± 0.08	1.51 ± 0.08	1.16 ± 0.06	0.71 ± 0.05	0.39 ± 0.02	0.31 ± 0.01
5	3.81 ± 0.12	2.49 ± 0.06	1.83 ± 0.10	1.38 ± 0.05	0.86 ± 0.04	0.43 ± 0.04	0.30 ± 0.04
6	3.32 ± 0.16	2.40 ± 0.11	1.69 ± 0.09	1.54 ± 0.08	0.88 ± 0.06	0.48 ± 0.03	0.29 ± 0.03
7	2.70 ± 0.11	2.10 ± 0.12	1.38 ± 0.12	1.63 ± 0.10	0.95 ± 0.08	0.55 ± 0.03	0.31 ± 0.02
8	1.84 ± 0.10	1.83 ± 0.09	1.03 ± 0.06	1.77 ± 0.06	1.01 ± 0.07	0.63 ± 0.06	0.29 ± 0.02
9	1.62 ± 0.07	1.70 ± 0.10	0.79 ± 0.09	1.86 ± 0.07	1.12 ± 0.11	0.74 ± 0.05	0.30 ± 0.03
10	1.41 ± 0.09	1.65 ± 0.06	0.73 ± 0.07	1.92 ± 0.11	1.19 ± 0.09	0.87 ± 0.04	0.32 ± 0.03
11	1.26 ± 0.12	1.56 ± 0.08	0.69 ± 0.11	1.89 ± 0.09	1.08 ± 0.12	0.99 ± 0.08	0.33 ± 0.04

Table 6b. Tyrosine hydroxylase activity for aab^cb^c genotypes.

Age	pmoles dopamine formed/min/ml.homogenate					
	<u>CC</u>	<u>Cc^{ch}</u>	<u>Cc</u>	<u>c^{ch}c^{ch}</u>	<u>c^{ch}c</u>	<u>cc</u>
0	0.58 ±0.04	0.58 ±0.02	0.49 ±0.04	0.50 ±0.03	0.46 ±0.03	—
1	0.72 ±0.03	0.63 ±0.05	0.52 ±0.06	0.58 ±0.05	0.49 ±0.05	—
2	0.95 ±0.06	0.77 ±0.08	0.63 ±0.03	0.71 ±0.08	0.51 ±0.03	—
3	1.65 ±0.09	1.44 ±0.06	0.95 ±0.06	1.18 ±0.07	0.73 ±0.06	—
4	4.36 ±0.09	2.91 ±0.11	2.30 ±0.09	1.66 ±0.09	0.99 ±0.08	—
5	5.80 ±0.13	4.03 ±0.09	2.99 ±0.13	2.09 ±0.08	1.25 ±0.09	0.33 ±0.02
6	4.63 ±0.11	3.76 ±0.13	2.48 ±0.15	2.35 ±0.10	1.37 ±0.13	0.32 ±0.04
7	4.21 ±0.07	3.15 ±0.08	2.23 ±0.11	2.44 ±0.11	1.41 ±0.10	0.29 ±0.04
8	2.90 ±0.07	2.77 ±0.10	1.49 ±0.13	2.65 ±0.08	1.45 ±0.07	0.30 ±0.03
9	2.49 ±0.09	2.56 ±0.12	1.42 ±0.09	2.76 ±0.10	1.49 ±0.11	0.31 ±0.02
10	2.33 ±0.06	2.40 ±0.09	1.41 ±0.09	2.93 ±0.13	1.58 ±0.09	0.32 ±0.04

Table 6c. Tyrosine hydroxylase activity for aabb genotypes.

Age	μmoles dopa formed/min/ml. homogenate						
	<u>cc</u>	<u>Cc</u> ^{ch}	<u>Cc</u>	<u>c</u> ^{ch} <u>c</u> ^{ch}	<u>c</u> ^{ch} <u>c</u>	<u>c</u> ^e <u>c</u> ^e	<u>cc</u>
0	0.79 ±0.06	0.76 ±0.06	0.69 ±0.07	0.70 ±0.04	0.42 ±0.06	0.33 ±0.04	---
1	0.95 ±0.03	0.83 ±0.07	0.74 ±0.09	0.77 ±0.08	0.54 ±0.03	0.30 ±0.07	---
2	1.29 ±0.07	1.31 ±0.06	0.82 ±0.11	0.89 ±0.09	0.61 ±0.05	0.29 ±0.06	---
3	2.30 ±0.07	2.09 ±0.09	1.30 ±0.06	1.44 ±0.06	0.69 ±0.05	0.33 ±0.09	---
4	5.53 ±0.09	3.71 ±0.06	2.99 ±0.10	2.13 ±0.11	0.99 ±0.08	0.42 ±0.06	---
5	7.41 ±0.08	4.50 ±0.10	3.71 ±0.09	2.69 ±0.07	1.20 ±0.07	0.56 ±0.05	0.32 ±0.03
6	6.74 ±0.12	4.85 ±0.12	3.45 ±0.06	3.13 ±0.12	1.47 ±0.11	0.67 ±0.08	---
7	5.79 ±0.11	4.43 ±0.13	2.98 ±0.11	3.30 ±0.10	1.61 ±0.09	0.79 ±0.09	---
8	3.92 ±0.13	3.87 ±0.09	2.32 ±0.08	3.61 ±0.11	1.78 ±0.12	0.91 ±0.07	0.31 ±0.04
9	3.46 ±0.09	3.66 ±0.11	1.79 ±0.13	3.83 ±0.09	1.93 ±0.12	1.20 ±0.09	---
10	3.11 ±0.11	3.39 ±0.13	1.58 ±0.12	3.92 ±0.13	2.16 ±0.08	1.57 ±0.09	---

After 9 days of age the skin became increasingly more difficult to homogenise. When homogenates were examined closely separate hairs could be distinguished: it appeared that the homogenisation process was effective on skin tissue only. The enzyme activities shown above therefore probably apply only to skin. Attempts to homogenise shaved hairs alone were partially successful. Tyrosine hydroxylase activity

was found to be present, but probably owing to the varying degrees of homogenisation obtained, was very variable and it was not possible to obtain any meaningful results. Since black, cordovan and brown albinos all show activities in the same range, while other genotypes show significantly different activities, cordovan and brown having approximately $1\frac{1}{2}$ and 2 times the activity of black for the same genotype at the c-locus, it was concluded that the low level of activity seen in albinos was not due to the presence of a tyrosine hydroxylase of very low activity, but either to non-specific enzyme activity or to the presence of a small amount of liver-type tyrosine hydroxylase. Evidence will be presented later to show that the tyrosine hydroxylase activity found in liver is due to a different enzyme under different control from that in the skin. The albino data are not complete owing to shortage of animals so that where necessary aaBBcc values will be used to fill in the gaps.

(iii). Tyrosine hydroxylase activity in dilute and dilute-lethal.

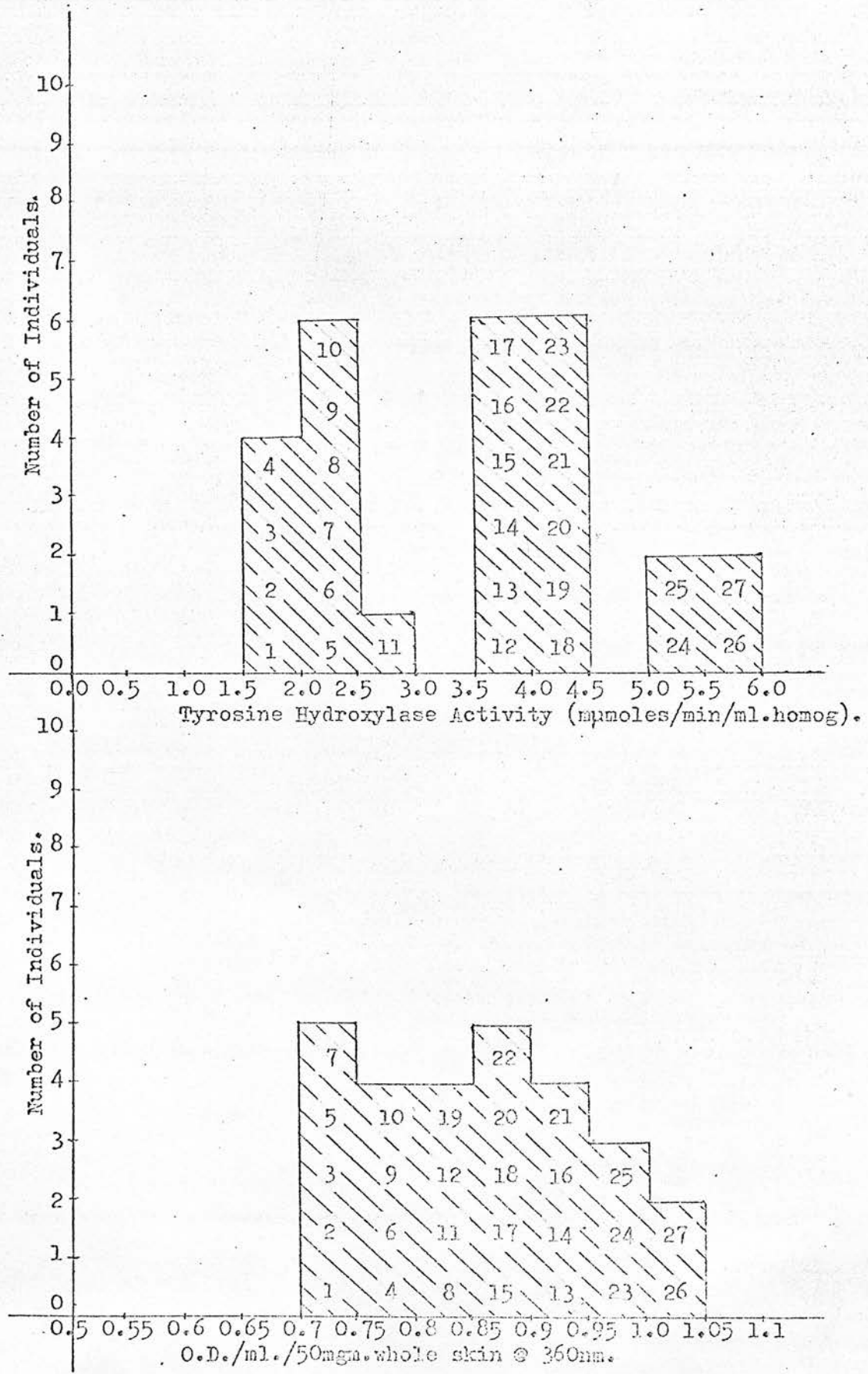
Tyrosine hydroxylase activity was measured in 5day aaBBCCdd and aaBBCCd¹d¹ skin and liver and found to be not significantly different from normal.

Table 7. tyrosine hydroxylase in DD, dd and d¹d¹.

genotype	μmoles dopa formed/min/ml.homog.	
	skin	liver
<u>DD</u>	3.81 ±0.12	1.51 ±0.03
<u>dd</u>	3.76 ±0.09	1.43 ±0.03
<u>d¹d¹</u>	3.70 ±0.13	1.49 ±0.01

At 5days dd and d¹d¹ animals already appear grey, but the neuromuscular abnormalities associated with older d¹d¹ animals are barely noticeable.

Fig. 6. Tyrosine Hydroxylase Activity and O.D. values for all ♂ Offspring tested from Matings segregating for λ^C .



(iv). Tyrosine hydroxylase activity and O.D. values of aaBBCCc.

aaBBCCc animals were produced from crosses between females carrying Cattanach's translocation and autosomally aaBBCCc and C57 males. Four classes of phenotypically indistinguishable male offspring were therefore expected: XY;aaBBCCc, XY;aaBBCC, X^CY;aaBBCCc and X^CY;aaBBCC (see p.24). All male offspring from 10 litters produced by three separate matings were taken at 5 days for determination of melanin content and tyrosine hydroxylase activity. 25mgm. skin was used for melanin extraction in order to leave sufficient for the enzyme assay. The results given below, however, have been corrected accordingly.

Table 8. Tyrosine hydroxylase activity and O.D. content of offspring from matings segregating for X^C.

Individual no.	Tyrosine hydroxylase activity	O.D. value	Individual no.	Tyrosine hydroxylase activity	O.D. value
1	1.90	0.73	15	3.81	0.86
2	1.82	0.73	16	3.98	0.91
3	1.92	0.74	17	3.84	0.89
4	1.99	0.76	18	4.01	0.88
5	2.04	0.74	19	4.16	0.84
6	2.12	0.76	20	4.12	0.89
7	2.22	0.73	21	4.28	0.94
8	2.40	0.82	22	4.25	0.89
9	2.46	0.77	23	4.39	0.95
10	2.41	0.79	24	5.02	0.95
11	2.70	0.80	25	5.29	0.98
12	3.61	0.84	26	5.59	1.01
13	3.78	0.93	27	5.82	1.01
14	3.90	0.90			

(v). Determination of K_M for tyrosine of tyrosine hydroxylase from skin and liver.

K_M 's were determined for tyrosine hydroxylase from liver and skin of aaBBCC, aaBBc^{ch}c^{ch}, aaBBc^ee^e, aaBBcc, aab^cb^cCC, aab^cb^cc^{ch}c^{ch}, and aabbCC animals. It was found that if label was added in proportion to substrate concentration to maintain constant specific activity the activities at low substrate concentrations were too small to measure accurately. The same quantity of label was therefore added to each different concentration of substrate and correction made afterwards for differences in specific activity. All animals used were 5days ~~or 10days~~ old. Additional determinations were carried out for aaBBCC and aaBBc^{ch}c^{ch} at 10days. Results are shown in table 9.

Table 9. K_M 's for tyrosine of tyrosine hydroxylase from skin and liver of black, cordovan and brown genotypes.

Genotype	Age in Days	K_M (mM tyr.)		V_{max} liver
		skin	liver	
<u>aaBBCC</u>	5	0.461 ±0.009	0.783 ±0.003	2.86 ±0.02
<u>aaBBCC</u>	10	0.480 ±0.013	not measured	not measured
<u>aaBBc^{ch}c^{ch}</u>	5	1.147 ±0.008	0.770 ±0.005	2.88 ±0.04
<u>aaBBc^{ch}c^{ch}</u>	10	1.233 ±0.011	not measured	not measured
<u>aaBBc^ee^e</u>	5	5.100 ±0.002	0.801 ±0.010	2.79 ±0.01
<u>aaBBcc</u>	5	*	0.776 ±0.006	2.82 ±0.03
<u>aab^cb^cCC</u>	5	0.474 ±0.004	0.789 ±0.007	2.88 ±0.01
<u>aab^cb^cc^{ch}c^{ch}</u>	5	1.216 ±0.009	not measured	not measured
<u>aabbCC</u>	5	0.455 ±0.012	0.767 ±0.009	2.91 ±0.05

*activity too low for K_M determination

Fig. 7a. Heat Deactivation of Tyrosine Hydroxylase.

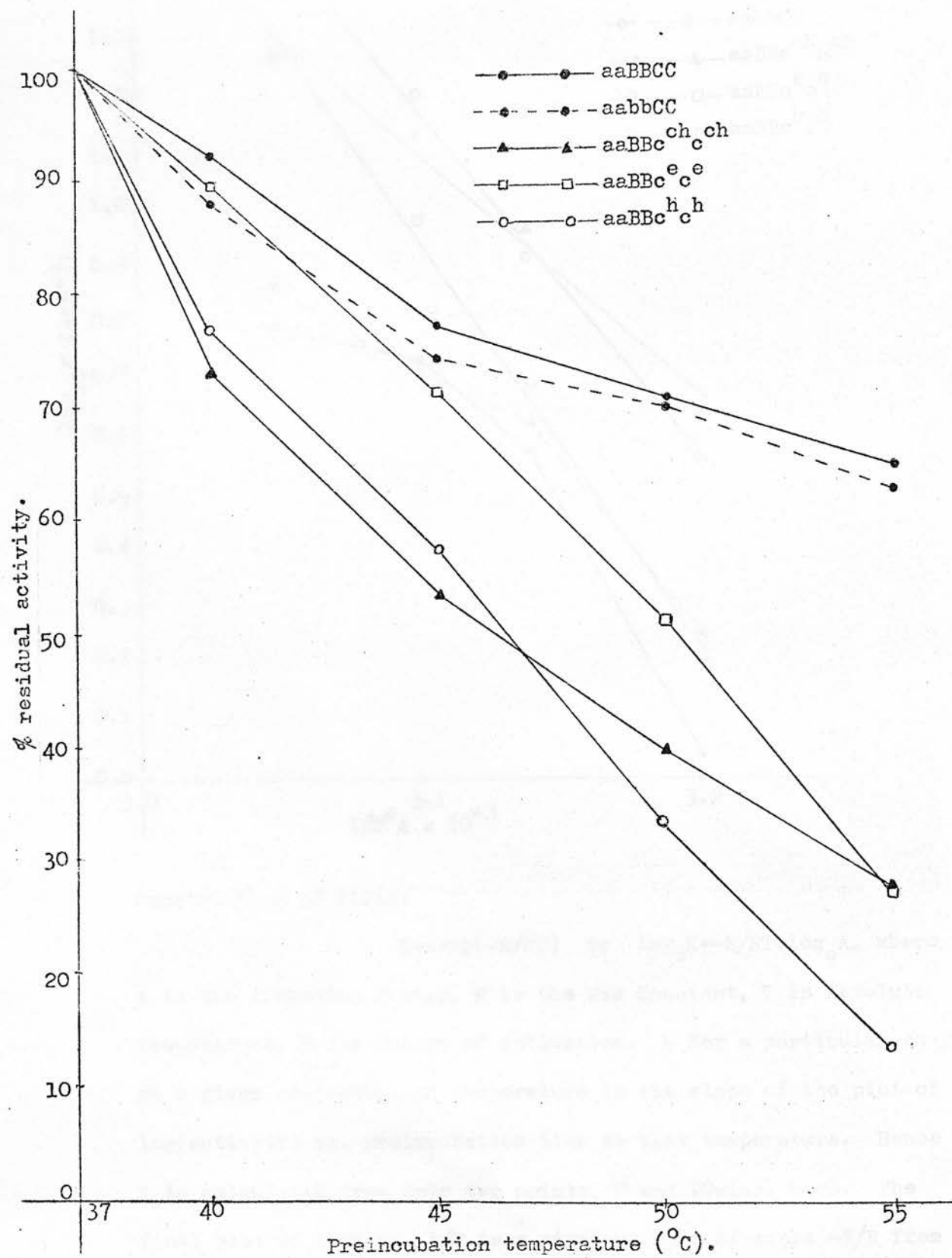
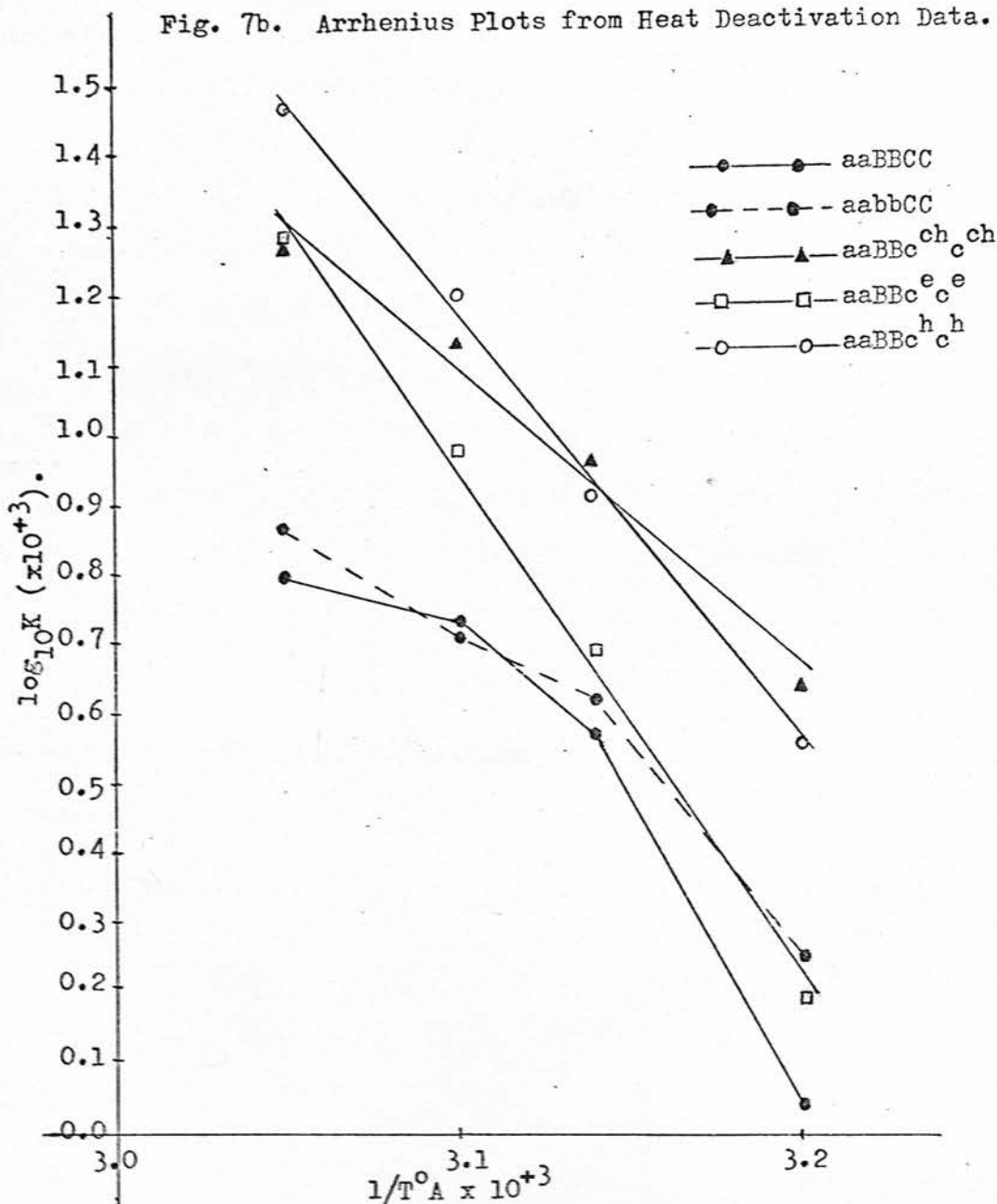


Fig. 7b. Arrhenius Plots from Heat Deactivation Data.



Construction of Plots:

$$K = A \exp(-E/RT) \quad \text{or} \quad \log_e K = -E/RT + \log_e A, \quad \text{where } A \text{ is the Arrhenius Number, } R \text{ is the Gas Constant, } T \text{ is Absolute Temperature, } E \text{ the Energy of Activation. } K \text{ for a particular enzyme at a given preincubation temperature is the slope of the plot of } \log(\text{activity}) \text{ vs. preincubation time at that temperature. Hence } K \text{ is calculated from only two points, 0 and 20mins. here. The final plot of } \log K \text{ vs. } 1/T \text{ is a straight line of slope } -E/R \text{ from which } E \text{ may be calculated.}$$

A is the Arrhenius Number, R is the Gas Constant, T is Absolute Temperature, E the Energy of Activation. K for a particular enzyme at a given preincubation temperature is the slope of the plot of log(activity) vs. preincubation time at that temperature. Hence K is calculated from only two points, 0 and 20mins. here. The final plot of logK vs. 1/T is a straight line of slope -E/R from which E may be calculated.

(vi). Heat deactivation of tyrosine hydroxylase.

Homogenates of aaBBCC, aaBBc^{ch}c^{ch}, aaBBc^hc^h, aaBBc^ec^e and aabbCC skin homogenates were pre-incubated for 30mins. at 37°, 40°, 45°, 50° or 55°C and then assayed normally at 37°C. Tyrosine hydroxylase from himalayan (c^hc^h) animals were included in these experiments as it has previously been reported to be heat labile (Coleman 1962). Himalayan animals are light fawn on the body but ears, nose, tail and feet are much darker, sometimes almost black. It is supposed that pigment is able to be formed in the extremities because the temperature is lower than that of the rest of the body. Himalayan animals had not been included in previous experiments because of the difficulties involved in assaying satisfactorily an enzyme of low activity which is decaying during the incubation period of the assay: results would not be able to be compared with those from other genotypes. Residual activities are expressed as percentages of activity at 37°C in table 10. The results are presented graphically in fig. 7a. All animals used were 5 days old and percentages were calculated from the means of three separate sets of four replicates.

Table 10. Heat deactivation.

Genotype	% activity at 37°C			
	40°C	45°C	50°C	55°C
<u>aaBBCC</u>	92.5	77.3	70.4	64.8
<u>aaBBc^{ch}c^{ch}</u>	73.6	53.2	39.3	27.7
<u>aaBBc^hc^h</u>	77.0	57.1	33.1	13.4
<u>aaBBc^ec^e</u>	89.5	71.3	50.8	26.8
<u>aabbCC</u>	88.3	74.5	69.9	62.7

Arrhenius plots could not be constructed from these results (see fig.7b), for aaBBCC and aabbCC.

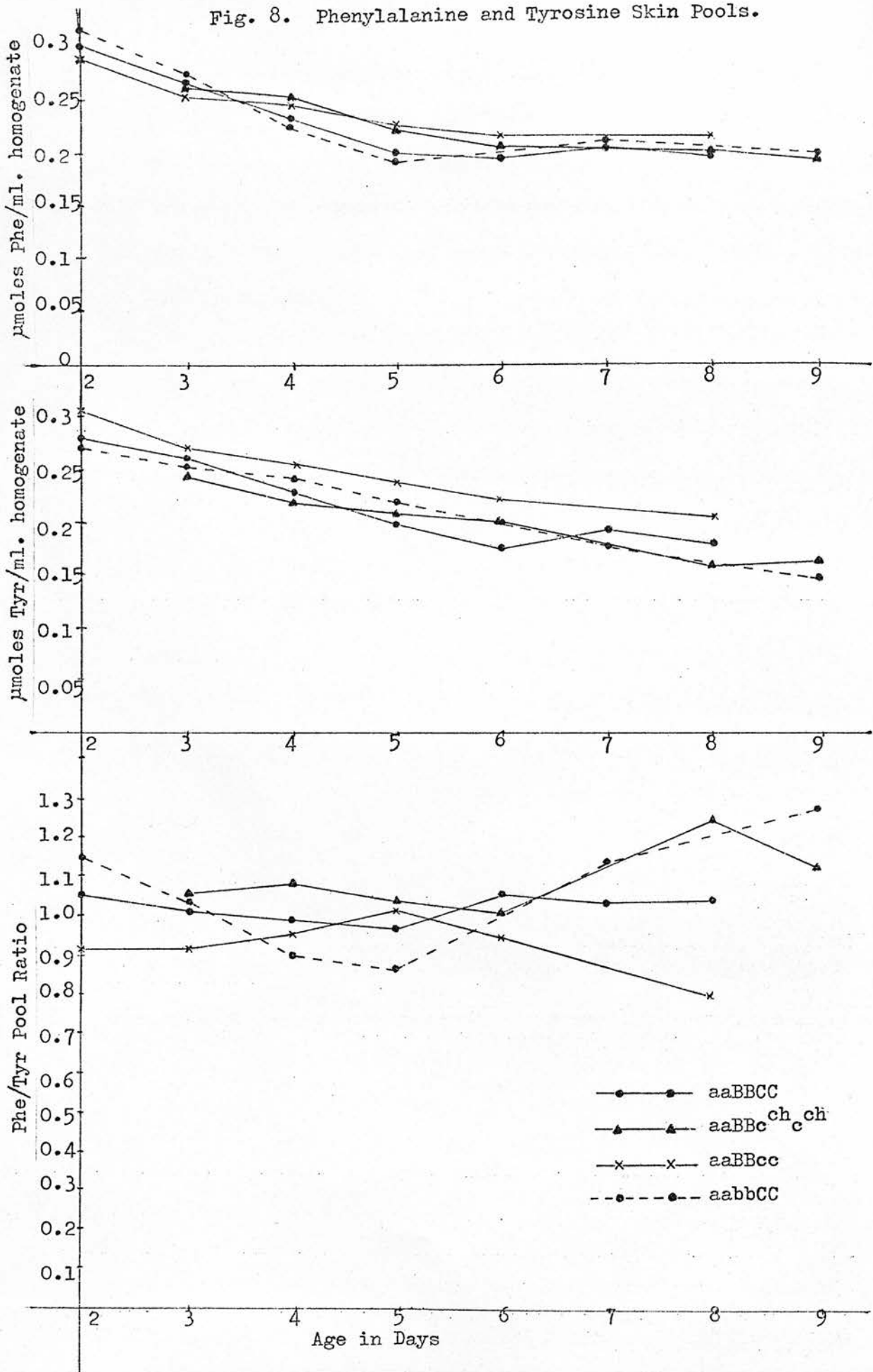
IV. Dopa Oxidase Activity.

Attempts to measure dopa oxidase activity by monitoring the production of dopaquinone or dopachrome spectrophotometrically were not successful. Since it is necessary to have a clear reaction solution for a continuous spectrophotometric assay attempts were made to release tyrosinase activity into the supernatant by incubation of the skin homogenate with different concentrations of Triton-X 100, mild heat treatment and repeated freezing and thawing but in all cases activity levels were too low to measure accurately in the genotypes which showed greatest tyrosine hydroxylase activity and immeasurably small for the lower alleles. Increasing dopa concentrations had little effect and addition of cupric ions as CuSO_4 appeared to facilitate non-enzymic oxidation, but it was found that the O.D. increase and blackening of the reaction solution was due to precipitation of black cupric oxide. Solutions of mushroom tyrosinase adjusted to give levels of tyrosine hydroxylase activity similar to those of aaBBCC animals were successfully assayed for dopa oxidase activity by this method. Supernatants from liver homogenates which showed measurable tyrosine hydroxylase activity had no dopa oxidase activity when assayed spectrophotometrically.

Attempts were made to carry out the assay on whole homogenates, then after terminating the reaction, the mixture was centrifuged and the supernatant examined spectrophotometrically but this was again unsuccessful. It was at first thought that the non-enzymatic conversion of dopachrome to melanin was proceeding so quickly that all dopachrome formed had disappeared during the preparation of the supernatant for spectrophotometric examination. Makower (1971), however, reported that formamide had a stabilising effect on the oxidation products of dopa. Formamide was therefore added at the termination of the reaction

but no improvement was observed. Another method of assay would be the measurement of incorporation of radioactive dopa into melanin but an assay depending on the formation of a product several steps further on in the same pathway would be unsuitable for the purposes of this project. The attempt to assay dopa oxidase activity in skin was then abandoned.

Fig. 8. Phenylalanine and Tyrosine Skin Pools.



V. Intermediate metabolite pools.

Changes with age in free phenylalanine and tyrosine concentrations in skin homogenates and the phenylalanine/tyrosine ratio for aaBBCC, aaBBc^{ch}c^{ch}, aaBBcc and aabbCC are shown in fig. 8. No dopa peak was found, which indicates that free dopa in the extract must have a concentration of the order of nanomoles/ml. Dopa added to skin extracts gave normal identifiable peaks. The failure to detect endogenous dopa therefore could not be due to oxidation during the analyser run. These results suggest that the dopa formed is oxidised immediately in the melanosomes without being released into the surrounding cytoplasm. Other intermediates of the melanin pathway would not be expected to be seen in a system using ninhydrin as colour reagent as the amino group is lost further on in the pathway (see fig. 1).

Table 11a. Free phenylalanine in skin homogenates.

Genotype	μ moles Phe/ml. homogenate							
	2days	3days	4days	5days	6days	7days	8days	9days
<u>aaBBCC</u>	0.304	0.270	0.233	0.202	0.194	0.206	0.199	---
<u>aaBBc^{ch}c^{ch}</u>	---	0.269	0.253	0.224	0.208	---	0.204	0.189
<u>aaBBcc</u>	0.293	0.253	0.248	0.227	0.216	---	0.214	---
<u>aabbCC</u>	0.318	0.275	0.226	0.196	---	0.209	---	0.195

Table 11b. Free tyrosine in skin homogenates.

Genotype	$\mu\text{moles Tyr/ml. homogenate}$							
	2days	3days	4days	5days	6days	7days	8days	9days
<u>aaBBCC</u>	0.286	0.264	0.233	0.204	0.179	0.195	0.186	---
<u>aaBBc^{ch}c^{ch}</u>	---	0.251	0.222	0.213	0.202	---	0.161	0.165
<u>aaBBcc</u>	0.319	0.274	0.258	0.241	0.226	---	0.262	---
<u>aabbCC</u>	0.275	0.263	0.248	0.223	---	0.181	---	0.149

Table 11c. Phenylalanine/Tyrosine pool ratio.

Genotype	Phe/Tyr ratio							
	2days	3days	4days	5days	6days	7days	8days	9days
<u>aaBBCC</u>	1.06	1.02	1.00	0.98	1.08	1.05	1.07	---
<u>aaBBc^{ch}c^{ch}</u>	---	1.07	1.10	1.05	1.03	---	1.27	1.15
<u>aaBBcc</u>	0.92	0.92	0.96	1.02	0.96	---	0.82	---
<u>aabbCC</u>	1.15	1.05	0.91	0.88	---	1.15	---	1.30

VI. Tyrosine Aminotransferase Activity.

Tyrosine aminotransferase was assayed in liver and skin of a selection of genotypes at various ages to see how significant the flux to hydroxyphenylpyruvate was in comparison with that to dopa and whether or not levels of transamination bore any relationship to the levels of tyrosine hydroxylase activity for age and genotype. Results are tabled below.

Table 12. Tyrosine aminotransferase in skin and liver.

Genotype	Age in Days	TAT (μ moles/min/ml. homog.)	
		skin	liver
<u>aaBBCC</u>	2	3.85	12.92
	5	3.34	11.97
	7	3.67	13.50
	8	3.48	11.06
<u>aaBBc^{ch}_c^{ch}</u>	5	3.91	10.76
	7	3.73	12.39
<u>aaBBcc</u>	2	3.59	11.84
	5	3.44	12.96
	8	3.62	10.99
<u>aabbCC</u>	3	3.69	11.62
	5	3.72	11.65
	6	3.55	12.08
	7	3.84	13.01

DISCUSSION.I. The d-locus and Phenylalanine Hydroxylase.

The results presented here do not support the claims (Coleman 1960; Rauch & Yost 1963) that the dilute locus is associated with phenylalanine hydroxylase activity and that dilute-lethal mice provide an animal model for phenylketonuria. The phenylalanine hydroxylase activities in 3-week-old DD, dd and d¹d¹ animals were not significantly different from each other, no differential inhibiting effect of the particulate fraction was detected and, in addition, blood phenylalanine levels measured by Dr. G. Bulfield in this laboratory did not show any elevation. (Dr. G. Bulfield, personal communication). These observations agree with those of Woolf (1963), Zannoni et al (1966) and Mauer & Sideman (1967) who found no increase in blood phenylalanine and no abnormality in liver phenylalanine hydroxylase activity. In their assays neither Coleman nor Rauch & Yost used the pteridine cofactor found by Kaufman (1958) to be associated with the in vivo hydroxylation of phenylalanine. Without cofactor, phenylalanine hydroxylase activity was found to be very low in all genotypes in the present study.

Woolf et al (1970) suggested that observations of high blood phenylalanine and its derivatives and depressed activity observed in dying dilute-lethal mice may be secondary effects of starvation, as similar phenomena have been observed in kwashiorkor, and it is possible that dilute-lethal mice die after weaning because their neuromuscular disturbances are too severe to allow them to eat normally. This condition would explain the observation in this investigation that dilute-lethal animals had livers of high protein content: a starving animal would first absorb liver glycogen and fat before breaking down protein. As all dilute-lethal animals used in the present study were

21 days old and their average lifespan was found to be 29 days when left with the mother it is possible that they were not suffering from advanced starvation, which could account for the failure to observe any disturbances in phenylalanine metabolism.

Russell (1948) calculated the volume of pigment per standard volume of hair in aaBBCCDD and aaBBCCdd genotypes by measurements of pigment granules and found that the dilute animals had 78.5% of the volume of wild-type. The pigment granules of dilute animals were also abnormally large owing to irregular clumping. The melanin content estimated by the spectrophotometric method used here showed dilute animals to have 97.5% and dilute-lethal 94.0% of wild-type. It is possible that the inaccuracies necessarily involved in measuring large irregular clumps of pigment caused Russell's value to be an underestimate. Even were this not so, however, it is more probable that the apparent pigment dilution observed in dilute and dilute-lethal is due to the abnormal distribution of pigment granules rather than to reduction of pigment. The blue-grey appearance of these mice might be compared to the silver-grey of agouti chinchilla (A-BBc^{ch}c^{ch}), where the lack of phaeomelanin in the subterminal agouti band in the hair causes dark brown hairs to appear grey. The hypopigmentation associated with human phenylketonuria is reported to be due not to lack of tyrosine but to inhibition of tyrosine hydroxylase activity by high phenylalanine levels (Miyamoto & Fitzpatrick 1957). Dilute and dilute-lethal animals were found to have normal levels of tyrosine hydroxylase activity at 5 days when they already appear grey (p. 44).

Recent detailed investigations of the kinetic properties of phenylalanine hydroxylase from dilute-lethal mice have shown that a normal quantity of the enzyme is present but that it has an altered

affinity for competitive and non-competitive inhibitors which is not expressed under normal conditions of diet (Zannoni & Morani 1970).

This has been contradicted by the recent results of Treiman and Tourian (1973) who reported that the specific activity of $\underline{d^1d^1}$ phenylalanine hydroxylase was two-fold higher than normal, that the K_M values for phenylalanine and tetrahydropterine are identical for mutant and normal enzymes and that three distinct peaks for each of the homozygotes $\underline{DD}, \underline{dd}$ and $\underline{d^1d^1}$ are seen on electrophoresis, the ratios varying for each genotype. The most abundant peak in $\underline{d^1d^1}$ is formed by an unstable protein.

The situation with respect to the connection between the d-locus and phenylalanine hydroxylase is hence still unclear, although it now appears certain that the dilute-lethal mouse is not phenylketonuric, nor is the syndrome akin to any of the variant forms of phenylketonuria described in man. Since the experiments described in this study did not yield results which would have made further investigation relevant to the aim of the project, the possibility of the hydroxylation of phenylalanine as a step in the melanin pathway at which variation might be introduced was not considered further.

II. The Effects of Allelic Substitution at the b- and c-loci on melanin production

The data from the spectrophotometric assay broadly support the

visual impressions formed from observation of the adults in that O.D. values from light-coloured animals are smaller than those from darker ones. The fact that aabbCC animals have smaller O.D. values than aab^cb^cCC and aaBBCC also suggests at a first glance that the colour differences between these animals are due to the formation of different amounts of pigment rather than to a different distribution of similar amounts, provided that the eumelanin in each of these genotypes is of the same structure and the same compound is being measured in each case (see pp. 31-32). A further instance of agreement of O.D. values with observation is the delayed appearance of values significantly higher than those of albino in aaBBc^ec^e and aabbc^ec^e. Russell (1946) in a histological study of single hairs of these light-coloured genotypes described a 'pigmentation lag' -i.e. no pigment granules were observed in the tips of the hairs. When examined in detail, however, the O.D. values show marked departures from this general agreement.

Where the wild-type C allele is present a trend in O.D. values is observed which is independent of the genetic constitution at the b-locus and not apparent in the visual assessment of the adult phenotype. At most ages, the genotypes CC, Cc^{ch}, and Cc show a progressive decrease in O.D. value. At 5days, for example, aaBBCC, aaBBCC^{ch} and aaBBCCc animals have O.D. values of 0.81, 0.75 and 0.72 respectively. This difference is still present at 10days between aaBBCC and aaBBCCc, the O.D. values being 3.45 and 3.20. Although differences of this magnitude are not statistically significant at any one day, their consistency over a number of days suggests that this trend is a real

one. For cordovan animals the $\underline{c}^{ch}c^{ch}$ homozygote is also included and for brown the $\underline{c}^{ch}c^{ch}$ homozygote and the $\underline{c}^{ch}c$ heterozygote. As the adults of each genotype appear equally pigmented, these differences must either have disappeared by the adult stage or be indistinguishable to the eye. O.D. values were therefore obtained for animals at 3 weeks, when growth of the first coat has been completed. \underline{aaBBc}^{ch} and \underline{aaBBc} were found to have 96.8% and 90.1% of \underline{aaBBCC} respectively, compared with 92.6% and 88.9% at 5 days, while brown (\underline{aabb}) animals were all indistinguishable by O.D. value at 3 weeks except for $\underline{aabb}c^{ch}$ which had 85.4% of the \underline{aabbCC} value at 5 days and 91.7% at 3 weeks.

From these results it appears that differences of up to about 10% in O.D. value for the more intensely pigmented genotypes with respect to the c-locus are not visually distinguishable for a given genotype at the b-locus, although between the different b-locus mutants there are significant differences in maximum pigmentation intensities. In the case of black animals it could be suggested that a visual threshold has been reached with \underline{C} so that any further increases in pigment content cannot be detected by eye, but the same explanation cannot also apply to the less intensely pigmented brown animals whose O.D. values lie between those of $\underline{aaBb}c^{ch}c^{ch}$ and $\underline{aaBb}c^{ch}c$, which are easily visually distinguished. The only two genotypes which differ by less than 10% and are visually distinguishable are $\underline{aabb}c^e$ and $\underline{aaBb}c^e$, the latter having an O.D. value of 95% of the former and being unmistakably lighter. (fig. 2). As alleles at the b-locus are involved here, however, the direct comparison of the two genotypes may not be meaningful. The fact that a visual saturation appears to be reached when the \underline{C} allele, and also in the case of brown the \underline{c}^{ch} allele, is present, but for different levels of pigment content according to the b-locus allele

present, could indicate that in genotypes of around maximum pigment intensity some of the pigment is hidden - perhaps in inner layers within the melanosome - and that this can occur at different absolute pigmentation levels. Russell (1948) reported that pigment granules of brown mice were round rather than oval, as in black mice, and brown rather than black, with half the diameter of normal black granules. The b allele had no effect on pigment granule number. Moyer (1961) described the effects of various coat colour mutations on the ultrastructure and distribution of pigment granules in the eye. Although he confirmed Russell's observations as to shape, relative size and colour, no ultrastructural differences were seen. In a more recent study, Hearing et al (1973) reported that melanin was deposited uniformly on the filaments of both black and brown melanosomes but that the melanin in brown melanosomes had a flocculent appearance in some areas. Ultrastructurally, therefore, the b-locus appears to affect melanosome shape and, if not the chemical structure of the melanin, its attachment or deposition on the melanosome filaments. A different arrangement of melanin within a smaller melanosome in brown animals might therefore contribute to the apparent visual saturation at O.D. levels similar to those found in visually distinguishable black genotypes.

With extremely light-coloured animals, $\underline{c}^e c$ and $\underline{c}^D c^D$ (platinum), which are distinguishable from albino only by direct comparison, spectrophotometric measurements failed to indicate the presence of any pigment so that at the lower end of the scale the eye appears to be more sensitive than the quantitative method of assessment employed. At the upper end of the scale, however, it is evident that the extraction and photometric measurement of 'melanin solutions' affords a finer

assessment of pigment content than the visual grading methods used previously (Dunn, 1936) which described the C allele as simply 'dominant'. Whether the eye shows a non-linear discrimination for this colour series or whether distribution of pigment within the melanosome accounts for the discrepancies between observation and measurement cannot be decided from these data. Table 13 below compares visual assessments of colour intensity of whole dried pelts from adult mice (in most cases around 30 weeks of age) with spectrophotometric measurements of the same pelts and the 5day values from tables 1a and c (p.33). The visual assessments were made by 19 people, who were asked to assign a grade between 0 and 10 to each pelt. The values shown below are means of their assessments converted to percentages of wild-type (aaBBCC).

Table 13. Comparison of visual and spectrophotometric assessments.

Genotype	5day O.D.		adult O.D.		visual assessment	
	black(<u>BB</u>)	brown(<u>bb</u>)	black	brown	black	brown
<u>CC</u>	100.0%	42.9%	100.0%	45.6%	100.0%	73.3%
<u>Cc</u>	87.2%	45.7%	--	41.3%	--	72.2%
<u>c^{ch}c^{ch}</u>	60.0%	40.0%	68.2%	40.9%	87.7%	75.5%
<u>c^{ch}c^e</u>	--	--	45.4%	38.7%	54.4%	73.3%
<u>c^{ch}c</u>	21.4%	34.3%	30.2%	36.1%	44.4%	61.1%
<u>c^ec^e</u>	1.4%	2.8%	8.9%	11.6%	23.3%	30.0%
<u>c^ec</u>	--	--	0.1%	0.0%	2.2%	3.3%
<u>cc</u>	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

O.D. values have been adjusted for albino values by subtraction of the relevant albino O.D. to bring them into line with the visual

grading where albino was fixed at 0. Evidence that the small O.D. value of albino is not due to the presence of small quantities of melanin comes from the work of Hearing et al (1973) who examined the fine structure of the albino eye and failed to find any of the pigmented melanosome stages, but distinguished unpigmented structures similar to the premelanosomes found in the pigmented eyes in the early stages of pigment formation. This suggests that a more accurate measure of the pigment content of other genotypes would be obtained by subtracting the albino value from each O.D. measurement.

The increase in O.D. value of skin and hair from birth to 10 or 11 days for black, cordovan and brown genotypes is shown in figs. 4a,b and c (facing p.33). O.D. values for all genotypes are approximately doubled between 7 and 10 days during a rise in the rate of increase of O.D. which is very marked for the more heavily pigmented genotypes. It should be pointed out here that the observed O.D. values are not directly comparable for different ages since they are expressed per unit weight of skin, which makes no allowance for follicle density, and therefore number of hairs present in a given weight of skin as the skin grows. Any detailed quantitative investigation of absolute increases must take this into account, ideally by measuring amount of pigment produced by a single follicle or a known constant number of follicles. An attempt to approximate to the latter was made by calculating the increase in volume of an initial (0 day) unit weight of skin, up to 11 days of age, and is described in detail on pp. 81-84. Dry (1926) reported that follicle initiation was completed by about 7 days of age, so that subsequently follicle density would be expected to

decrease as the skin continues to grow. Correction factors were calculated for 6 days of age onwards, as it is at this age that CC tyrosine hydroxylase activity apparently begins to decline, and are shown in table 14a (p.82). The effect of using this rough correction on aaBBCC and aaBBc^{ch}c^{ch} O.D. values is shown in table 14c (p.83) and presented graphically in fig. 10b (facing page 84). The 7 to 10 day increase in O.D. is magnified by correction, but its onset appears to be more gradual, starting at 5 days rather than 7.

Russell (1946) examined pigment granule number, size and shape in the hair of various mouse coat colour mutants and found that for mutants of the b- and c- loci the mean size and number of granules per unit volume of hair did not change significantly along the length of the hair. This implies that unless the pigment associated with the melanosome can be increased without increasing the size of the melanosome, O.D. increase must be associated with hair growth in order to maintain a constant number of melanosomes per unit volume of hair. Russell's data on pigment granule size do not include heterozygotes of the C allele with the lower alleles. Her estimates of pigment granule volume for aaBBCC, aaBBc^{ch}c^{ch}, aabbCC and aabbc^{ch}c^{ch} give values of 52% of aaBBCC for aaBBc^{ch}c^{ch} and 90% of aabbCC for aabbc^{ch}c^{ch}, for a region of hair produced during the sixth day after birth. The percentages calculated from the O.D. values in tables 1a and 1c (pp.33,35) obtained in the present study are 58% and 86% for aaBBc^{ch}c^{ch} and aabbc^{ch}c^{ch} respectively at 6 days. (The 6 day albino O.D. was subtracted before percentages were calculated). For a given b-locus genotype, therefore, it appears that pigment granule size in chinchilla animals is an approximate indication of O.D. value. This suggests

that the major part of the increase in O.D. after 7 days must be associated with an increase in melanosome production and thus with hair growth.

There are two ways in which an increase in total hair growth may be brought about - either the growth rate of the hairs must increase, or the total number of hairs growing simultaneously must increase. Data on rates of hair growth in the mouse are scarce. Fraser (1951) described hair growth rate as constant, while Priestley & Rudall (1964) measured growth rates of rat hair from 0 to 20 days and found growth to be exponential until 12 days and higher than the rate for mouse recorded by Fraser. It seems more probable, as the shape of the graph in fig. 10b does not fit an exponential increase, that growth of new hair is contributing to the rapid rise in O.D., than that it is due simply to growth of existing hair. Dry (1926) reported that underfur growth was initiated after guard hair growth, underfur follicle initiation continuing until 7 days after birth while guard hair follicle development was completed by 3 days after birth. (Underfur hairs constitute 84% of the coat by number and guard hairs 16%, but are smaller than guard hairs and therefore contain less pigment). It is therefore suggested that the rapid rise in O.D. observed between 7 and 10 days of age reflects the melanin synthesis associated with underfur growth. The data available on hair growth, however, are not detailed enough for possible relationships between melanin synthesis and appearance of the different hair types to be investigated.

III. Evidence that the c-locus is the structural locus for skin tyrosine hydroxylase.

In diploid organisms the first indication that a mutation affecting enzyme activity is at the structural locus for that enzyme is intermediacy of activity at the enzyme level in the heterozygote. In more general terms, where two homozygotes have distinguishable forms of enzyme, the heterozygote will show both in equal proportion unless the altered structure of the enzyme itself affects its controlability. There is now a large body of evidence to support the universality of this type of codominance in higher organisms (Childs & Young 1963; Harris 1964). The well-known and major exception to the rule is the nucleolar mutation in Xenopus, a deletion of the ribosomal RNA genes, where heterozygous embryos synthesise as much rRNA as homozygous normal embryos (Gurdon 1967). In addition to the demonstration of heterozygote intermediacy it must also be shown that the enzyme protein produced by different alleles differs in primary structure. Paigen (1971) lists five criteria for this: physical denaturation, electrophoretic mobility, kinetic constants, immunological variation and peptide mapping, of which two were attempted in the present study with tyrosine hydroxylase.

Alleles at both the b- and c-loci affect the tyrosine hydroxylase activity of skin tyrosinase. aabbCC animals have twice as much in vitro activity as aaBBCC and aab^cb^cCC about one and a half times as much. Fitzpatrick & Kukita (1959) and Coleman (1962) also found from studies of in vitro incorporation of ¹⁴C-tyrosine into insoluble skin compounds that the activity of aabbCC skin slices was twice that of aaBBCC. The data in tables 6a,b and c (pp.41-43) for tyrosine hydroxylase activity show that these proportional increases in activity are maintained irrespective of genotype at the c-locus

except in the case of albino. Phenotypically B is dominant to b^c which is dominant to b and in the heterozygotes Bb^c, Bb and b^cb dominance at the enzyme level was also observed.* To test whether or not this effect could be due to the production of an inhibitor by the B and b^c alleles, assays were performed on mixtures of homogenates of the skins of homozygotes but no inhibition was detected. In contrast, the tyrosine hydroxylase activity of genotypes heterozygous for c-locus alleles is in every case near the average of the two parents. Figs. 5a-f (facing p.41) compare the experimental heterozygote values with the parental averages. Heterozygote intermediacy was also observed by Coleman (1962) for all the heterozygotes at the c-locus that he examined (aaBBCC, aaBBCC^{ch}, aaBBc^{ch}c^{ch}_{pp} and aaBBc^{ch}c^{ch}_{cpp}).

Two other types of assay have been used for mouse skin tyrosinase. The first study on mouse skin pigment forming abilities was made by Russell & Russell (1948) who used dopa as a substrate for adult skin sections which had been frozen during preparation. After incubation in an approximately 2.5mM dopa solution and phosphate buffer the sections were examined for blackening of the area around the hair follicles and graded visually. A black pigment was always formed regardless of whether the section was from yellow, brown or black skin. The genes A^y and b were found to have no effect on dopa oxidase activity and heterozygote intermediacy was not observed (as theory could predict if the reaction product being measured was the end-product of the pathway, melanin itself). The formation of black pigment by yellow skin can be explained by suggesting that the cysteine-containing compounds now believed to be necessary for phaeomelanin had been diluted to such an extent by the volume of

*Note: The data on tyrosine hydroxylase activities in heterozygotes at the b-locus were unfortunately lost during the writing of this thesis so that this and the following statement are qualitative recollections.

the incubation mixture that they were no longer effective in diverting the eumelanin pathway. Coleman (1962) showed that A^y_a skin slices had about one-third of the ability of aa skin to incorporate tyrosine into melanin, and tyrosine hydroxylase assays on recessive yellow (ee) skin during the present study also showed activity levels of about one-third normal. This is irreconcilable with Russell & Russell's report that the presence of A^y had no effect on the extent of darkening of the hair follicles, as their method was sensitive enough to pick up differences in activity as great as 70%. Their observation that the b allele also had no effect on dopa oxidase activity could be due to the limitations of their method: if BB hair follicles turn densely black during incubation a visual threshold is reached such that it would be impossible to distinguish any further degrees of blackness. A dopa oxidase activity greater than normal would not therefore be detected. The reports that b increases the level of tyrosinase activity, however, come from assays which used tyrosine as substrate and are therefore in some way assaying tyrosine hydroxylase activity, not dopa oxidase activity. It is not clear why the melanin formed should be black rather than brown.

Foster (1951) used oxygen consumption with tyrosine as substrate to assay the pigment-forming ability of pooled skin homogenates of 5-9 day old mice. The skin was frozen to facilitate homogenisation. As oxygen is required for at least four steps in the melanin pathway (fig.1 facing p.7), differences in oxygen consumption cannot be associated with a block in any specific step. It was demonstrated that the formation of pigment from tyrosine and simultaneous oxygen consumption was enzyme-dependent by showing that these two

phenomena did not occur in the presence of boiled skin, D-tyrosine and phenylthiourea, which removes copper. Oxygen absorption was greatest in black skin and in brown and yellow skin an inhibitor was detected, by means of interaction experiments with black skin, which reduced oxygen uptake. Cupric ions enhanced all reactions. In albino skin a small amount of oxygen uptake was seen which was increased by addition of cupric ions but no pigment was formed. Albino homogenates were not active towards dopa, as were all other homogenates.

Although the addition of cupric ions was found to have no effect on tyrosine hydroxylase activity in the present investigation a black pigment was formed in the tubes of all genotypes tested and also in control tubes, which contained TCA treated skin. This darkening could be due to two processes: (i) the enhancement of dopa oxidase activity and (ii) the non-enzymatic production of black copper oxide. The dark colour seen in the control tubes could be formed by the second process, further evidence for which was obtained when the development of a similar colouration was observed on omitting tyrosine from the reaction mixture. This explanation does not apply to Foster's observations, however, as his controls containing cupric ions did not develop any colouration. His observation that albino homogenates were active towards tyrosine but not dopa could indicate the presence in skin of liver-type tyrosine hydroxylase which is normally masked by skin tyrosinase in pigmented genotypes. Under the conditions of assay of tyrosine hydroxylase activity used in this study, however, cupric ions had no effect on liver tyrosine hydroxylase, although again a black precipitate was observed. Foster did not distinguish between

homozygotes and heterozygotes at the b- and c-loci.

The results of neither of these assays are strictly comparable with those of the more specific tyrosine hydroxylase assay as in each several steps intervene between enzyme and product measured so that the activities observed are the net result of several interactions. Taken with the tyrosine hydroxylase activities found for the b-locus alleles, however, Foster's results suggest that the b allele has its effects on melanin reduction later in the pathway, although his claim that the b allele produces a diffusible inhibitor of oxygen uptake by black skin is difficult to reconcile with b's being recessive to B. Coleman (1962) and Fitzpatrick & Kukita (1959) also used a 'pathway' assay by measuring the incorporation of labelled tyrosine into insoluble skin constituents and found that bb skin showed a higher incorporation than BB, a result which agrees with the tyrosine hydroxylase activities but, if the insoluble skin compounds are largely melanin, contradicts the O.D. values obtained for these genotypes. Coleman also repeated his experiments in vivo with similar results.

From the observation that all the c-locus alleles examined exhibit codominance for tyrosine hydroxylase activity, while the b-locus alleles, although affecting tyrosine hydroxylase activity, show no heterozygote intermediacy, it was decided to investigate further the possibility that the c-locus is the structural locus for tyrosine hydroxylase. Since the quantitative aspects of the project require the determination of kinetic constants, K_M determinations were carried out for tyrosine hydroxylase from the skin and liver of BB, b^cb^c and bb and CC, c^{ch}c^{ch} and c^ec^e genotypes. A survey by Langridge (1968) of several hundred E.coli mutants

unable to utilise lactose showed that while most had a small but measurable percentage of residual activity only 4-6% showed alterations in substrate affinity, suggesting that mutations are more likely to affect V_{\max} than K_M . From these data failure to detect a change in K_M would not necessarily imply that the primary structure of the enzyme molecule was unaffected. The data presented in tables 6&9 (pp. 41-3,46) show that the effect of different c-locus alleles is to change both the V_{\max} and K_M of skin tyrosine hydroxylase whereas the b-locus alleles affect V_{\max} only. Liver tyrosine hydroxylase has a different V_{\max} and K_M which is unaffected by alleles at the b- and c-loci, the activity even in albinos being normal. As dopa is required for catecholamine synthesis and albinos show no general neurological abnormalities which might be associated with reduced catecholamine levels it follows that dopa must be produced in adequate amounts in the albino so that an alternative tyrosine hydroxylating system might be expected. The K_M values therefore support the suggestion that the c-locus is the structural locus for tyrosine hydroxylase, but also imply that liver tyrosine hydroxylase is an independently produced and controlled enzyme.

Since the tyrosine hydroxylase activity in albino skin remains at a constant level from birth to 11 days and is not affected by b-locus alleles it is suggested that the activity present may be due to liver-type enzyme which is normally masked by the presence of much greater amounts of the skin-type enzyme in pigmented genotypes. This is supported by the data on tyrosine hydroxylase activity in supernatants from fresh skin homogenates (table 5a, p.38), where the level of activity found is close to that of albino. Most of the tyrosine hydroxylase activity of the liver was located

in the supernatant. Assays on supernatants from albino skin homogenates showed about 78% of the total activity to be in the supernatant although a considerable variation was found. Unfortunately the level of activity was too low for K_M determinations to be made. If, however, albino skin tyrosine hydroxylase activity is due to the presence of liver enzyme its failure to form melanin suggests that liver tyrosine hydroxylase has no associated dopa oxidase activity.

Relative thermolability is probably the most sensitive criterion for detection of changes in primary protein structure. Langridge (1968) constructed β -galactosidase molecules differing in known amino acid residues and found 70% to have altered heat stabilities. From a survey of known haemoglobin mutants Lehmann & Carrell (1969) estimated that substitution of a polar for a non-polar amino acid would have a drastic effect on protein conformation (and hence thermolability) for about 50% of residues. In addition to this type of change, which affects hydrophobic bonding, tertiary structure would also be affected by changes in ionic bonding, hydrogen bonding and disulphide linkages which all increase the probability of any single substitution having a detectable effect. In contrast, differences in electrophoretic mobility, which depend on amino acid substitutions altering molecular charge have been estimated to occur for 25% of substitutions in haemoglobin. (Lehmann & Carrell 1969).

In the case of tyrosine hydroxylase, however, although alleles at the c-locus were found to alter heat stability, the results (table 10 p.47) showed wide variation and it was not possible to construct Arrhenius plots from the data obtained (fig.7b) for CC enzyme. Many more determinations would have been required in order to determine whether or not this was due to experimental error and sufficient

numbers of animals were not available. Fig. 7a, however, does indicate that the tyrosine hydroxylase activity of aaBBCC and aabbCC animals is affected less by heat than that of the other three c-locus alleles, c^{ch}, c^h and c^e investigated.

Menon & Haberman (1970), using essentially the same tyrosine hydroxylase assay as used in the present study, reported that the activity (of BBCC enzyme) in mouse melanoma was increased 2.5 to 3 times by preheating melanosomal and microsomal fractions to 60°C or 70°C for 15mins. At 80°C and above no activity was detected. These effects were more marked in the melanosomal fraction. It was found in the present study that aaBBCC tyrosine hydroxylase activity in skin homogenates was almost completely destroyed by heating at 70°C for 15mins. but in three isolated cases, in aaBBc^ec^e and aabbc^{ch}c^{ch}, activity was increased by up to 50% by preincubation at 45°C. This phenomenon was not consistent or repeatable. These conflicting results from skin and melanoma are probably not directly comparable since the crude skin homogenates may contain factors affecting tyrosine hydroxylase activity which are not present in a concentrated preparation of melanosomes.

If the himalayan colouration of light cream body with darker extremities (ears, nose, feet, tail) is due, as is popularly believed, to the presence of a heat-labile tyrosinase which is fully active only in those parts of the body where the temperature falls below 37°C, the response of the tyrosine hydroxylase of aaBBc^hc^h animals to heat might be expected to be greater than that of aaBBc^{ch}c^{ch} and aaBBc^ec^e. Coleman (1962) reported that the incorporation of ¹⁴C-tyrosine into aaBBc^hc^h skin slices was decreased by 70% at 55°C in the first 20-30mins. of heating, whereas

incorporation of normal skin decreased only 10%. After preincubation at 55°C for 30mins. tyrosine hydroxylase activity in the present study was found to have decreased by 86.6% and 35.2% for aaBBc^hc^h and aaBBCC respectively. The activities of aaBBc^{ch}c^{ch} and aaBBc^ec^e were also decreased by 72.3% and 73.2% by the same treatment, however. The effect of temperature on pigment production in vivo was investigated by keeping the animals at 22°C and 32°C from 3 weeks of age to 7 weeks and observing the differences in pigmentation intensity of the second coat, which is grown during this time. In addition, small areas just behind the head were plucked free of hair and the new growth thus initiated observed. In himalayan animals the differences were distinct: animals kept at 22°C were fawn in body colour with black extremities and those kept at 32°C had a light cream body colour with brown extremities. When the latter group of mice were returned to 22°C they gradually darkened. There therefore seems to be no doubt that temperature affects the in vivo production of pigment in himalayan mice. It was also observed during this experiment that aaBBc^ec^e animals showed a tendency to darken at the higher temperature: at normal temperatures, these mice were distinguishable from other genotypes of similar pigment intensity by their white noses, and pink, unpigmented ears, tails and feet.

The failure to detect an increased sensitivity to heat of tyrosine hydroxylase of himalayan animals could be due to two reasons. First, the mutation might affect the dopa oxidase activity of himalayan tyrosinase rather than the tyrosine hydroxylase with respect to heat stability. Secondly, to produce the difference in pigment intensity seen between the body and the extremities,

the tyrosine hydroxylase of himalayan mice must show great sensitivity to the small temperature range about 37°C which is likely to be encountered in vivo. It is therefore possible that at 37°C the enzyme is already largely inactivated and the finding that it is apparently no more sensitive to further increases in the temperature of preincubation than tyrosine hydroxylase from chinchilla and extreme dilution may be a reflection of this. An enzyme inactivated at 37°C cannot, of course, be meaningfully assayed at this temperature as it will be decaying during the assay period. Changing the assay temperature, however, affects reaction velocity. To compare the relative activities of the alleles at a lower assay temperature it would have been necessary to repeat all the above determinations. This was not possible owing to shortage of time and animals.

In conclusion, the results obtained indicate that the tyrosine hydroxylase activity associated with the recessive alleles c^{ch}, c^h and c^e is less stable towards heat than enzyme from normal skin homogenates, but the decay curves produced are too irregular for any detailed comparison of heat stabilities to be made. The tyrosine hydroxylase of himalayan mice is not markedly more heat-labile than that of the other mutants. The b-locus had no effect on the behaviour of CC tyrosine hydroxylase towards heat.

The results discussed so far support the suggestion that the c-locus is the structural locus for tyrosine hydroxylase: c-locus alleles cause changes in V_{\max} , K_M and heat stability and codominance is observed in all heterozygotes. Figs. 5a-f (p.41), however, show that the change with age of activity is different for each homozygote. CC tyrosine hydroxylase activity, for example, reaches a maximum around 5 days after birth and then gradually declines, while c^{ch}c^{ch} activity increases steadily up to 10 days, by this time being higher than CC activity. This phenomenon by itself would be most easily explained by suggesting that the c-locus is responsible for the production of some molecule with a regulatory function which has an effect on a tyrosine hydroxylase molecule determined by another locus. The steep increase in activity from 3 to 5 days observed for wild-type tyrosine hydroxylase might be due to an induction or activation process which is impaired in chinchilla and absent in albino. Alternatively, the lack of activity in albino might be due to a very efficient repression or inhibition which is not present in wild-type. The changes in K_M and heat stability could be explained by suggesting that the regulation occurs at the enzyme level by altering the enzyme molecule, perhaps by the regulatory molecule becoming an integral part of the enzyme molecule, and is therefore more accurately described as an activation or inhibition process. On this theory, however, there is no reason to expect the very close agreement between observed tyrosine hydroxylase activity in heterozygotes and that calculated on the expectation of codominance. The above theory still applies if it is suggested that it is the differences in the structure of the tyrosinase molecule itself, caused by mutations at the c-locus,

which affect its activation or inhibition by altering its ability to interact with regulatory molecules. In this way, the two species of enzyme molecule present in the heterozygote would be regulated independently and codominance would still be expected.

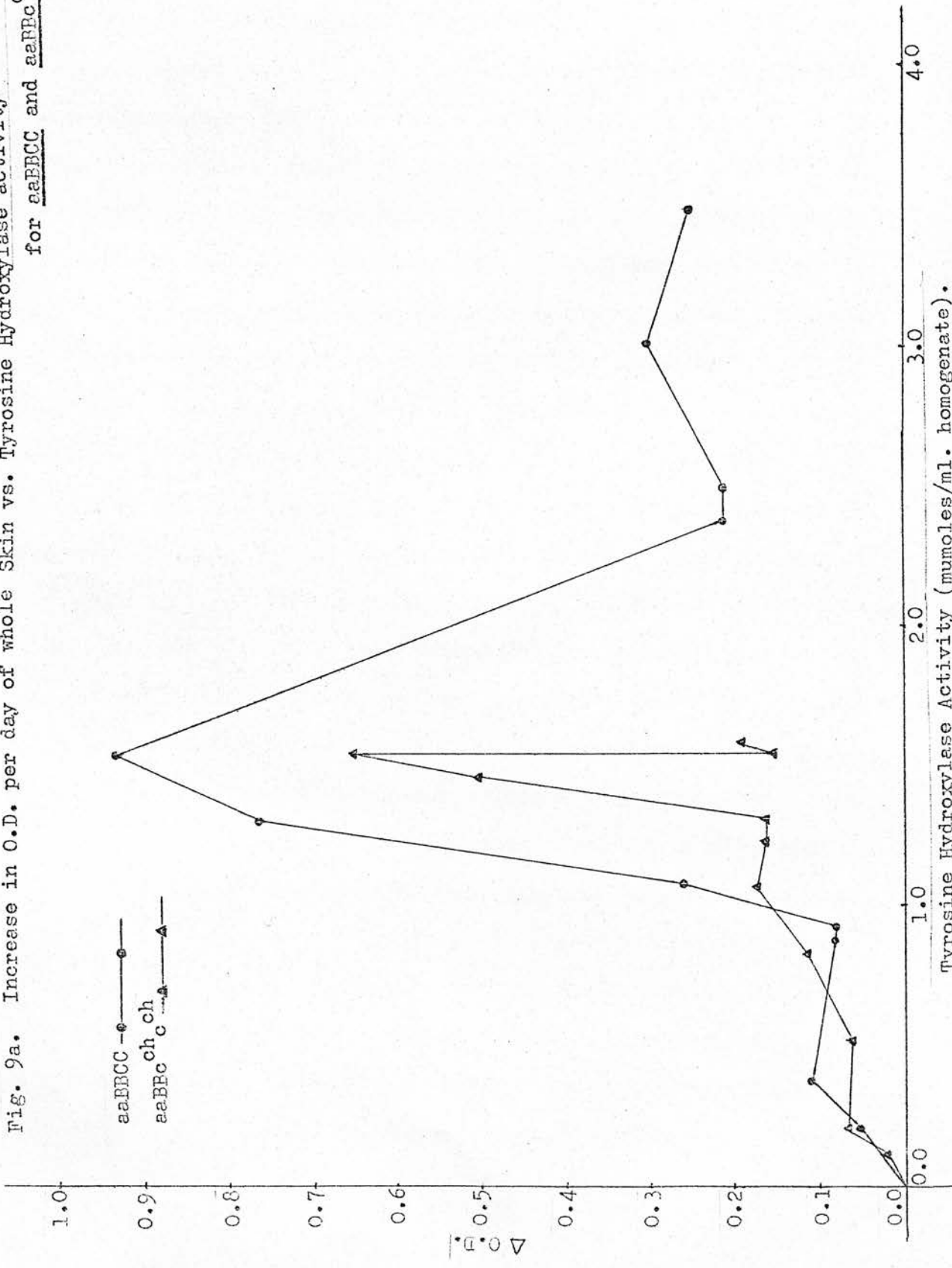
There are several reports from studies on invertebrates of regulation of tyrosinase at the enzyme level. Whittaker (1973) showed by histochemical tests and inhibition of protein synthesis that tyrosinase is stored after its synthesis for several hours before the initiation of melanogenesis in the presumptive pigment cells of ascidian embryos. In this case the stored molecules appear to be active and are released in active form by cell disruption. Mechanical cell-disruptive processes and proteolysis have also been reported to release tyrosinase in grasshopper eggs (Bodine et al 1937), silkworms (Ashida 1971) and Drosophila (Horowitz & Fling 1955). Lee & Lee (1971) studied the effect of long-term administration of the polypeptide melanocyte-stimulating hormone (MSH) on melanogenesis in adult Rana pipiens. Tyrosinase here was found to be evenly distributed over pigmented and non-pigmented skin in an inactive form which was activated in vitro by trypsin. MSH had the effect of inducing melanogenesis and the suggestion was made that this might be achieved through the release of a trypsin-like proteolytic intracellular tyrosinase activator. A proteolytic activation in Rana pipiens was also observed by McGuire (1970). Similar studies on tyrosinase development in mouse coat-colour mutants might yield interesting information on post-translational enzyme control.

There is at present one recent report (Hearing 1973) which concludes that the c-locus is a regulatory locus for tyrosinase.

Homogenates of eyes from black and albino mice were fractionated and tyrosine hydroxylase activity determined in the melanosome fraction. Treatment of albino homogenates by dialysis against water, mildly heating, or addition of triton-X 100 increased tyrosine hydroxylase activity five-fold. Addition of albino homogenate to black resulted in an inhibition of activity. When these experiments were repeated on skin homogenates in the present study no indication of the presence of any inhibitor in albino skin could be found. It can be seen from fig.2 (facing p.30) that eye colour does not appear to be closely related to skin colour: animals as light as $\underline{c^e c}$ have black eyes. $\underline{c^h h}$ animals have purple eyes and another c-locus allele, $\underline{c^p}$ (platinum), when homozygous produces a very light pigmentation as in $\underline{c^e c}$ and is only distinguishable from albino by direct comparison, but in contrast to $\underline{c^e c}$, $\underline{c^p c^p}$ animals have pink eyes. Both genotypes have similar levels of tyrosine hydroxylase activity in the skin, $\underline{c^p c^p}$ being slightly higher than $\underline{c^e c}$. This observation indicates different conditions of pigment formation in eye and skin but no simple explanation can be put forward for a single allelic substitution which suppresses pigment formation in skin by production of a mutant enzyme molecule and in the eye by production of a diffusible inhibitor. Unfortunately, there are no data on the tyrosine hydroxylase activity of the black/albino heterozygote eye.

The data obtained in this study on the behaviour of tyrosine hydroxylase activity in various coat-colour mutants indicate that the c-locus is the structural locus for tyrosine hydroxylase. The effects of the changes in the properties of tyrosine hydroxylase on melanin production in mutants of the b- and c-loci will now be discussed.

Fig. 9a. Increase in O.D. per day of whole Skin vs. Tyrosine Hydroxylase activity for aaBBCC and aaBBc ch ch c.



IV. The Relationship between Tyrosine Hydroxylase Activity and Melanin Production in the Melanin Pathway.

It is clear from a comparison of figs. 4 and 5 (pp.33,41), which show the changes with age of O.D. values and tyrosine hydroxylase activity for skin plus hair of allelic combinations at the b- and c-loci, that the maximum rate of O.D. increase does not coincide with the highest level of enzyme activity. In all genotypes examined maximum O.D. increase, suggested on pp. 60-62 to be due to underfur development, occurs between 7 and 10 days, while in aaBBCC, aab^cb^cCC and aabbCC, for instance, a peak of enzyme activity is found at 5 days, and in the corresponding chinchilla homozygotes maximum activity is reached at 10 days. In both cases a comparison of enzyme activity at each day and increase in O.D. value over the previous 24hrs. gives no sensible result, as shown in fig. 9a opposite.

On pp. 10-11 a picture of melanogenesis is presented in which melanin is deposited around a protein matrix, with which tyrosine hydroxylase activity is associated, to form a pigmented melanosome. This process takes place in the follicular melanocytes, from which 'mature' melanosomes are secreted into the cells surrounding the base of the growing hair. In the present study, homogenisation of skin plus hair, prior to tyrosine hydroxylase assay, left hairs intact. Hair alone, however, was homogenised to variable degrees, and the presence of tyrosine hydroxylase activity demonstrated, which supports the close association of tyrosinase activity and the melanosome. Mottaz & Zelickson (1969) in an electron microscopic study of melanosome transfer reported that melanosomes in the hair were larger than those in the melanocyte, which was taken as an indication that

Fig. 9b. O.D. vs. Age for aaBBCC and aaBBc^{ch}c^{ch} Whole Coat and Shaved Skin.

aaBBCC —●— whole skin —○— shaved skin
aaBBc^{ch}c^{ch} —▲— whole skin —△— shaved skin

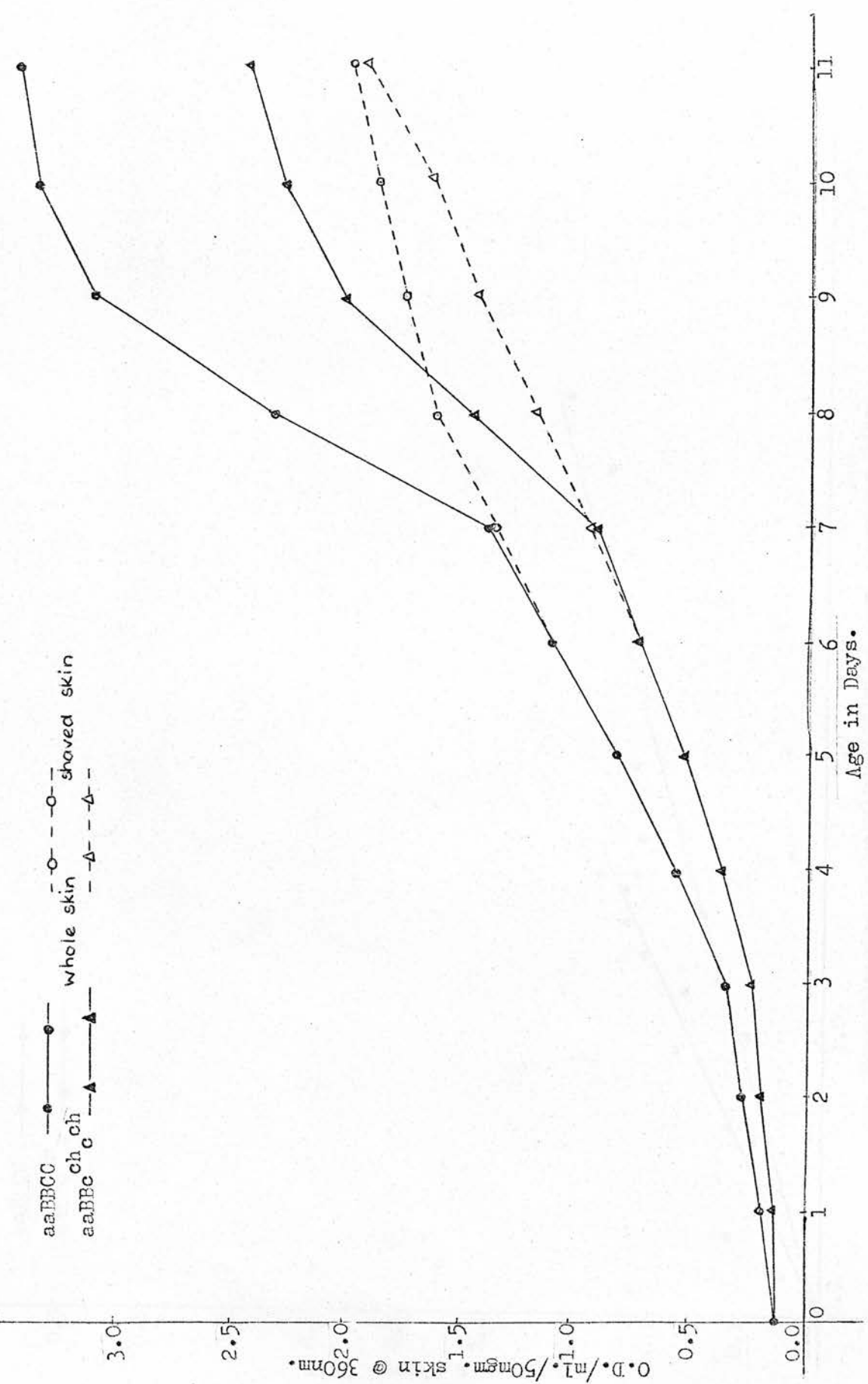


Fig. 9c. Increase in O.D. per Day of Shaved Skin vs. Mean Tyrosine Hydroxylase Activity over Preceding 24hrs.

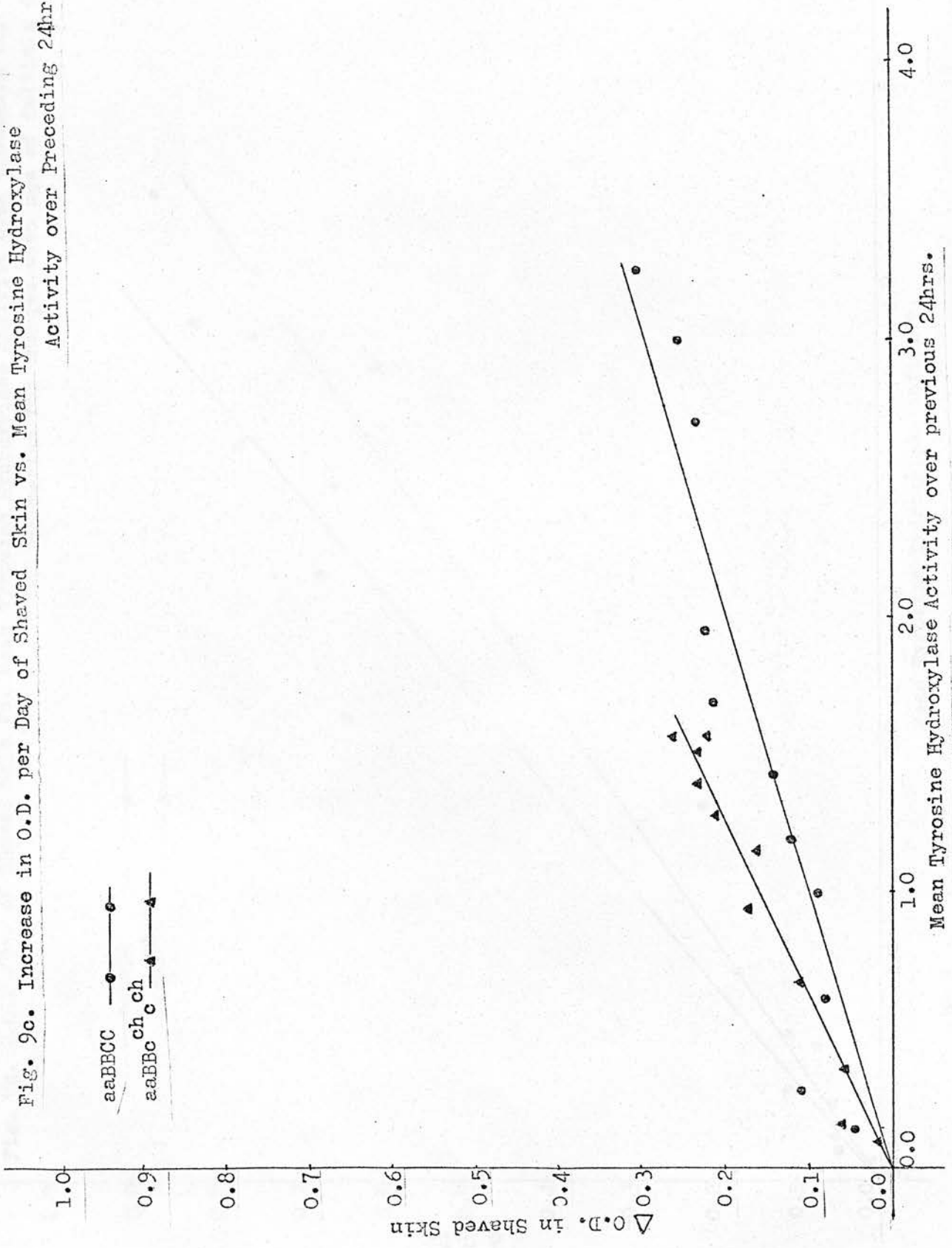
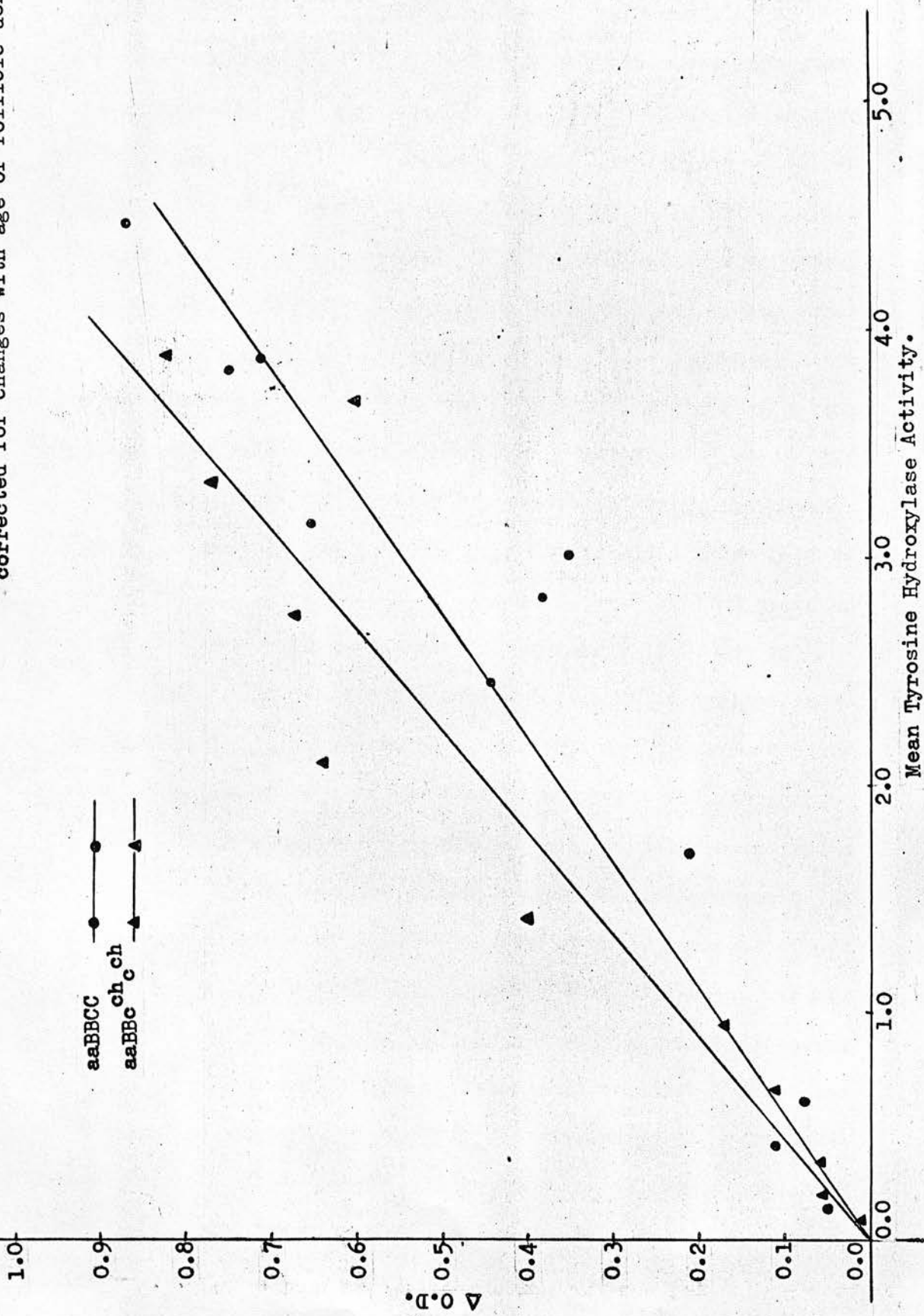


Fig. 9d. Δ O.D./Day of Shaved Skin vs. mean Tyrosine Hydroxylase Activity over preceding Day corrected for changes with age of follicle density.



growth (melanin production) continues after transfer. The duration of this synthesis must depend on substrate supply, which is not likely to continue after keratinisation of the hair shaft and formation of septae.

An attempt to assess the relative contributions of skin and hair to pigment content of the coat was made by shaving the coat before dissection from the animal from 6 days of age onwards. The results are shown in table 1d (p.36) and fig. 9b opposite, and show that the rapid 7-10 day O.D. increase is due mainly to the hair. Figs. 9c and 9d indicate a possible linear relationship between skin O.D. increase over 24hrs. and the mean tyrosine hydroxylase activity for the period. All values in fig. 9d were corrected for changes with age of follicle density (described in detail on pp. 82-84), since O.D. increase and mean tyrosine hydroxylase activity are calculated from the values of two consecutive days.

The immediate implication of a direct proportionality between shaved skin tyrosine hydroxylase activity and O.D. increase is that the skin enzyme activity is responsible for skin pigmentation only, as these measurements do not take into account the loss of enzyme activity and pigment from the skin to the hair by melanosome transfer. The proportion of the daily increase in O.D. of the hair due to melanosome transfer or in situ melanosome growth is not known, and the table on the following page shows that there is no simple relationship between skin tyrosine hydroxylase activity, or its decline in aaBCC, and hair O.D. increases.

Δ O.D./Day in hair & mean skin Tyrosine Hydroxylase for aaBBCC & aaBBc^{ch}c^{ch}.

Age (days)	aaBBCC hair						aaBBc ^{ch} c ^{ch} hair			
	uncorrected			corrected			uncorr.		corr.	
	O.D. incr.	mean TH activity	mean TH decrease	O.D. incr.	mean TH	mean TH decr.	O.D. incr.	mean TH	O.D. inc.	mean TH
6-7	0.00	2.71	0.56	0.00	4.35	-0.46	0.00	1.28	0.00	2.10
7-8	0.69	1.97	0.74	1.04	3.83	0.52	0.23	1.40	0.48	2.76
8-9	0.62	1.43	0.54	2.01	3.15	0.68	0.42	1.52	1.02	3.35
9-10	0.14	1.20	0.23	0.39	2.82	0.33	-0.03	1.58	-0.02	3.72
10-11	-0.01	1.01	0.19	0.16	2.46	0.36	-0.11	1.58	-0.20	3.87

It is therefore not possible to make any suggestion as to a relationship between tyrosine hydroxylase activity in the skin and the amount of pigment in the hair. It was difficult to shave the skin completely and, of course, a portion of the keratinised hair shaft is left behind, but hair could not have been plucked as follicle damage would have resulted. A more refined technique, ideally based on investigation of single or small groups of follicles and hairs, is obviously needed here.

The preceding discussion on the evidence for the c-locus as the structural locus for tyrosine hydroxylase considered the possibility that the different patterns in change with age of enzyme from different c-locus mutants are due to differences in response of the mutant molecules to activating agents. There are reports, however, that tyrosinase is inactivated by accumulation of melanin. Miyamoto & Fitzpatrick (1957) found that suspensions of 10 day old, dark brown, chick retinal pigment epithelium had 7 times the tyrosinase activity of 17 day old, black, suspensions. Lee & Lee noted that elevation of melanin content of Rana pipiens was accompanied by a parallel decrease in extractable tyrosinase activity. Seiji &

Fukuzawa (1972) studied the effects of melanisation on the dopa oxidase activity, measured by oxygen uptake, of melanosomes isolated from mouse melanoma. Dopa oxidase activity decreased with incubation and was not recovered by transferring to fresh dopa solutions. No significant dopa oxidase activity was released from melanosomes melanised either in vivo or in vitro by deoxycholate treatment, sonication, or trypsin digestion. It was concluded that the inactivation process was probably an irreversible blocking of the active centre of tyrosinase, rather than a progressive sequestration of the enzyme molecules by the building up of impermeable layers of melanin around the melanosome. Both black and chinchilla hair in the present study were found to have tyrosine hydroxylase activity which did not appear to be greatly increased by treatment with triton-X 100, but the variable degrees of homogenisation obtained made meaningful comparisons between separate experiments impossible. Activation of tyrosine hydroxylase in melanosome-containing fractions of mouse melanoma by membrane-disrupting procedures is also reported by Menon & Haberman (1970). Since the investigation was carried out on pigment-forming organelles which were, or had been, active in pigment production, the process described is probably not strictly one of activation but a release of tyrosine hydroxylase by melanosome disruption.

It would obviously be desirable to compare the rate of decline in extractable tyrosine hydroxylase activity in aaBBCC and its absence in aaBBc^{ch}c^{ch} animals with in vivo melanin production to see whether any of the difference in development of enzyme activity with age can be attributed to a difference in response to melanisation. There are three possible mechanisms which could

produce an apparent non-competitive inhibition situation during melanogenesis: first, removal of the enzyme molecules into the hair with melanosome transfer; second, the burying of the enzyme molecule under layers of melanin so that it becomes inaccessible to substrate, and, third, non-competitive inhibition proper, where melanin or its precursors interact with the enzyme molecule to render it inactive. Phenylalanine has been shown to be a competitive inhibitor of tyrosine hydroxylase (Miyamoto & Fitzpatrick 1957), but it seems unlikely that the decline in extractable aaBBCC tyrosine hydroxylase activity is caused by this type of competitive inhibition as the addition of tyrosine to the assay mixture would effectively compete against the phenylalanine. In addition, the phenylalanine/tyrosine skin pool ratios shown in table 11c (p.51) and fig. 8 do not increase with age.

For competitive and non-competitive inhibition the relationship between enzyme activity and amount of inhibitor present is given by the following expressions:

$$\text{competitive}$$

$$V = \frac{V_{\max} S}{S + K_M (1 + I/K_I)}$$

or:

$$\frac{1}{V} = \frac{1}{V_{\max}} \cdot (1 + K_M/S) + \frac{K_M}{K_I \cdot V_{\max}} \cdot I$$

$$\text{non-competitive}$$

$$V = \frac{V_{\max} S / (1 + I/K_I)}{(S + K_M)}$$

or:

$$\frac{1}{V} = \frac{1}{V_{\max}} \cdot (1 + K_M/S) + \frac{(S + K_M) \cdot I}{K_I \cdot V_{\max} \cdot S}$$

where V is enzyme activity in the presence of the inhibitor, S is substrate concentration, I inhibitor concentration and K_I the inhibition constant. If melanin (as assayed by O.D. measurements) acts as an in vivo inhibitor of tyrosine hydroxylase activity (assayed in vitro), a plot of 1/activity vs. O.D. value should give

Note: Changes with age in follicle number per unit area of skin were measured by Slee (1962) directly from follicle counts in 1.2mm. lengths of sagittal skin sections. These data show a maximum follicle density at 4 days after birth for skin from the sacral region and 5 days for the lumbar region. Slee also measured epidermal and dermal thicknesses: the epidermis was found to reach a maximum of 42.5 μ at 4 days, but the dermis was found to fluctuate randomly between 65 and 125 μ and individual values are not given. Estimations of follicle number per unit volume as required for the calculation of correction factors for tyrosine hydroxylase activity cannot therefore be made from these data. The area correction factors alone, however, calculated by the method described opposite, are compared below with those calculated from the follicle counts using: (a) the 4 day value as a basis for comparison and (b) the 5 day value as in table 14a.

		Age			
		4	5	7	9
(a)	Slee's data	1.00	1.27	1.54	1.74
	area corr.	1.00	1.26	1.52	1.79
(b)	Slee's data	--	1.00	1.19	1.37
	area corr.	--	1.00	1.32	1.56

A very close agreement is seen in the first two sets of values, suggesting that the effect of follicle initiation on follicle density after 4 days is insignificant compared with interstitial skin growth and that corrections should probably therefore have been made for tyrosine hydroxy-activity and O.D. values from 4 days rather than 5 days of age.

a straight line, providing that V_{\max} remains constant over the period of inhibition. This means that the total amount of tyrosine hydroxylase present at 5 or 6 days, when the decline in activity begins, must not be added to by further activation or induction.

Another problem is that both enzyme activity and O.D. value are expressed per standard weight of skin, but melanogenesis is restricted to the follicles. Dry (1926) reported that follicle initiation was complete by 7 days of age. The skin, however, continues to grow, so that the number of hair follicles and thus the number of sites of tyrosine hydroxylase activity and melanin production per given weight of skin will decrease. The decline in tyrosine hydroxylase activity from 5 to 10 days as measured by an assay on a given weight of skin may therefore not reflect the situation within the hair follicle, but rather the number of follicles taken for assay. A crude attempt to correct for changes with age of follicle density and thus to estimate the tyrosine hydroxylase activity within the follicle was made by estimating the growth in volume up to 11 days of a given 5 day volume of skin. The relative increase in thickness of skin with age was found by weighing 1cm.^2 areas of dorsal skin and the surface area increase calculated from body weight measurements using the relationship $\text{surface area} \propto (\text{volume})^{0.73}$ (Blaxter 1967), which, if skin density remained constant, can also be expressed as: $\text{s.a.} \propto (\text{body weight})^{0.73}$. Volume increase was calculated by multiplying thickness increase and surface area increase. The correction factors obtained are shown in table 14 on the following page.

Table 14a. Calculation of follicle density correction factor for growing skin.

Age (days)	gm/cm ² skin	(A) proportional increase in skin thickness	body wt. (gm.)	surface area (wt) ^{0.73}	(B) proportional increase in skin s.a.	correct. factor = (A)x(B)
0	0.027		1.30			
1	0.032		1.50			
2	0.038		1.75			
3	0.047		2.10			
4	0.059		2.55			
5	0.072	1.00	3.10	2.29	1.00	1.00
6	0.089	1.24	3.75	2.62	1.14	1.41
7	0.101	1.40	4.55	3.02	1.32	1.85
8	0.104	1.44	5.15	3.31	1.45	2.09
9	0.107	1.49	5.70	3.57	1.56	2.32
10	0.104	1.44	6.15	3.77	1.65	2.38
11	0.106	1.47	6.50	3.92	1.71	2.51

Tyrosine hydroxylase activities and O.D. values for whole coat and shaved skin for aaBBCC and aaBBc^{ch}c^{ch} from 5 to 11 days before and after correction are shown in tables 14b and 14c on the following pages. Figs. 10a and 10b compare corrected and uncorrected patterns of development with age of tyrosine hydroxylase activity and O.D. for aaBBCC and aaBBc^{ch}c^{ch}. Albino values were subtracted before correction in all cases, the appropriate albino value having already been subtracted from the uncorrected tyrosine hydroxylase activities and O.D. values shown in tables 14a and 14b.

Table 14b. Correction of aaBBCC and aaBbc^{ch}c^{ch} tyrosine hydroxylase activities for changes with age of follicle density.

age	correct. factor	aaBBCC		aaBbc ^{ch} c ^{ch}	
		tyr. hydr. activity	corrected activity	tyr. hydr. activity	corrected activity
0		0.10		0.06	
1		0.20		0.12	
2		0.38		0.22	
3		0.89		0.52	
4		2.51		0.85	
5	1.00	3.51	3.51	1.08	1.08
6	1.41	3.03	4.27	1.25	1.76
7	1.85	2.39	4.42	1.32	2.44
8	2.09	1.55	3.24	1.48	3.09
9	2.32	1.32	3.06	1.56	3.62
10	2.38	1.09	2.59	1.60	3.81
11	2.51	0.93	2.33	1.56	3.92

Table 14c. Correction of aaBBCC and aaBbc^{ch}c^{ch} whole coat and shaved skin O.D. for changes with age of follicle density.

age	correct. factor	aaBBCC				aaBbc ^{ch} c ^{ch}			
		whole coat		shaved skin		whole coat		shaved skin	
		O.D.	cor.O.D.	O.D.	cor.O.D.	O.D.	cor.O.D.	O.D.	cor.O.D.
0		0.00				0.00			
1		0.05				0.02			
2		0.16				0.08			
3		0.24				0.14			
4		0.45				0.25			
5	1.00	0.70	0.70			0.42	0.42		
6	1.41	1.00	1.41			0.58	0.82		
7	1.85	1.21	2.24	1.23	2.28	0.74	1.37	0.79	1.46
8	2.09	2.14	4.47	1.45	3.03	1.25	2.61	1.02	2.13
9	2.32	2.90	6.73	1.59	3.68	1.90	4.40	1.25	2.90
10	2.38	3.16	7.50	1.71	4.06	2.09	4.98	1.47	3.50
11	2.51	3.24	8.10	1.80	4.50	2.24	5.60	1.73	4.32

Fig. 10a Comparison of $aaBbCC$ and $aaBbCc^{ch}$ tyrosine hydroxylase activities corrected and uncorrected for change with age of follicle density.

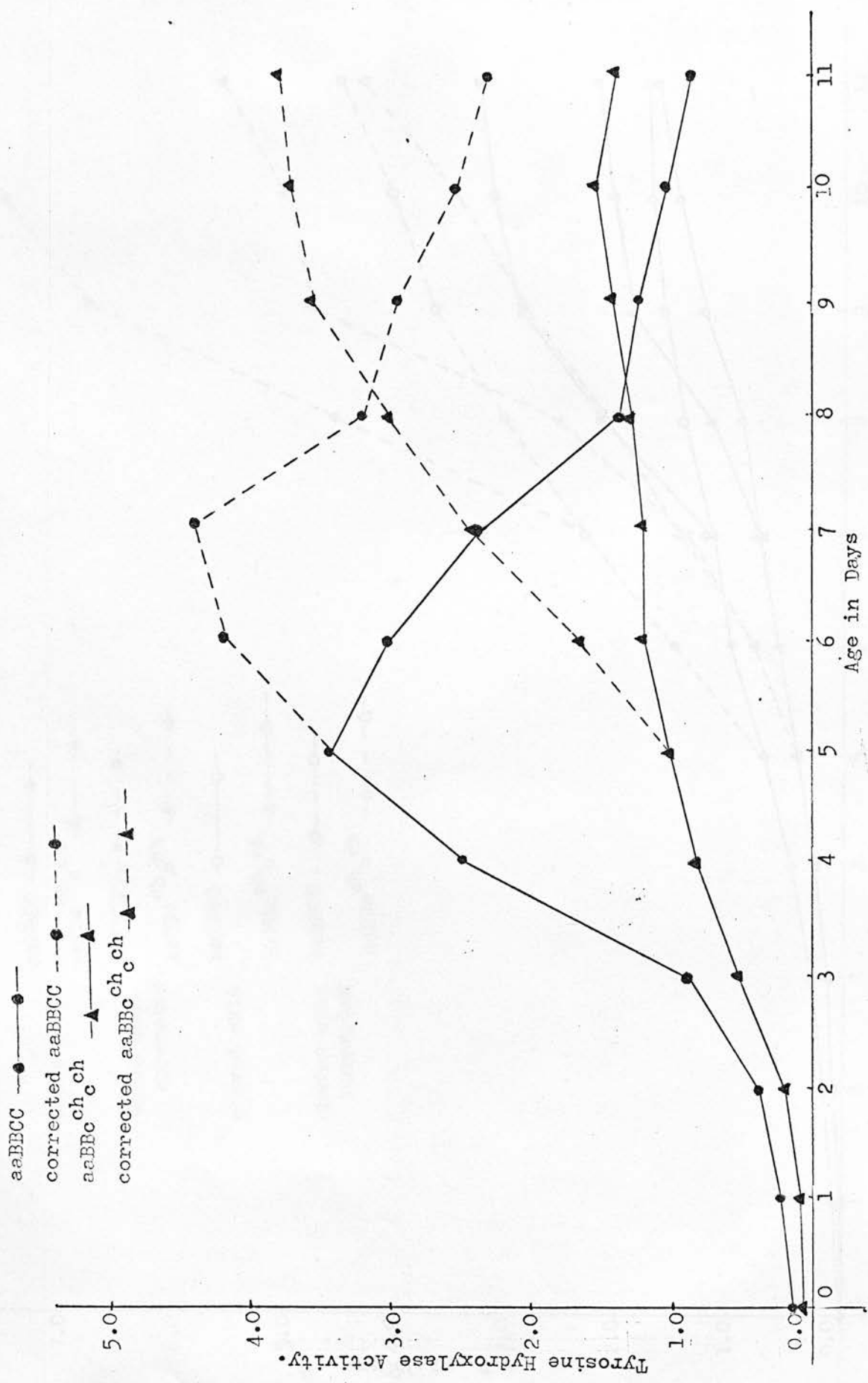


Fig. 10c. Inhibition Plot for aaBBCC and aaBBc^{ch}c^{ch} Skin Tyrosine Hydroxylase by Skin Melanin.

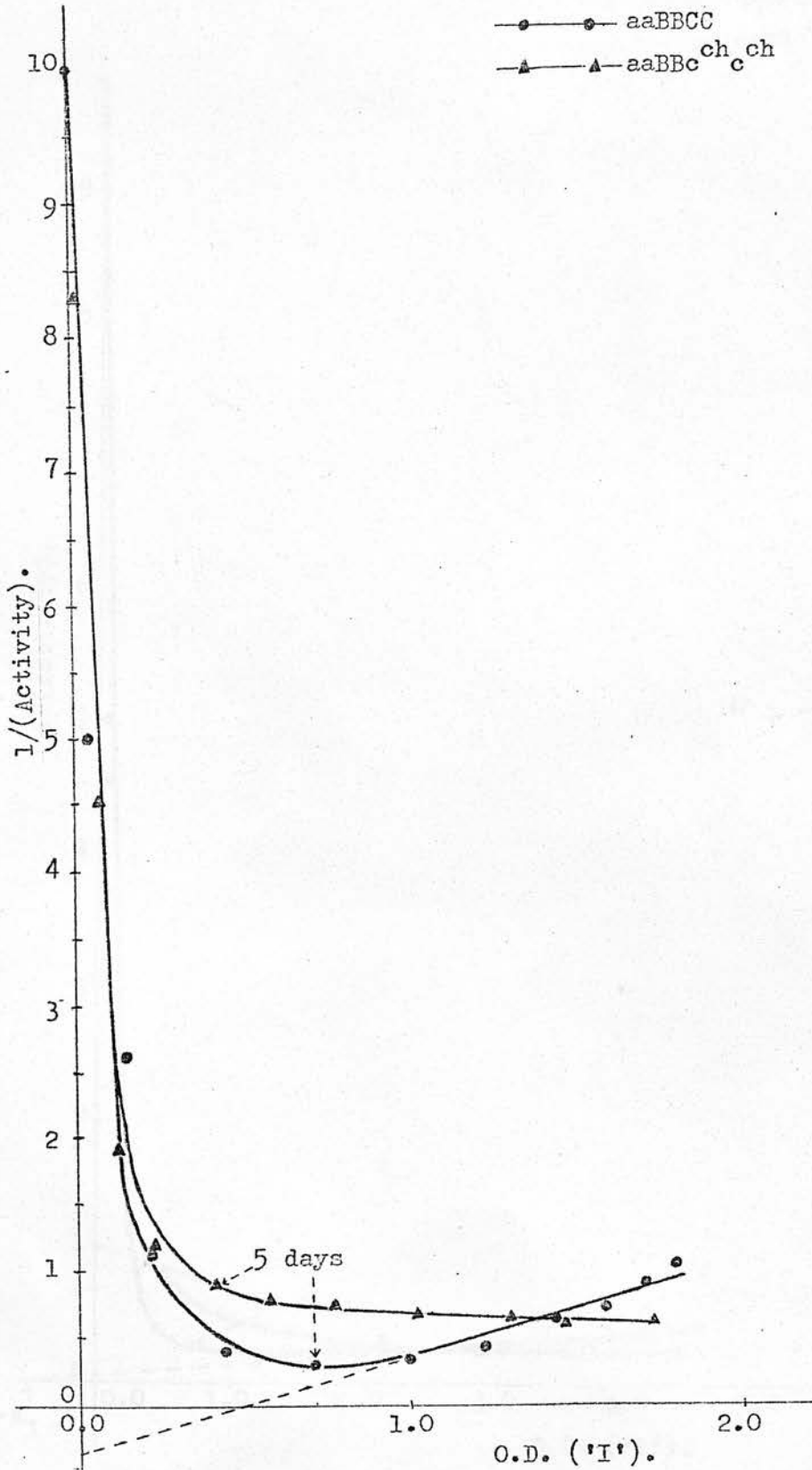


Fig. 10d. Inhibition Plot for aaBBCC and aaBBc^{ch}c^{ch} Skin Tyrosine Hydroxylase by Skin Melanin, corrected for changes with age of follicle density.

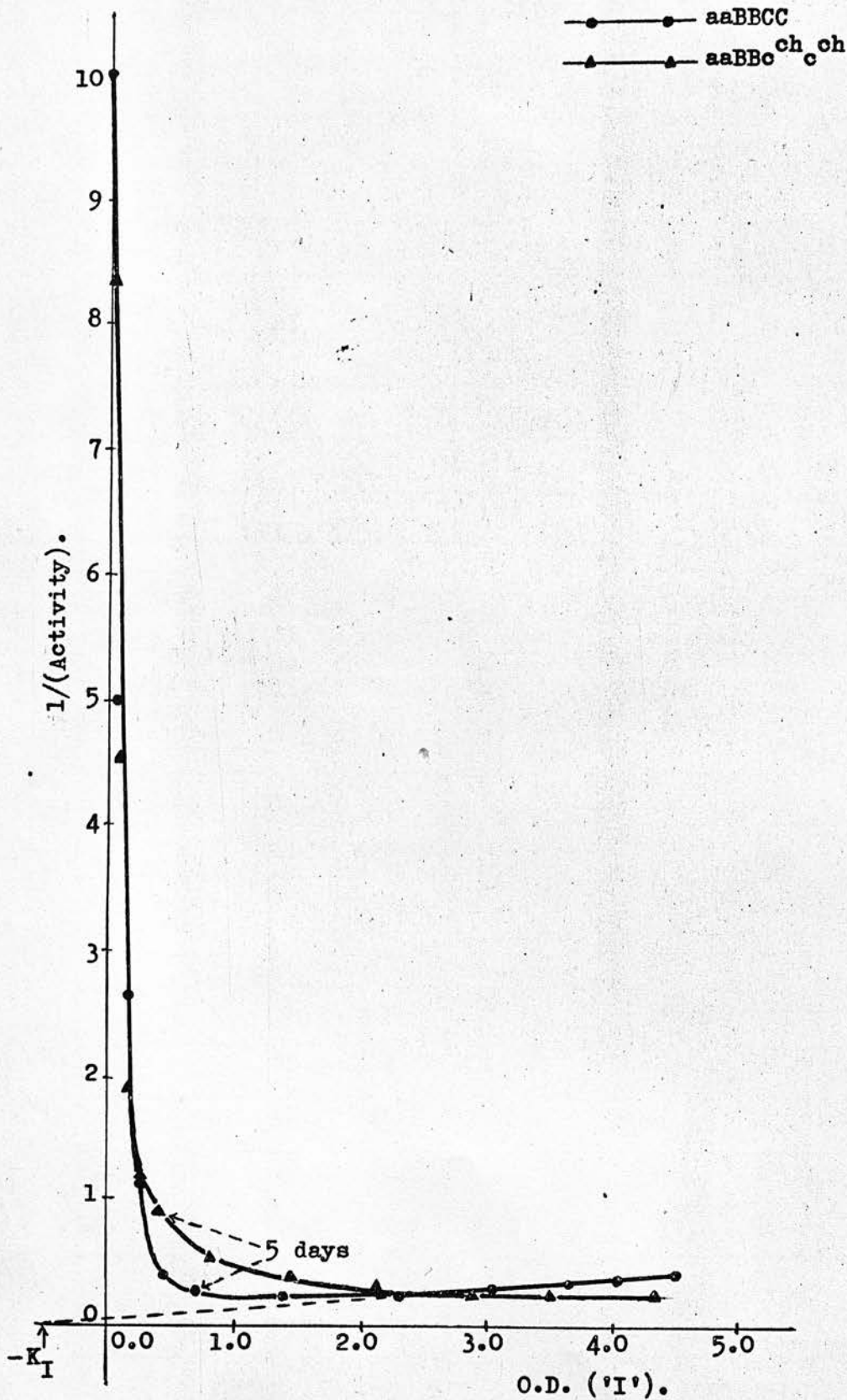


Fig. 10a shows that the effect of correcting aaBBCC tyrosine hydroxylase activity for changes with age of follicle density is to shift the peak of activity from 5 days to 7 days, while O.D., shown in fig. 10b, continues to rise. A direct comparison between enzyme activity and O.D. would not be affected by this correction but an inhibition plot, which uses reciprocal activity against O.D., will obviously be altered. This is shown by figs. 10c and 10d, in which the inhibition plots for aaBBCC and aaBBc^{ch}c^{ch} skin tyrosine hydroxylase by skin melanin are shown (a) with enzyme and O.D. values corrected only for albino activity and O.D. and (b) enzyme and O.D. values also corrected for changes with age of follicle density. The O.D. values of shaved skin were used for these plots so that the effect of skin melanin on skin tyrosine hydroxylase activity could be seen more clearly.

Figs. 10c and 10d both show that, from the point at which tyrosine hydroxylase activity declines in aaBBCC, a linear relationship is seen between reciprocal activity and skin O.D.. The projection of this line, however, in fig. 10c gives a positive intercept on the abscissa, although it is seen from the inhibition equations on p. 80, the abscissa intercept should be negative. This suggests that some factor or factors other than melanin accumulation are contributing to the decline in tyrosine hydroxylase activity in aaBBCC. Fig. 10d has a negative intercept, indicating that one of these factors could be the decrease in follicle density owing to skin growth. It is thus evident that the ideal terms in which to express enzyme activity and O.D. content would be per follicle, rather than per unit weight of skin or skin protein. The above calculations for correction for changes in follicle density are

obviously only very approximate and therefore an accurate investigation of an inhibitory relationship between melanin and tyrosine hydroxylase cannot be made until melanin content and enzyme activity can be measured differently.

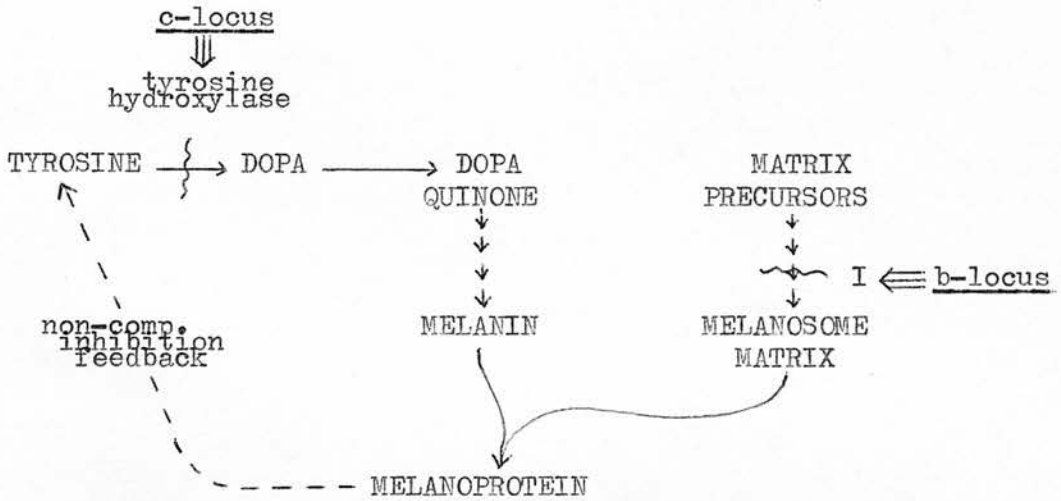
These considerations, however, do not invalidate the differences between aaBBCC and aaBBc^{ch}c^{ch} in the pattern of development of tyrosine hydroxylase activity with age. Although a detailed analysis of inhibition is not possible using the results of this investigation, some general speculations can be made as to how inhibition of tyrosine hydroxylase by melanin could cause the difference in enzyme development. First, apparent inhibition of tyrosine hydroxylase due to removal of melanosomes to the hair should affect both black and chinchilla in equal proportions and therefore cannot be considered as a source of difference. Second, the sequestration of tyrosine hydroxylase within the melanosome by building-up of melanin, or, third, non-competitive inhibition proper, could be made to explain the apparent absence of inhibition in chinchilla as follows: wild-type tyrosine hydroxylase makes melanin more quickly than the chinchilla enzyme so that the wild-type molecules are inhibited before those of chinchilla, where, because of the slower rate of melanin synthesis, inhibitors are not able to build up to an effective concentration before melanosomes are transferred to the hair.

This explanation cannot account for the behaviour of tyrosine hydroxylase in the Cc^{ch} heterozygote, however. Fig. 5b (p. 41) shows that the level of tyrosine hydroxylase activity here is very close to the parental mean. If the enzyme population of each melanosome is a mixture of both C-type and c^{ch}-type molecules, the

melanin produced by the former should inhibit the latter and the pattern of tyrosine hydroxylase activity would be expected to approach that of the Cc heterozygote. Heterozygote intermediacy could be achieved if each enzyme molecule were covered separately by a surrounding of melanin of its own production, or if each melanosome contained only one enzyme molecule. It follows, however, that if this were so, the pigment content of Cc would be half that of CC. Table 1a (p.33) shows that the O.D. value of aaBBcc is over 85% of that of aaBBCC for all ages investigated. The fourth explanation for the apparent absence of an inhibition response of chinchilla tyrosine hydroxylase to melanin is that, owing to its different primary structure, the configuration of the mutant enzyme molecule is such that its K_I for melanin or its precursors is very much higher than that of the wild-type molecule and is not reached in vivo.

The effect of the b allele on tyrosine hydroxylase is to increase activity by a factor of approximately 2 (tables 6a,c pp. 41,43; figs. 5a,b,e,f) without significantly affecting K_M for tyrosine (table 9, p.46). O.D., however, is decreased by approximately half (tables 1a,c pp.33,35; figs. 4a,c). Moyer (1961) suggested that the b-locus affected melanosome structure: the melanosomes of bb genotypes are smaller than those of BB genotypes and spherical. Coleman (1962) reported that the formation of insoluble skin compounds from labelled tyrosine in aabbCC was twice that of aaBBCC both in vitro and in vivo, which suggests that twice as much melanin (or immediate precursors) is actually formed and remains stable for some time. If the melanosome structure is affected by the b allele such that melanin produced cannot adhere

firmly to the matrix and can diffuse away and possibly be degraded, the tyrosine hydroxylase molecules associated with the melanosome will not be inhibited as effectively as in wild-type.



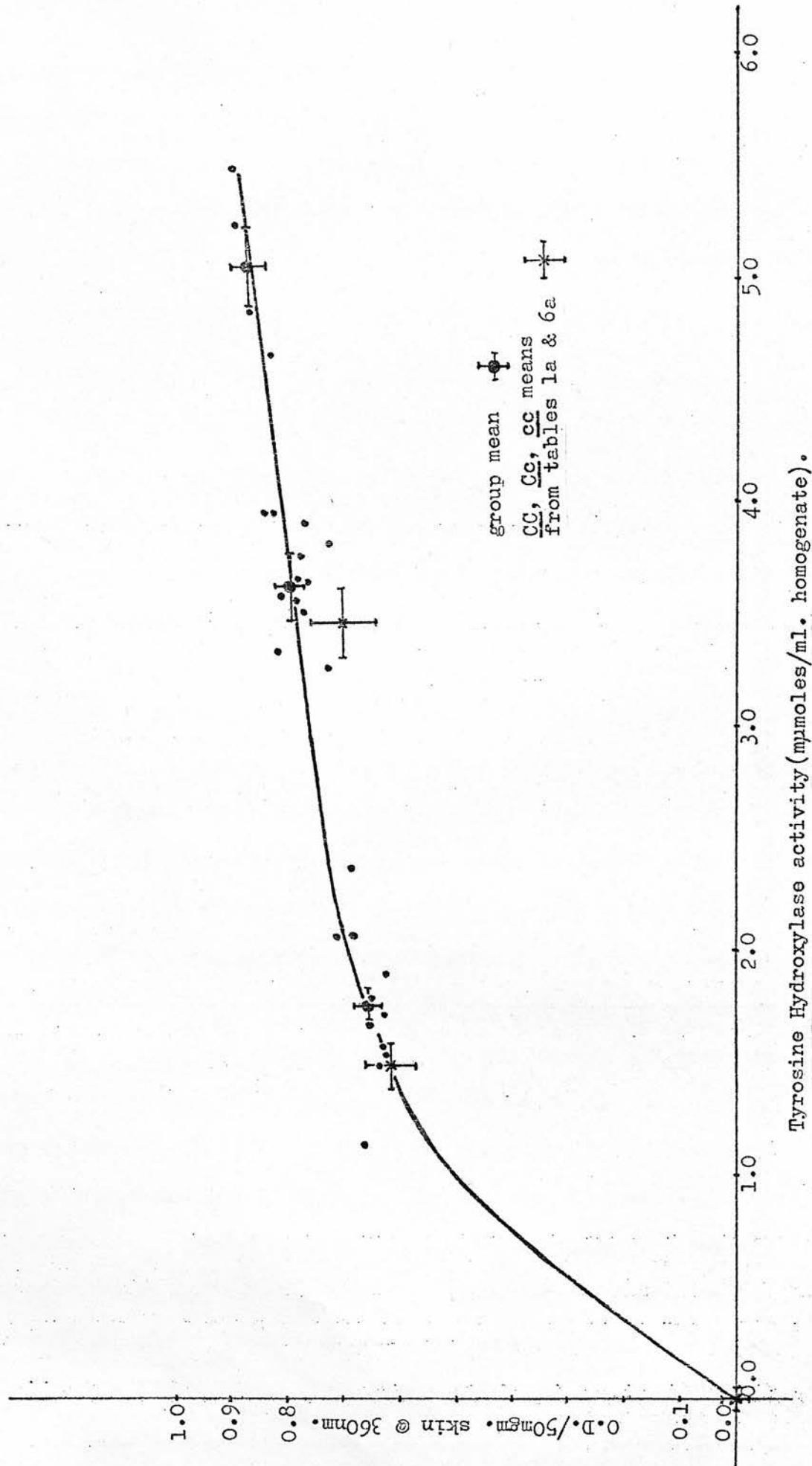
If $O.D. \propto 1/Activity$ as in the inhibition plot, then a halving of O.D. will cause a doubling of activity. Unfortunately inhibition plots for aab^cb^cCC and aabbCC could not be constructed as O.D. values for shaved skin were not obtained. A similar mechanism of action is suggested for the b^c allele also. It will be noticed in fig. 5e (facing p.41), however, that the activity of aabbCC tyrosine hydroxylase is higher than that of aaBBCC at birth when the melanin content is measured as zero.

The main purpose of this project was to compare the extractable melanogenic enzyme activity of various genotypes with the phenotype, melanin content, to see if any agreement with a theoretically-predicted relationship could be found. The equation for this relationship is:

$$F = \frac{S_o - S_n / (K_1 K_2 K_3 \dots K_n)}{\frac{1}{E_1} + \frac{1}{E_2 \cdot K_1} + \frac{1}{E_3 \cdot K_1 K_2} \dots + \frac{1}{E_n \cdot (K_1 K_2 \dots K_{n-1})}} \quad (\text{see p.16})$$

where E is the ratio V_{\max}/K_M determined by in vitro assay, S_o the in vivo substrate concentration, S_n product concentration and K the equilibrium constant for a single step in the pathway. For the melanin pathway, S_o could be phenylalanine or tyrosine and S_n is melanin itself. The skin pool measurements (fig.8, p.50) show both phenylalanine and tyrosine to be maintained at levels which change with age rather than genotype: the small daily fluctuations cannot be associated in any consistent way with tyrosine hydroxylase activity. Tyrosine aminotransferase activities (table 12, p.52) suggest that the similar tyrosine levels in the various genotypes are not achieved by deamination of excess tyrosine. Whatever the mechanism of pool regulation, the value of S_o can be considered a constant for present purposes. If melanin were removed as soon as it appeared, or if the amount of melanin made were insignificant compared with S_o , S_n could be ignored. It is not possible to measure melanin content in μmoles as its molecular weight as an irregular polymer cannot be calculated so S_o and S_n cannot be compared in the same terms, but the fact that melanin is deposited as an insoluble compound probably means that, in effect, it can be ignored as its precipitation will prevent its affecting the backward

Fig. 11a. Dose Response using X-translocated C Allele.



reaction. This leaves the reciprocal term containing E as the variable with which F (melanin production over 24hrs.) is to be compared.

After 5 days of age in wild-type, tyrosine hydroxylase may be inhibited by melanin so comparisons must be made before this age. Comparisons for later ages must also ignore melanin production in the hair as the assay method measured skin tyrosine hydroxylase activity only. It was therefore decided to compare the increase in O.D. of skin between 4 and 5 days with the ratio V_{\max}/K_M for the mean enzyme activity over this period. V_{\max} is obtained from the Lineweaver-Burke plot.

Fig. 11a shows the curve obtained when O.D. value is plotted against V_{\max} for the offspring of matings segregating for the X-translocated C allele. 4 classes of male offspring were expected from the mating of aaBBCCc;X^CX females with aaBBCC;XY males, namely, aaBBCCc;XY (1 dose C), aaBBCC;XY (2 doses C), aaBBCCc;X^CY (2 doses C) and aaBBCC;X^CY (3 doses C). 27 male offspring were produced from three such matings and O.D. determinations and tyrosine hydroxylase assays were carried out on individuals rather than pooled skins of the same genotype as attempts to introduce an X-linked marker in order to distinguish animals not carrying the translocation had failed. The mating scheme is presented in detail on p.24. Because of this limitation activity and O.D. determinations were able to be made at 5 days of age only. The enzyme activity and O.D. values obtained are shown in table 8 (p.45) and are presented in histogram form in fig. 6. The ordinate in fig. 11a is thus the O.D. accumulation over 5 days rather than 24hrs.

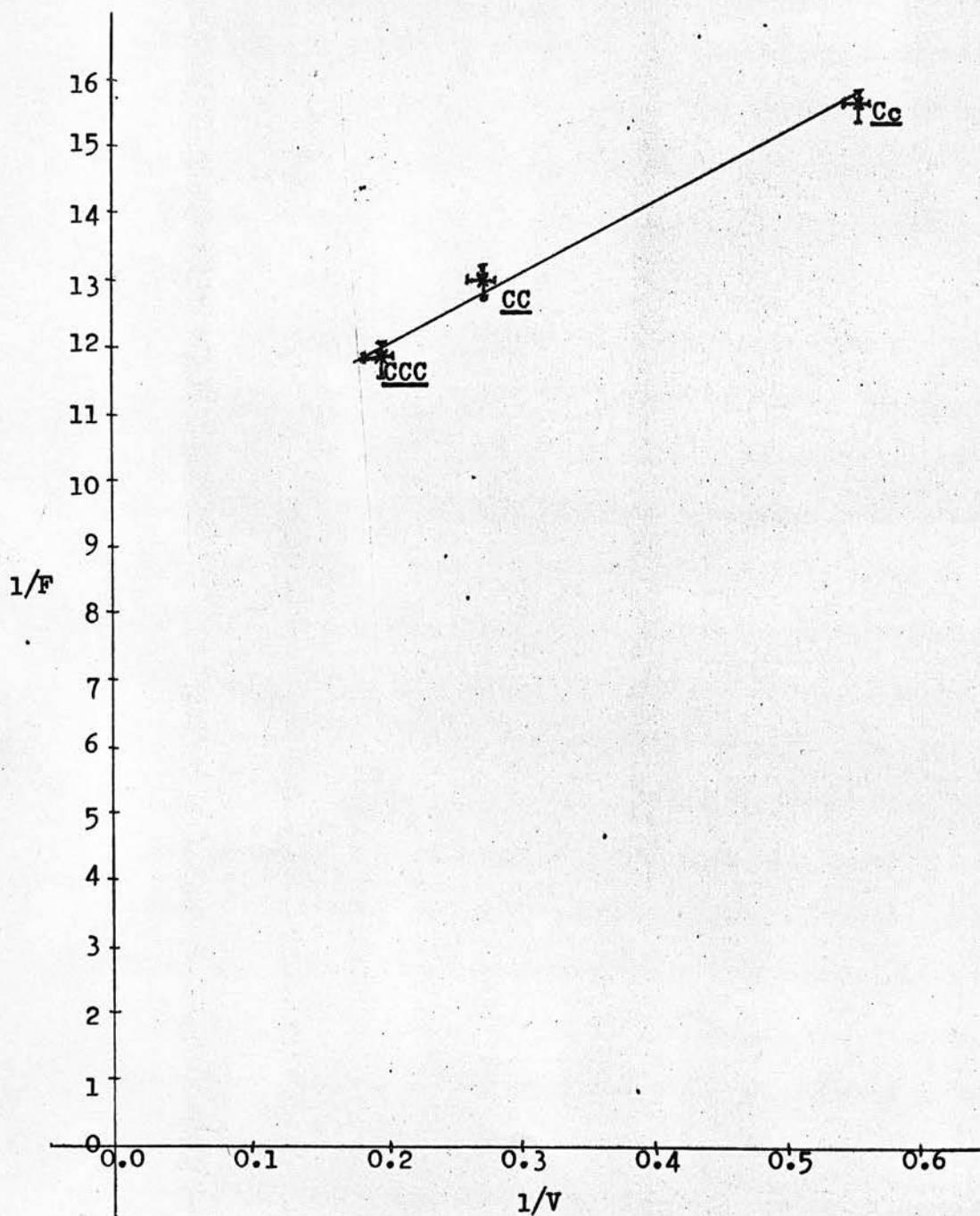
The obvious advantage in varying the dose of a single allele

as a method of varying enzyme activity is that the need to take K_M differences into account is eliminated. In higher animals methods of introducing such variation is limited and the use of translocations rather than aneuploids probably better as less genetic material is involved and the probability of metabolic disturbance owing to combined interference of many other linked genes is reduced. Had an autosomal translocation of the C allele been available, it might have been possible to produce doubly homozygous individuals with 4 doses of the C allele, but in the present case the C dosage of aaBBCC;X^CX^C females, had they been viable, would have been expected to be affected by Lyonisation and the investigation was therefore confined to males.

The histogram in fig. 6 (p.45) shows the data to fall into three separate groups for enzyme activity (means 1.8, 3.7 and 5.1), but to be indistinguishable by O.D. value. Fig. 11a shows the position of each individual separately when O.D. is plotted against enzyme activity and also the mean of the three classes and those of the pooled data from known aaBBCCc and aaBBCC animals from tables 1a and 6a. The means for individuals and pooled data overlap within the range of $\pm 2x$ standard error and are therefore not considered to be significantly different, although the mean of the former is higher than that of the latter in both cases and the standard errors are smaller. The higher means could reflect some hybrid vigour introduced with the translocation, which was maintained on an outbred background, and the larger standard error of the C57 animals an increased susceptibility to environmental fluctuations which can accompany inbreeding.

If the first two groups of points represent 1 dose and two

Fig. 11b. $1/F$ vs. $1/V$ for 1, 2 and 3 doses of the C allele with aaBB.

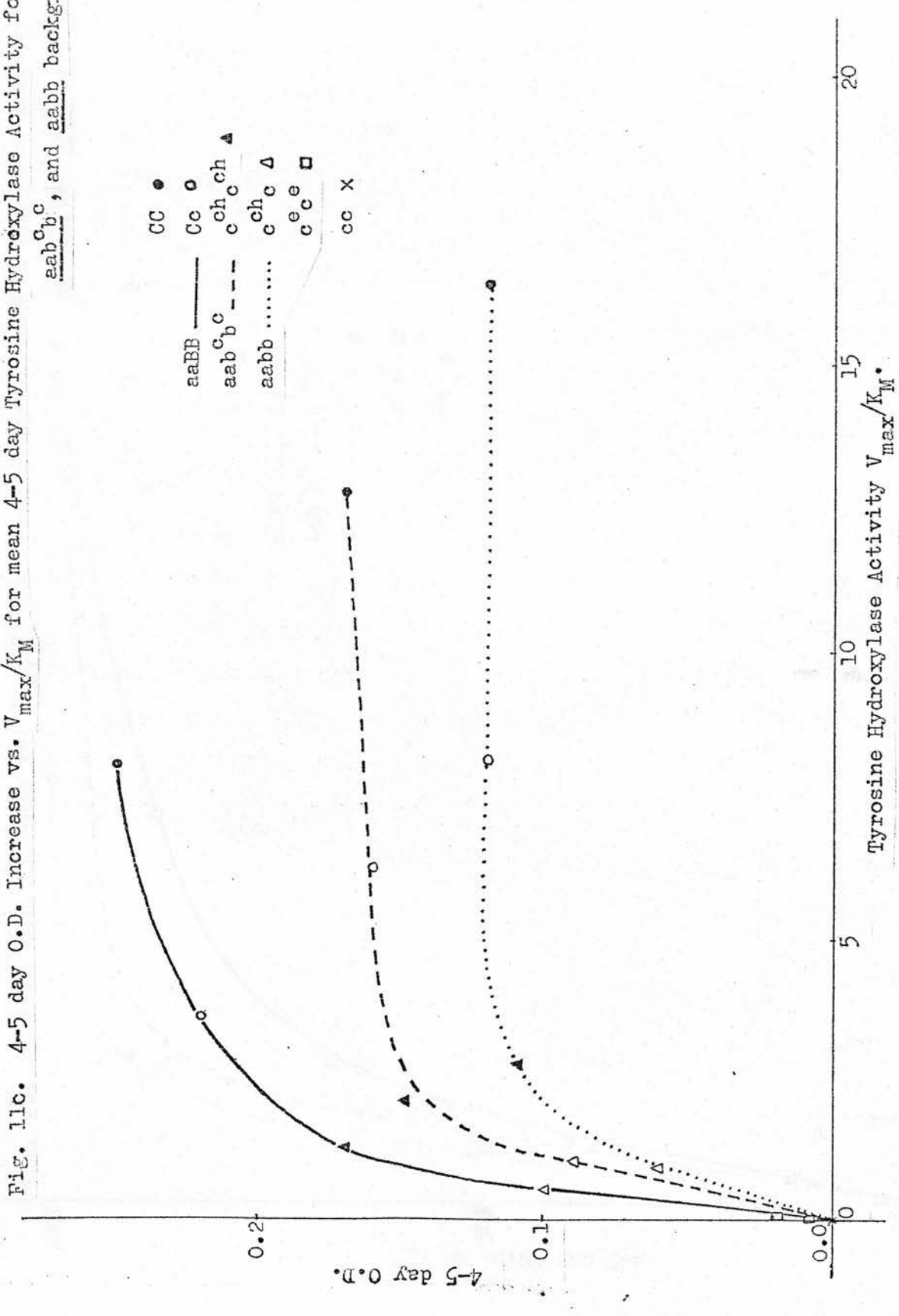


doses of the C allele respectively, the third group of 4 individuals of highest tyrosine hydroxylase activity probably represents the 3 dose aaBBCC:X^CY class. The numbers in the groups do not fall into the 1:2:1 ratio expected but this discrepancy could be due to the reduced viability and high pre-natal mortality rate of the translocation males.

It is obvious that the points indicate a non-linear relationship between O.D. and tyrosine hydroxylase activity as theoretically predicted. It can be seen, furthermore, from the flux equation on p.88 that the relationship between flux (F) and enzyme activity as expressed by the ratio V_{\max}/K_M (E) is essentially a Michaelis type one if only V_{\max} is changing for a particular enzyme as then $1/F \propto 1/E$, or $1/F \propto 1/V$. Fig. 11b opposite shows the double reciprocal plot of $1/O.D.$ vs. $1/V$ for the means of the three data groups suggested to represent aaBBcc, aaBBCC and aaBBCCC. The three points indicate a linear relationship between the reciprocal values of 5-day O.D. and tyrosine hydroxylase activity. It can therefore be concluded that, for dosage from 1 to 3 of the C allele in the diploid genome, enzyme activity is proportional to gene dose and when the wild-type activity is exceeded the wild-type pigment intensity is also exceeded. There is thus at these levels no indication of a regulation mechanism constraining the limits of tyrosine hydroxylase activity or melanin production.

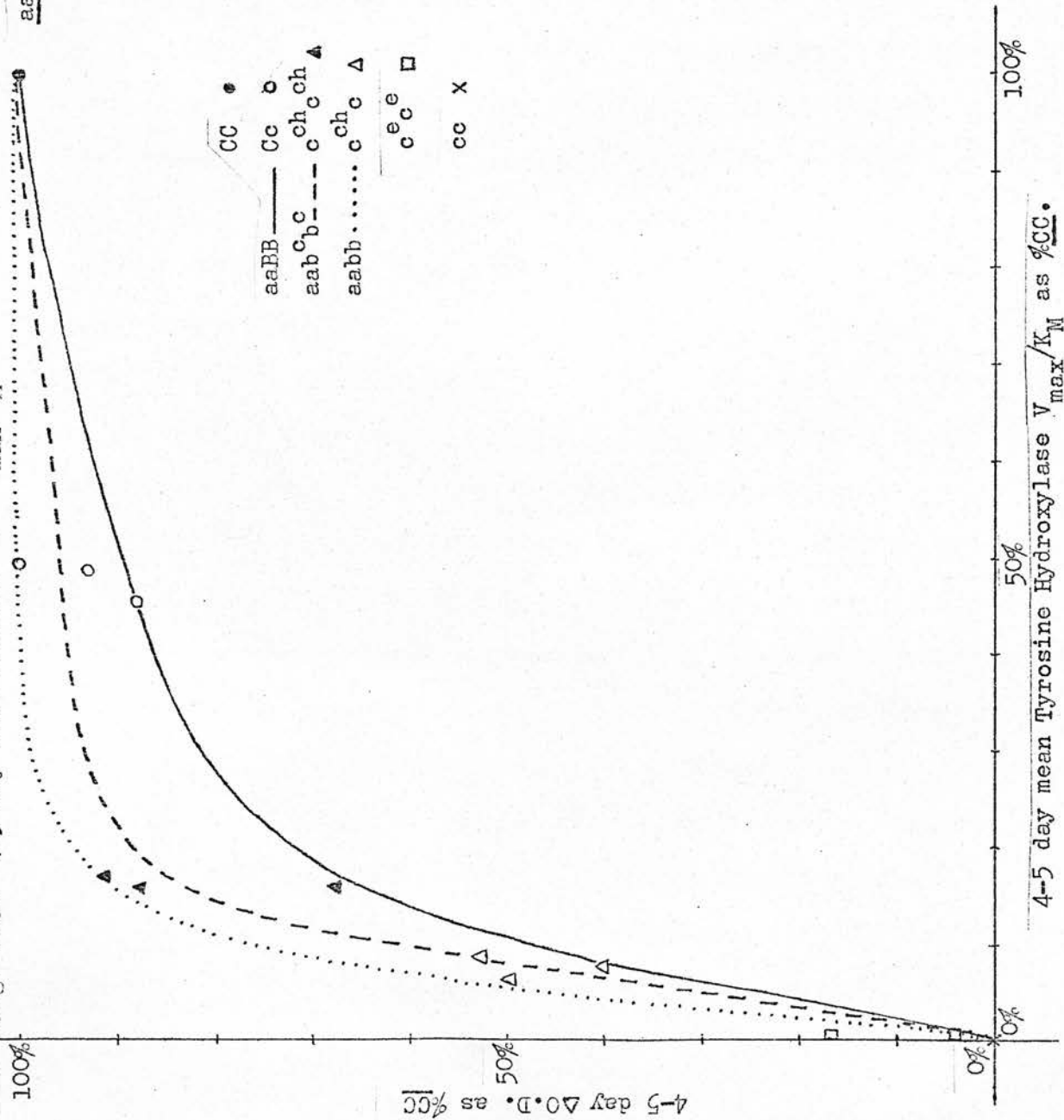
Fig. 11c (facing p.92) shows the relationship between the O.D. increase between 4 and 5 days and the mean tyrosine hydroxylase V_{\max}/K_M for the same period for the allelic combinations CC, Cc, c^{ch}c^{ch}, c^{ch}c, c^ec^e, and cc (values adjusted so that cc = 0,0) on aaBB, aab^cb^c and aabb backgrounds. Again the points for each

Fig. 11c. 4-5 day O.D. Increase vs. V_{max}/K_M for mean 4-5 day Tyrosine Hydroxylase Activity for aabb, aab^cb^c, and aabb backgrounds.



aabb ——— ●
aab^cb^c - - - ▲
aabb ○
aabb - · - · - ◻
 CC ●
 Cc ○
 ch ch ▲
 c c c
 ch c c Δ
 e e e
 c c c ◻
 cc X

Fig. 11d. 4-5 day O.D. Increase vs. V_{max}/K_M for mean 4-5 day Tyrosine Hydroxylase Activity for aaBB, aa^c_b^c, and aa^c_b^c as % of CC.



4-5 day mean Tyrosine Hydroxylase V_{max}/K_M as %CC.

series fall into a curve such that increasing enzyme activity has proportionately decreasing effects on O.D. The point at which increase in enzyme activity has no effect on O.D. value is not quite reached in aaBB, but in aab^cb^c and aabb the curve levels off quite sharply around c^{ch}c^{ch} levels. The comparative effect of this difference in the shapes of the curves on phenotype in the three series is perhaps more clearly seen in fig. 11d where all values for each series are expressed as a percentage of CC. It can now be seen that a given reduction of enzyme activity causes progressively smaller effects on aaBB, aab^cb^c, and aabb respectively, which is precisely the type of effect which would be predicted from the theoretical observations (pp. 17-18). As the value of E becomes larger owing to increased V_{max} , so the reciprocal term $1/E$ which appears in the flux equation becomes smaller and has a smaller proportional effect on the flux. When $1/E$ is small, changes in its value therefore have less effect on the flux than when it is large, i.e. when E itself is small. A 50% decrease in tyrosine hydroxylase, for instance, would appear to cause an approximately 12% decrease in 4-5 day O.D. production in aaBB, 5% in aab^cb^c and has no effect in aabb. This effect is more marked at lower levels of enzyme activity where the curves are steeper: at 10% CC activity O.D. production is 50% CC in aaBB, 65% in aab^cb^c and 80% in aabb; at 5% CC activity O.D. production is 25%, 35% and 50% of CC for aaBB, aab^cb^c and aabb respectively. The observation that aabbc^ec^e animals are darker in colour than aaBBc^ec^e (see fig. 2) now has a theoretical explanation.

Two further points should be made concerning the applicability of the flux equation in these cases, however. The equation is

derived from a simplification of the expression relating enzyme activity to substrate and product concentrations and kinetic parameters, as follows:

$$\text{if } v = \frac{V/M_1(S_0 - S_1/K)}{1 + (S_0/M_1 + S_1/M_{-1})}$$

where S_0 is substrate concentration, S_1 is product concentration, K is the equilibrium constant, V is V_{\max} and M_1 and M_{-1} are the K_M 's for the forward and backward reactions respectively, the denominator approaches unity if the enzyme is unsaturated: i.e. if S_0 is very much smaller than M_1 and S_1 is very much smaller than M_{-1} . The tyrosine skin pool measurements for skin homogenates gave estimates of 0.2 umoles tyrosine per ml. homogenate (table 11b, p.51). Homogenates were prepared by adding 3x the skin weight in mls. of homogenising fluid to the skin. Assuming a skin density of unity, the approximate concentration of tyrosine in skin can be estimated at 0.8mM, which is of the same order as the K_M values obtained (0.46mM for CC and 1.15mM for c^{ch}c^{ch}). Whether or not the enzyme sees a similar concentration in vivo cannot be known, but it is doubtful from these data that S_0 will be very much less than M_1 in vivo, and the ratio S_0/M_1 is not a constant but varies for the enzyme produced by each c-locus allele. The dopa pool, S_1 , is not detectable in vivo, either because it is too small (of the order of nanomoles) or because the dopa formed on the tyrosinase molecule remains attached to the molecule until it is oxidised to dopa quinone. If M_{-1} , which was not calculated, was also very small, the ratio S_1/M_{-1} could have significant magnitude. The approximation of the denominator in the present case is therefore not proved to

be justified.

The second point is that the c-locus mutations may also alter dopa oxidase activity, if, as has been shown to be the case in lower organisms, tyrosine hydroxylase and dopa oxidase activity are contained in a single protein molecule. It is therefore possible that two consecutive steps in the melanin pathway have been altered and owing to the failure in this investigation to assay dopa oxidase activity in the skin the extent of the effect on the second step is not known and may not be proportional to the effect on the first step.

Nevertheless, the data obtained do indicate that the theoretically-predicted relationship applies to the melanin pathway at least between 4 and 5 days of age. At later ages the situation becomes confused as CC activity declines relative to other genotypes.

One previous attempt to compare melanogenic enzyme activity with coat pigment has been made. Russell & Russell (1948) measured dopa oxidase activity in previously frozen skin sections by visual estimation of the degrees of blackening obtained when the sections were incubated in dopa. Assays were performed on skin in which the mid-region of the hair was being formed so that dopa oxidase activity could be compared with the pigment volume estimates of this region of the hair made by E.S. Russell (1946, 1948). The relationship between enzyme activity and pigment volume which can be derived from the data presented in the paper is roughly linear, as might be expected if the melanogenic enzyme is assayed in terms of the end-product of the pathway.

CONCLUSION.

The aim of this investigation of melanogenesis was to measure the effects upon the intermediate metabolite pools and the end-product of the biosynthetic pathway of changes in the levels of activity of the enzymes involved in order to attempt to describe the pathway as a system responding to changes in its various components, with special reference in this particular case to the biochemical basis of phenotypic dominance.

Three enzymes are involved, at three consecutive steps, in the conversion of phenylalanine to melanin - namely, phenylalanine hydroxylase, tyrosine hydroxylase and dopa oxidase (see fig.1, p.7). In the first instance it was hoped to alter the levels of activity of all three enzymes by using mutations at the d- and c-loci. Phenylalanine hydroxylase activity, however, was found to be unaffected by the different d-locus alleles, contrary to the report of Coleman (1960), and also all other available mutations associated with changes in pigmentation or neurological abnormalities. Tyrosine hydroxylase activity was successfully assayed in skin homogenates but all attempts to demonstrate the presence of dopa oxidase activity using this method of preparation failed. It was felt that since the effects on in vivo melanin production of in vivo variation of enzyme activity (assessed by in vitro assay) were being measured, the assay procedure should involve as few extraction steps as possible. For the same reason assays were performed at the 'physiological' pH of 7.4 although this may not be the pH optimum of the enzyme, nor the pH at which it works in vivo, since melanin synthesis occurs only where tyrosine hydroxylase activity is associated with melanosomes, whose membranes may

maintain a unique biochemical milieu. It is not known whether or not melanosome integrity was destroyed by homogenisation.

Tyrosine hydroxylase activity was found to be affected by alleles at both the b- and c-loci. The b-locus alleles \underline{b}^c and \underline{b} both increase V_{\max} when homozygous, but when heterozygous are completely recessive to \underline{B} . The c-locus alleles \underline{c}^{ch} , \underline{c}^e and \underline{c} all affect V_{\max} and K_M and also heat inactivation rates. A further indication that the c-locus could be the structural locus for the tyrosine hydroxylase molecule comes from the activity levels of heterozygotes which are in all cases very close to the parental mean (figs. 5a-f, facing p. 41). The c-locus alleles also affect the pattern of development of activity with age, however, a phenomenon which can be reconciled with heterozygote intermediacy and K_M changes in two ways: (i) alteration of the response of the mutant enzyme molecule to activating agents owing to changes in recognition sites, for example, and (ii) a different K_I for melanin. There is as yet no evidence that an activation process is involved in the development of tyrosine hydroxylase activity in the skin of the mouse. An investigation of the effects of MSH on tyrosine hydroxylase activity of the various mutants might yield some information on this question. On the other hand, a non-competitive inhibition relationship between skin melanin content and enzyme activity was demonstrated for aaBBCC (figs. 10c and 10d, facing p. 84). There is, of course, no reason why these two processes should be mutually exclusive, but if both are involved simultaneously the situation becomes complex for quantitative analysis.

It was also pointed out that the expressing of melanin content or enzyme activity measurements per unit weight of skin or skin

protein does not give a consistent estimation of the melanin production or enzyme activity of the follicles, since follicle density changes as the skin grows. Direct comparisons between the different genotypes at any one particular age are not affected, but if meaningful and accurate statements are to be made about changes with age in O.D. or enzyme activity, or if comparisons between O.D. and enzyme activity are not direct, as in the inhibition plot where the relationship between O.D. and $1/(\text{activity})$ is considered, then the terms in which these measurements are expressed must bear a direct relationship to the situation in the follicle itself. The ideal solution would be to perform micro-assays on single isolated follicles, but another technically much simpler solution would be to use adult skin for assay. A new cycle of hair growth in already existing follicles can be initiated by plucking the hair: growth then proceeds in skin in which the follicle density remains constant. A more efficient method of homogenisation would be required for adult skin, however.

The relationship between melanin production and tyrosine hydroxylase activity is not a simple one: the rate of accumulation of melanin in the hair cannot be directly related to either the tyrosine hydroxylase activity of the skin, or, in the case of aaBBCC, its rate of decline in the skin (see table on p.78). At 4 or 5 days of age, however, when melanin is still in the follicle tissue, hair growth being in the very early stages, the relationship between tyrosine hydroxylase activity and melanin content has been found to be a non-linear one of the type predicted by the theoretically-derived equation of Kacser & Burns (p,88 & figs. 11a-d). Using the b-locus alleles b^c and b to obtain higher background levels of tyrosine

hydroxylase activity, c-locus alleles were seen to have less marked effects on phenotype although their proportional effects on enzyme activity did not change. This phenomenon is also in agreement with the predictions of the flux equation, where the effects of reducing enzyme activity are seen to depend on the initial magnitude of the $1/E$ term for that enzyme. Substitution at the b-locus of alleles which raise enzyme activity and thereby reduce the magnitude of $1/E$ for tyrosine hydroxylase would be expected to render the proportional reductions of activity superimposed by the lower c-locus alleles less effective on flux. The phenotypic dominance relationships of the c-locus alleles when in combination with the different b-locus alleles (see fig. 2) are thus explained as a natural consequence of the responses of the underlying biosynthetic pathway to the changes in tyrosine hydroxylase activity caused by the alleles themselves.

The use of multiple allelic series as a means of varying in vivo enzyme activity has the disadvantage that structurally different enzyme molecules will be involved if the alleles are at the structural locus of the enzyme. It has been seen in the present study that the c-locus alleles affected both K_M for tyrosine of tyrosine hydroxylase and the pattern of development of tyrosine hydroxylase activity with age. It was very fortunate that for this locus a triploid dosage of the c allele could be obtained by using Cattanach's translocation. Enzyme activity was found to be still proportional to gene dose and thus it can be concluded that there is at these levels of activity no mechanism to regulate the limits of the activity. If the translocation were to be bred into aab^cb^c and aabb backgrounds a considerably wider range of tyrosine hydroxylase activity is potentially obtainable, and it might therefore be

instructive to do this to see whether the linear relationship between tyrosine hydroxylase activity and gene dose obtains at these higher levels of activity.

Phenylalanine and skin pool measurements failed to show any relationship between pool size and tyrosine hydroxylase activity (tables 11a,b,c pp. 50,51; fig. 8). Tyrosine aminotransferase activities are also so constant (table 12,p.52) that it appears unlikely that skin pool levels are maintained by deamination of excess tyrosine. The apparent insensitivity of pool levels to tyrosine hydroxylase activity may be a reflection of the organisation and distribution of the pools within the melanocyte and their availability to the enzyme.

Another interesting point arising from this investigation is the demonstration that the tyrosine hydroxylase of the liver, which is not concerned in the production of melanin, has a different K_M for tyrosine and that its level of activity bears no relationship to genotype at the c-locus or the b-locus. It was suggested that the residual activity of albino could be due to a low level of expression of the liver-type enzyme as no detectable melanin is produced. Extraction and purification of the enzyme molecules from both sources might indicate whether the failure of the liver to produce melanin is due to an inhibition of the dopa oxidase activity of the tyrosine hydroxylase molecule or whether dopa oxidase activity is associated only with the skin enzyme molecule.

Although this first attempt at a quantitative investigation of a metabolic pathway in a higher organism has been restricted by the structural complexity of the system chosen, it has been seen that the effects on the end-product of the pathway of in vivo variation

of the activity of one of the enzymes involved follow those predicted by the theoretical model. In addition, the study of these quantitative aspects of melanogenesis has provided an explanation of the phenotypic effects of the c-locus alleles in their various combinations and also their interaction with those of the b-locus.

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