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PHENYLKETONURIA

An overall discussion of the disease and, in particular, examination of the value of the "Guthrie Microbial Inhibition Assay" diagnostic technique.

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Preamble.

Until the advent of easily performed, accurate and economic methods for detection of phenylketonuria, this disease was thought to be somewhat rare. Estimates of its frequency have varied between 2 to 6 per 100,000 to 10 per 100,000, and in order to determine the true incidence of the disease in Scotland complete neonatal screening would be necessary over a period of years. Such a project not proving feasible at this juncture, routine screening of all neonates born within Stebbill and Robroyton Hospitals and Redlands Hospital for Women, Glasgow, has been performed over a period of one year. A further programme of screening has been undertaken for all children in Special Schools and children and mentally retarded adults in Occupational Centres, where such are administered by the Corporation of the City of Glasgow. Surveys have also been undertaken in Lennox Castle Hospital, Lennoxtown, and the Royal Scottish National Institution, Larbert. In all the screening programmes, the "Guthrie Microbial Inhibition Assay" technique has been employed.

The aims of the various screening programmes have been:-

1. to determine if utilisation of this technique was feasible in discovering neonatal cases of the disease;
2. to determine if use of the technique could discover any hitherto-unsuspected cases of the disease;
3. to determine if the particular technique possessed any significant advantages over conventional methods of screening employing urinalysis;
4. to determine if it were possible to demonstrate the existence of regional frequency-variation of the disease.

Definition.

Phenylketonuria is a disease resulting from an inborn defect in the metabolism of the amino acid, phenylalanine, causing the development of mental retardation in the majority of untreated cases.

## Historical Background.

With the discovery of the original paper on inheritance in peas, the foremost British exponent of the Mendelian theory very quickly became W.M. Bateson who, in conjunction with many of his pupils, published a mass of work extending Mendelism to man and mammals. In addition to such publications he suggested certain lines of investigation to be followed by others. Among such was his friend Garrod (later Sir Archibald Garrod), to whom he made the suggestion that the disease, alcaptonuria, might be inherited as a simple Mendelian autosomal recessive trait. Garrod investigated this subject and, in the Croonian Lecture of 1908 delivered before the Royal College of Physicians, suggested the existence of a group of diseases caused by inborn metabolic defects. In 1909 he published the lecture under the title of "Inborn Errors of Metabolism" and discussed four such metabolic disorders: albinism, alcaptonuria, cystinuria and pentosuria. The correlating features of those diseases were, he pointed out:-

1. onset in the early days or weeks of life;
2. a pronounced familial tendency;
3. their relative benignity and compatibility with an approximately normal life-expectancy;
3. their occurrence with increasing frequency in the children of consanguineous marriages.

Garrod furthermore expounded his firmly-held belief to the effect that many more such diseases existed and would eventually be diagnosed. From such a prophesy, the state of affairs has now been reached when it has been stated by Stevens and Heber (1964) /

that almost ninety such diseases have been demonstrated.

Phenylketonuria was first described by the Norwegian physiologist, Dr. Asbjørn Følling (1934). Two retarded children were brought to Følling by their mother after she had consulted numerous other doctors regarding their mental and physical states and also regarding an odour which appeared peculiar to them. At birth, both children were apparently quite normal but had shown evidence of mental defect within several months. Furthermore, at about the age of six weeks, each had developed this noticeably-pronounced and unpleasant smell which was so evident that their father could not be in their presence for more than a very short period without developing an asthmatic attack (Centerwall and Centerwall, 1961). Følling noted that the urine from both children became green in colour when a solution of ferric chloride was added to it. Investigation of other somewhat clinically-similar patients in nearby institutions for the mentally retarded uncovered, within a period of five months, a further eight in whom a similar urinary reaction to ferric chloride was present. Of those ten cases, all of whom showed a ferric chloride urine-positive reaction, from a genetic standpoint it was outstanding that six were from three sibships. To the condition Følling gave the name "Imbecillitas Phenylpyruvica", having discovered that the substance present in the urine and causing the development of the green colouration with ferric chloride was phenylpyruvic acid, a phenyl ketone ( $C_6H_5-CH_2-CO-COOH$ ). In urine from unaffected subjects, he found this substance to be present only in insignificant amount.

Penrose (1935) and Jervis (1937; 1939) determined the inheritance of the disease and respectively suggested the adoption of the terms "Phenylketonuria" and Phenylpyruvic Oligophrenia" /

in its description. The usage of Fölling's original designation has now been discarded. Penrose initially suggested use of the term "Phenylpyruvic Amentia", but both this and the "Phenylpyruvic Oligophrenia" of Jervis have been rendered outmoded by more-or-less universal adoption of the short and succinct term, phenylketonuria.

Usage of the term phenylketonuria, while correctly referring the disorder to its classification of an inborn metabolic disorder denotes, by inference, diagnosis by urinalysis for detection of the phenyl ketone. It will be subsequently shown that it is not necessary for a person suffering from the disease to excrete phenylpyruvic acid in the urine. Basically, the criterion to be adopted for the diagnosis of the disease is the demonstration of an excess of phenylalanine in the blood, either with or without the presence of urinary phenylpyruvic acid in readily detectable amount. Adoption of such a criterion would semantically justify the adoption of the term "Hyperphenylalanaemia" but this will not be advocated. For purposes of convenience and brevity, that proposed by Guthrie (1961) is to be preferred, namely, "P.K.U.". Wherever this term is subsequently used in the text it will be held to denote the disease, phenylketonuria, or the adjective, phenylketonuric.

In spite of the discarding of Fölling's descriptive term, it must be clearly noted, as has been pointed out by Centerwall and Centerwall, 1961, that his work on the nature of the disease represented the first demonstration of a mental defect resulting from a basic biochemical disorder. Not only that, as they also pointed out, in collaboration with Norwegian colleagues, some of his other accomplishments included his being the first to devise a method for the determination of phenylalanine levels in serum and thus show that those levels are elevated in patients suffering /

from P.K.U., and also his being the first to demonstrate the increased urinary excretion of certain metabolites such as phenyllactic acid and of phenylalanine itself, in sufferers from the disease. Yet, in spite of the far-reaching pioneering studies undertaken by Fölling, more than thirty years afterwards the true reason for the development of the mental retardation has not been elicited.

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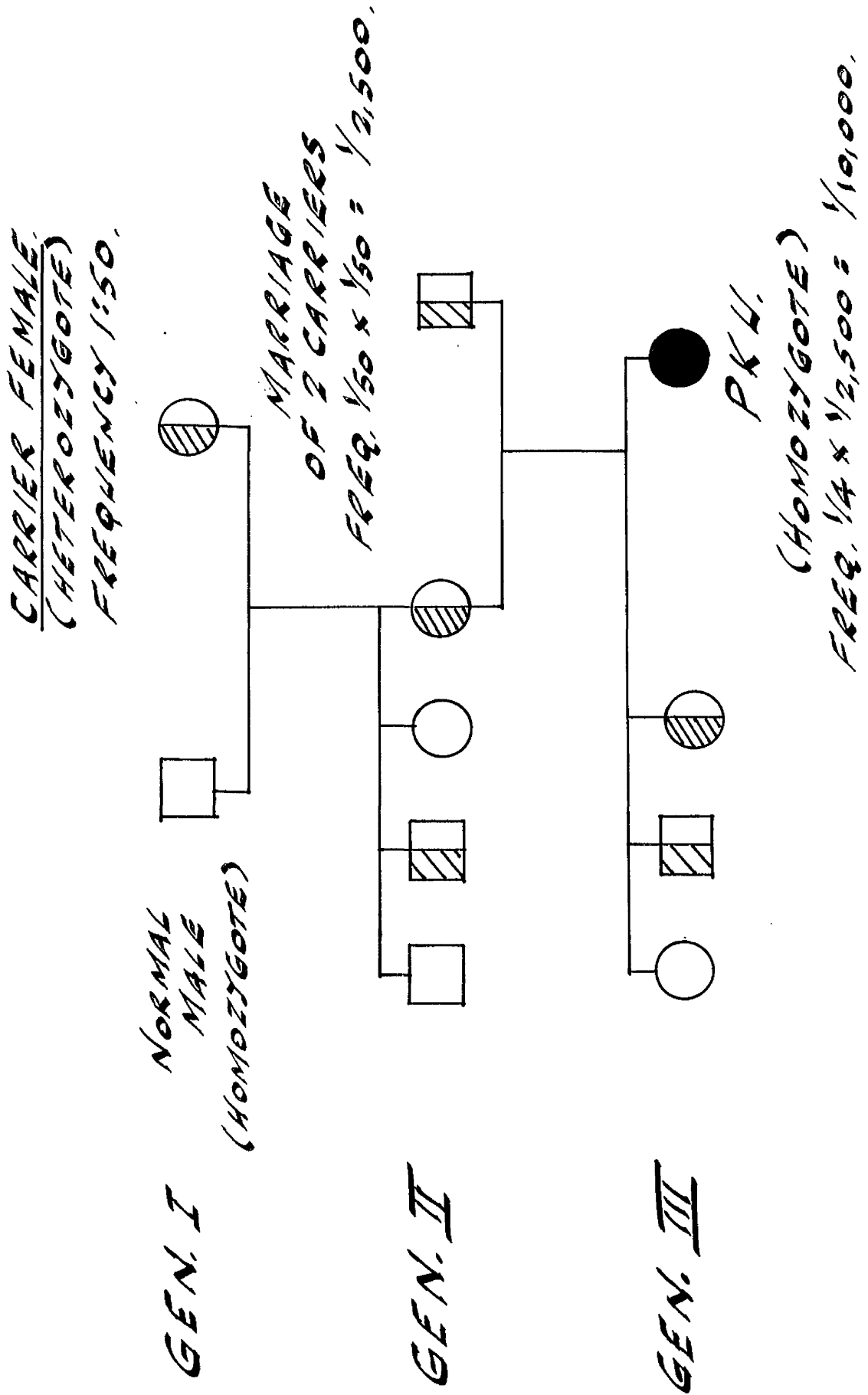
## Genetics and Incidence.

Phenylketonuria is an hereditary disease, known only to occur in man (Woolf, 1962), affecting both sexes equally and transmitted by an autosomal recessive gene (Penrose, 1935). Till quite recently it had been thought that this gene occurred in about 1 in 70 persons of European stock (Guthrie, 1961), but recent advances in methods of diagnosis of the disease coupled with the screening of sufficiently large numbers of infants have shown that this ratio is, in certain areas, at present estimated to be in the region of 1 in 50 (Guthrie and Whitney, 1964; MacCreedy, 1964, 1965; Carson, 1965). Since the chance of marriage between two carriers of the responsible gene - assuming such a heterozygote-incidence for P.K.U. to be 1 in 50 - will be 50 times 50, or 1 in 2,500, and since, theoretically, 1 out of every 4 children born of such a marriage will have homozygous P.K.U., it follows that the disease should occur approximately once in every 10,000 births in such populations. These calculations, together with a typical inheritance-pattern, are shown in Figure 1. Jarvis (1939) studied 200 cases of P.K.U. and concluded from the data which he obtained that the transmission of the disease was, on a quantitative basis, in accordance with the theory of monomeric recessivity, by establishing:-

1. that the ratio of affected to normal sibs in families with normal parents was 1 to 4 when the necessary statistical corrections had been applied; /

Figure 1.

(Modified from Guthrie, 1961)



- 9.
2. that the rate of consanguinity among the parents of affected individuals was significantly higher than normal;
  3. that the distribution of the character among the ascendant and collateral relatives followed the rules for monomeric recessivity.

Until Guthrie (1961) introduced his microbiological method for estimation of serum phenylalanine in detection of the disease on a large scale, methods based on the use of ferric chloride in urinalysis had been employed. It would be but natural that such surveys would be conducted in countries possessing a sophisticated culture and, for this reason, populations of European origin would be primarily those to have been investigated. It follows from this that the widely-held belief of the disease being more common in populations of European origin (Jarvis, 1954; Knox, 1960) could be fallacious. The figure of 2 to 6 P.K.U. patients per 100,000 advanced by Jarvis (1954) and Knox (1960) would, in the light of evidence submitted by Guthrie and Whitney (1964) and MacCreedy (1964; 1965), appear to be too low. Jarvis (1954) had organised a comprehensive survey of institutions for the mentally retarded on a world-wide basis and reported that 312 out of 48,536 patients (0.64 per cent) suffered from P.K.U. Accepting this figure as representative of the incidence of P.K.U. in the defective population and taking the figure of 1 per cent as being representative of the incidence of defectives in the general population, the incidence of P.K.U. in the general population was assumed to be in the region of 6 per 100,000. Again it must be emphasised that these figures, in consequence of populations of European origin having primarily been those from whom the data /

10.  
was obtained, refer to P.K.U. incidence in such populations. Previous estimates of the incidence of the disease made by Penrose (1946) and Munro (1947) with regard to the populations of England and Sweden, had suggested that in those countries it was between 2 and 3.5 per 100,000. With the realization that estimates of incidence of the disease based upon institutional sampling might be fraught with inaccuracies consequent upon socio-economic and other factors varying the rate of institutionalisation, it became apparent that there was a need for direct case-detection outwith such establishments. In consequence, detection-programmes utilising ferric chloride urinalysis were initiated and directed primarily towards neonate and child populations. With the introduction of such programmes it became evident that the P.K.U. homozygote-incidence for the areas in which they were conducted was in the region of 1 in 20,000 or thereabouts.

Such surveys were primarily initiated in Great Britain by various Local Health Authorities and, in the U.S.A., by States' Public Health Departments. In Great Britain, after the value inherent in initial pilot-surveys had quickly become apparent, the Medical Research Council set up its sub-committee on phenylketonuria in 1960. The stimulus provided by this body was such that within three years 87 per cent of Local Health Authorities were co-operating in neonatal screening programmes for the early detection of P.K.U. In the U.S.A., surveys of a similar sort have been conducted in many States. (The bibliography will detail a representative selection from both countries.) In addition to such large-scale screenings of neonates in Great Britain and the U.S.A., various other investigations into the incidence of the disease in institutions for the mentally retarded have been/

undertaken. Again it is not proposed that all such investigations should be detailed; the investigation undertaken by Mellon (1961) may be taken as typical and his findings noted. His investigation took place in Scotland and encompassed 828 defectives in mental deficiency hospitals and pupils at a special school and an occupational centre. Using "FRIENISTIX" test-strips (Ames Co.), he confirmed the diagnosis of 4 known cases and discovered a further 11 hitherto-unknown and subsequently-confirmed cases. In the conducting of this and other surveys, many problems, such as finding of false-positive results, became apparent and highlighted the need for a more accurate and fool-proof method of detection. As an illustration of one such problem, the experience of Farquhar et al. (1962) will be quoted.

In Edinburgh, beginning in 1960, routine screening for P.K.U. was carried out on 98.2 per cent of all neonates at the age of 6 weeks. The method initially employed was that whereby "FRIENISTIX" test-strips were pressed between the folds of a urine-impregnated napkin. No new cases were reported as a direct result of this screening. The only new case found, the younger sibling of a known P.K.U. child, had had a negative napkin test at the age of 6 weeks and again at the age of 8 months, by which time the older sibling had been diagnosed as having P.K.U. However, the testing of freshly-voided urine gave a positive result for the younger child at the age of 8 months with subsequent confirmation of the diagnosis of P.K.U. by demonstration of an abnormally high level of phenylalanine in the serum. Thus, in spite of all the screening which had been done, this case had escaped detection on two separate occasions of testing. Such a matter was, of itself serious, but when taken in conjunction with the average number/

of live-births per annum in Edinburgh and the then-accepted incidence of P.K.U. in a large British city (Boyd, 1961), it became apparent that, on statistical grounds, this child was the only P.K.U. homozygote who could have been born in Edinburgh in this two and one-half year period.

Guthrie (1961), using his own newly-developed technique, demonstrated an incidence for homozygous P.K.U. of 1 in 20,000 for part of New York State. From his findings in respect of this pilot-study, it appeared that there was no significant difference in the validity of results determined either by ferric chloride urinalysis or by microbiological estimation of serum phenylalanine.

Different conclusions, of great significance, were published by Guthrie and Whitney in 1964. With the co-operation of 29 States of the U.S.A., screening of 400,000 neonates had been undertaken utilising Guthrie's own method of detection and from this total there emerged 39 positive cases, all of whom were subsequently confirmed by biochemical methods. This finding led to postulation of a P.K.U. homozygote-incidence of 1 in 10,000 for the populations concerned and a heterozygote-frequency of 1 in 50, and this doubling of the previously-accepted incidence of the disease showed conclusively that formerly-used methods of detection had been inadequate and that at least one-half of detectable homozygous P.K.U. neonates were escaping such detection and presumably becoming the victims of irreversible mental retardation. The results obtained by Guthrie and Whitney showed that the Guthrie method was accurate and suitable for large-scale use. Utilising Guthrie's technique, MacCreedy (1965) has found 30 confirmed-positive cases in a total of 255,000 neonates, as a result of a mandatory screening programme for such neonates introduced by the/

State Legislature of Massachusetts. This result shows a P.K.U. homozygote-incidence of 1 in 8,500 of all births in this State and a calculated heterozygote-frequency of 1 in 46 for the population of the State.

The latest survey to be published is that of Carson (1965), to determine the incidence of P.K.U. among the mentally retarded population in part of Great Britain. By examination of the urine and/or serum for phenylalanine and other metabolites associated with the disorder, she discovered 69 persons suffering from the disease among 2,920 mentally retarded individuals in Northern Ireland. Such an incidence gives a prevalence of P.K.U. among the mentally retarded persons examined, of 2.4 per cent. She pointed out that, since only 55 per cent of the individuals known to be mentally retarded had been examined at the time of publication of the survey, such an incidence must not be accepted as the final one. Indeed, utilising known facts and prospective deduction, she postulated an incidence for P.K.U. in Northern Ireland in the region of 1 in 10,000. When taken in conjunction with the findings published by Guthrie and Whitney and also those published by MacCreedy, it thus seems that the incidence of the disease in the U.S.A. and Northern Ireland is closely similar.

The reported rarity of the disease among Jews (Jervis, 1954) was based on the absence of the condition among the children of Jews in the U.S.A. and the finding of only one previously-reported case in the literature, by Cohen and Kozinn (1949). Centerwall and Neff (1961) reported a family case-history in which 2 out of 3 Jewish children had P.K.U. and, between them, illustrated many of the frequently-encountered features of the disease. This report was, they thought, only the second reported instance in the/

World's medical literature in which one or both parents of P.K.U. children were Jewish. They reported that this Jewish mother and her relatives were proud of their racial background and believed they represented a pure Jewish strain. Nevertheless, Centerwall and Neff suggested that the possibility of some outside contamination of the Semitic strain could not be ruled out, since the mother herself had blonde hair, fair complexion and blue eyes.

Very shortly before Centerwall and Neff had published their findings, Cohen et al. (1961) had intimated a preliminary announcement to the effect that they had discovered many more Jewish children suffering from P.K.U. With different associates, Cohen published full details in 1962 to the effect that a survey of 1,000 children in institutions for the mentally retarded in Israel had disclosed the existence of 10 cases of P.K.U. among Jewish families of non-Ashkenazi origin, including two families from Yemen and one each from Iraq, Iran and Afghanistan. None were found among patients of Ashkenazi Jewish heritage. The total population of these institutions consisted of approximately equal proportions of Ashkenazi and non-Ashkenazi children. A frequency for P.K.U. of 1.0 per cent for non-Ashkenazi children was thus shown to exist in such institutions in Israel; a frequency, at that time, closely-comparable to the accepted frequency for P.K.U. children in British and American institutions of a similar sort. Cohen noted that pigment-defect was not necessarily a constant feature of the disease in the non-Ashkenazi Jews and its absence in the Ashkenazim could be explained by the occurrence of consanguineous marriages contrary to Mosaic Law among the "oriental" Jews or by a basic difference in gene-frequency between the two groups. He ascribed the reports on the rarity of the condition among Jews in /

Europe and the U.S.A. to the fact that the Ashkenazim comprise the majority of Jews in those continents. In this connection it thus becomes probable that Ashkenazi heritage is involved in the family reported by Centerwall and Neff.

Salam (1963) put forward the suggestion that the relative absence of reported cases of P.K.U. from among the indigenous population of the Middle East was perhaps due to lack of suitable screening programmes rather than to major differences in the geographical distribution of the disease. She found an Armenian child suffering from P.K.U. during routine screening of children with mental defect, utilising ferric chloride urinalysis, after only 202 such children had been screened. She subsequently found 3 additional cases among 60 inmates of an institution for retarded children - 2 Arab and 1 other Armenian. The disease has also been reported among Indians (Chatterjee, 1964), Chinese (Wu, 1964; Tu, 1964), Japanese (Fujiki et al., 1961; Tanaka et al., 1961), American Negroes (Engel, 1964; Katz, 1964) and a pure-blooded Ojibwa Red Indian (Partington, 1961). It would thus appear probable that the introduction of such simple and efficient diagnostic methods of population-screening, as is instanced by the Guthrie method, together with the enthusiastic prosecution of such screenings, will eventually demonstrate that no ethnic group is truly free from the disease.

The possibility of the existence of regional frequency-variation in the incidence of the disease in Great Britain was investigated by Carter and Woolf (1961). They compared the birth-places of parents and grandparents of a control series of patients. The P.K.U. patients numbered 38, the controls 303 and the index patients in each series were restricted to those referred to the/

Hospital for Sick Children, London, between 1949 and 1959 and resident in south-east England. The control series comprised cases of Hirschprung's disease, congenital dislocation of the hip and coeliac disease. The findings showed a high proportion of the parents and grandparents of the P.K.U. children to have been born in west-Scotland and Ireland. The frequencies of P.K.U. children with grandparents born in west-Scotland and Ireland, relative to the control patients, suggested that P.K.U. gene-frequency was about four times as high in the population of those parts of The British Isles as in the population of south-east England. A similar mapping of parents and grandparents of patients with P.K.U. was undertaken by Larson (1964) which seemed to reveal regional differences within the supposedly more homogenous population of south Sweden. He was able to demonstrate that there is an increased frequency for homozygous P.K.U. in districts of Sweden bordering upon Norway and also distributed on both sides of the ancient border between Sweden and Denmark. He also suggested, somewhat tentatively, that demographic data revealing a relatively low population-mobility, taken in conjunction with actually-observed consanguineous marriages among parents of affected patients, could be called upon to support the hypothesis that the inhabitants of these regions keep at some degree of volitional inbreeding. The question of such frequency-variation in Denmark may also be raised by perusal of a report by Lund and Wamberg (1964). This report, inter alia, noted the distribution of P.K.U. patients in 11 Danish Local Government Centres for the mentally retarded. Such centres receive patients only from the area in which they are established and, although attention was not drawn specifically to the fact, study of the data demonstrates that even in the small country of /

Denmark the possibility of such frequency-variation must be considered, it being readily apparent that there are indeed marked variations in the numbers of P.K.U. patients in the various Centres. Verification of this suggestion would necessitate investigation along the lines followed by Larson (1964) before it could be seriously considered.

One further genetic aspect which has received consideration is that first proposed by Penrose (1945) and Munro (1947) to the effect that there existed the possibility of a linkage mechanism between the ABO blood group locus and the P.K.U. locus. It had been suggested by both that such a loose linkage mechanism might exist, but Penrose (1951) in further investigations stated that evidence to this effect was inconclusive and that he could advance no evidence for linkage of the ABO and MN blood group antigen loci and the P.K.U. locus. <sup>e</sup>Renwick et al. (1960) and Hsia and Steinberg (1960) subjected the initial postulate to further investigation and both arrived at conclusions similar to those reached by Penrose in 1951. Hsia and Steinberg stated that, in P.K.U., "linkage with ABO, Rh or MNS as close as .1 is excluded" and that "linkage with K of .05 or less is excluded". They further noted that their data was insufficient to warrant putting forward any additional conclusions. It would thus appear that no evidence exists to demonstrate any linkage mechanism between the blood group loci and the P.K.U. locus.

Metabolic and Biochemical Aspects and Pathogenesis.

Fölling, in 1934, demonstrated the presence of phenylpyruvic acid in the urine of patients suffering from phenylketonuria. Since then, clarification of all the biochemical aspects of the underlying metabolic defect has not been accomplished speedily or yet with certainty. Bloek et al. (1940) found no significant difference in amino acid composition of the proteins of the blood and tissues in normal persons and P.K.U. patients. In the same year however, Jervis et al. (1940) were able to demonstrate an increase in the level of phenylalanine in the blood of P.K.U. patients after the ingestion of proteins, phenylalanine, phenylpyruvic acid and phenyllactic acid. They also demonstrated that ingestion of phenylalanine produced an increase in the amount of phenylalanine in the C.S.F. of such patients. Possibly of greater significance was the determination, on a quantitative basis, of phenylalanine and phenylpyruvic acid in the blood of 16 P.K.U. patients, showing phenylalanine content to be from 15 to 41 mg. per 100 ml. and phenylpyruvic acid to be absent. Jervis continued to investigate in this particular field and, in 1947, published findings in support of the hypothesis that the basic error responsible for the development of P.K.U. was a blockage in the conversion of phenylalanine to tyrosine. He showed that normal individuals, after the ingestion of phenylalanine, exhibited a temporary increase in blood levels of tyrosine and tyrosine-like substances; such was not demonstrable in patients suffering from P.K.U. In his unremitting pursuit of the unsolved biochemical problems Jervis (1950; 1952) noted that administration of a high-protein diet, phenylalanine or phenylpyruvic acid to P.K.U. patients caused an increased daily output of phenyl compounds and also, in a /

somewhat different context, that there appeared to be no correlation between the content of phenylalanine, phenylpyruvic acid and phenyllactic acid in the blood of such patients and their level of intelligence. In 1953 he contributed significantly to basic knowledge of the enzyme-defect responsible, by studying livers from normal non-P.K.U. persons and P.K.U. patients. He demonstrated, by colorimetric methods, that extracts from normal livers always brought about a significant degree of conversion of phenylalanine to tyrosine but that similar extracts from livers of P.K.U. patients failed to accomplish this conversion. From this, he concluded that a specific enzyme which would normally facilitate this conversion was absent from, or lacking in, the livers of P.K.U. patients. The demonstration of the absence, or lack, of such a specific enzyme, he postulated, clarified the observations that the inheritance of P.K.U. is determined by the transmission of a single recessive gene. He published a review of the subject in the following year (1954) and noted that two other theories had already been suggested as being responsible for the development of the condition. Those were, he stated, that:-

1. a block in phenylalanine metabolism at the stage of phenylpyruvic acid led to excretion of the unmetabolised acid;
2. an abnormal racemisation of phenylalanine took place, with the L-form (naturally occurring) being converted to the D-form by the patient suffering from P.K.U., then undergoing decamination by the kidneys and being excreted as phenylpyruvic acid.

Nevertheless, Jervis felt that his own hypothesis of single enzyme defect was the correct explanation. Subsequent developments in the main have upheld his theory and it is now accepted (LaDu, 1962)/

that the failure of P.K.U. patients to oxidise phenylalanine to tyrosine is due to diminished activity of the enzyme, phenylalanine hydroxylase, first isolated by Wallace et al. (1957) and Mitoma et al. (1957). This enzyme has been shown by Posner et al. (1961) to be soluble, i.e. nonmicrosomal.

As has been stated by Knox and Hsia (1957), it has not yet been clarified whether there is actually a diminished amount of enzyme in patients with the disease or if an abnormal form of the enzyme is present. Either of those conditions could result in diminished catalysis of the oxidative reaction of phenylalanine to tyrosine and lead to a high level of blood phenylalanine and a low level of tyrosine. Although Jervis must be given pride-of-place in the earlier studies to clarify the reason for the defect, many others have contributed significantly to overall knowledge of the problem.

In company with various associates, Armstrong (1954; 1955; 1956; 1957; 1958) investigated many aspects of the problem. He investigated the excretion of indole derivatives in P.K.U. and identified indoleacetic acid as the indole derivative present in greatest amount in P.K.U. urine; determined that P.K.U. patients excrete 100 to 400 per cent of the amount of orthohydroxyphenylacetic acid excreted by normal subjects, but could not relate this in any direct way to the occurrence of the mental defect, nor could he detect any abnormality in the metabolism of orthotyrosine - the probable precursor of orthohydroxyphenylacetic acid - in P.K.U. patients. He showed, in a newborn infant who subsequently developed the disease, that cord blood at birth showed a normal phenylalanine level which rose to 62 mg. per 100 ml. at 24 days after birth, with phenylpyruvic acid only appearing in the urine /

at 34 days, and also that phenylpyruvic acid excretion by P.K.U. patients is roughly proportional to fasting levels of blood phenylalanine. Furthermore, he demonstrated phenylpyruvic acid only to be detectable in urine when P.K.U. patients have blood phenylalanine levels in excess of 15 mg. per 100 ml. and that infants fed with artificial milks developed high levels of blood phenylalanine more rapidly than those who were breast-fed. From this last study he suggested that newborn sibs of known P.K.U. patients should be kept on a low-protein diet till blood phenylalanine levels could be estimated for positive diagnostic purposes. This particular study was one primarily initiated to observe the development of biochemical abnormalities in newborn P.K.U. infants in order to learn whether or not there were differences in the times of development of such abnormalities and also what factors might be incriminated as being responsible for such differences in timing. His findings supported the hypothesis that there may be marked differences in times of development and appearance of various biochemical abnormalities in different P.K.U. patients. Differences in time of appearance of phenylpyruvic acid, he felt, might be of especial significance, there being some indication that patients with atypical high mental ability might excrete less phenylpyruvic acid than usual. Should increased production of this acid be directly concerned with the damage to the C.N.S. which occurs in the majority of infants with P.K.U., production of it in large amounts by some infants at about one week of age but not until five weeks or later by others, might result in a great difference in the brain damage suffered in each of the respective cases. He concluded that differences in timing of development of biochemical abnormality finally established, might lead to great /

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differences in neurological damage and help to explain the lack of correlation between biochemical abnormality and mental ability often observed in older children suffering from P.K.U. With a more comprehensive knowledge of the biochemistry of the disease having become available, it is not now felt that this particular line of reasoning is truly valid.

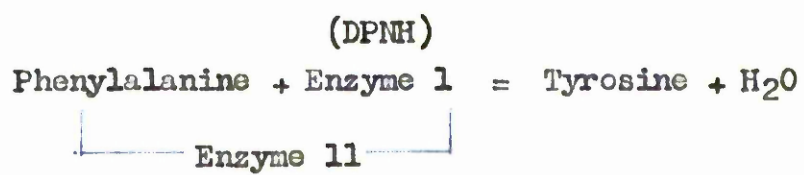
Berek et al. (1950) also demonstrated, in an investigation into the constancy of the metabolic error in P.K.U., that there was no correlation between the degree of mental deficiency and the concentration of phenylalanine in serum. Studying 18 patients, they showed that phenylalanine concentration in the C.S.F. varied between 6.1 and 8.2 mg. per 100 ml. They postulated that, although increased amount of phenylalanine in the blood results from metabolic error, its level in the serum is determined by the efficiency of the kidney tubules to reabsorb the amino acid. In this connection, recent work (Guthrie, 1965) has demonstrated that tubular reabsorption may be so efficient that even in cases of P.K.U. where serum phenylalanine levels may be in excess of 20 mg. per 100 ml., no phenylalanine is detectable in the urine.

An important finding was announced by Udenfriend and Cooper (1953) to the effect that a feasible procedure existed whereby phenylethylamine could be obtained enzymatically from amino acid mixtures containing phenylalanine, in sufficient quantity and purity for its radioactive measurement. Utilising this method, Udenfriend and Bessman (1953) were able to prove that hydroxylation of L-phenylalanine to tyrosine does take place in P.K.U., albeit to a limited extent. This suggested that absence of the enzyme from the livers of P.K.U. patients was not absolute or, alternatively but somewhat unlikely, that other pathways for the metabolism of /

25.  
phenylalanine existed which did not depend on the presence of this particular enzyme. A further suggestion was made by Hsia et al. (1956) who applied Pauling's concept of "molecular disease" to P.K.U., postulating a one-to-one gene-enzyme activity relationship, with part of the enzyme being present in an inactive form in the heterozygote with one abnormal gene and almost all of the enzyme being inactive in a homozygous patient possessing two abnormal genes. Knox and Messinger, however, were able to demonstrate two years later (1958) that P.K.U. heterozygotes had elevated basal levels of blood phenylalanine. They noted that approximately the same degree of disturbance of phenylalanine metabolism was present in P.K.U. heterozygotes under both basal and loading conditions, illustrating this by the fact that the serum phenylalanine levels in these individuals were approximately 1.5 to 2.5 times that of normal subjects when both the heterozygote and normal levels were measured under the same conditions of fasting or phenylalanine loading.

In consequence of these findings, Knox and Messinger stated that, apparently "the enzyme-defect caused by a single (P.K.U.) gene results in the same degree of inefficiency of the reaction at both low and high substrate concentrations". They put forward the view that this finding negated Hsia's concept of heterozygotes having diminished enzyme-activity with no reserve available to degrade loading doses by calling upon a fraction of the enzyme not usually used. While the balance of probabilities would appear to favour the view advanced by Knox and Messinger, there can be no doubt that the state of confusion resulting from the apparent impossibility of reconciling this view with that advanced by Hsia et al. can only be resolved by further investigations to /

Outline of method of action of double-enzyme  
system in formation of tyrosine from  
phenylalanine.



(TPNH + O + Tetrahydropteridine)

Figure 2.

determine if alternative enzyme-system metabolic pathways for phenylalanine do exist and are actually operative in vivo.

The currently-held view on the metabolism of phenylalanine in the normal subject has been stated by Hsia (1964). He stated that the hydroxylation of phenylalanine to tyrosine requires not one, but two, enzymes. The first, enzyme I, is labile, can be prepared from rat liver and requires reduced diphosphopyridine nucleotide (DPNH); the second, enzyme II, is stable, can be obtained from sheep liver extracts and requires, in addition to oxygen, reduced triphosphopyridine nucleotide (TPNH) and any one of several tetrahydropteridines (Kaufman, 1958). The second enzyme is not involved in the hydroxylation reaction but merely catalyzes a reaction which keeps the coenzyme in an active form for hydroxylation to take place. Figure 2 gives an outline of the mode of action of the double-enzyme system. It is now known that enzyme I is the component lacking from the complete enzyme system in P.K.U. This has been shown by the fact that when enzyme II (cofactor) is added to the liver homogenate from a P.K.U. patient, there is little conversion of phenylalanine to tyrosine. On the other hand, if enzyme I is added, conversion occurs at a rapid rate (Mitoma et al., 1957; Wallace et al., 1957). All of the metabolic abnormalities in P.K.U. can be accounted for by an enzyme block at the phenylalanine to tyrosine level. Following upon the concept of phenylalanine hydroxylation advanced by Hsia, when there is a block in the hydroxylation at the phenylalanine to tyrosine level, increased amounts of phenylalanine persist in the tissues and allow the formation to take place, in significantly large quantities, of metabolites normally present in traces only. Such metabolites reach levels high enough to be excreted in /

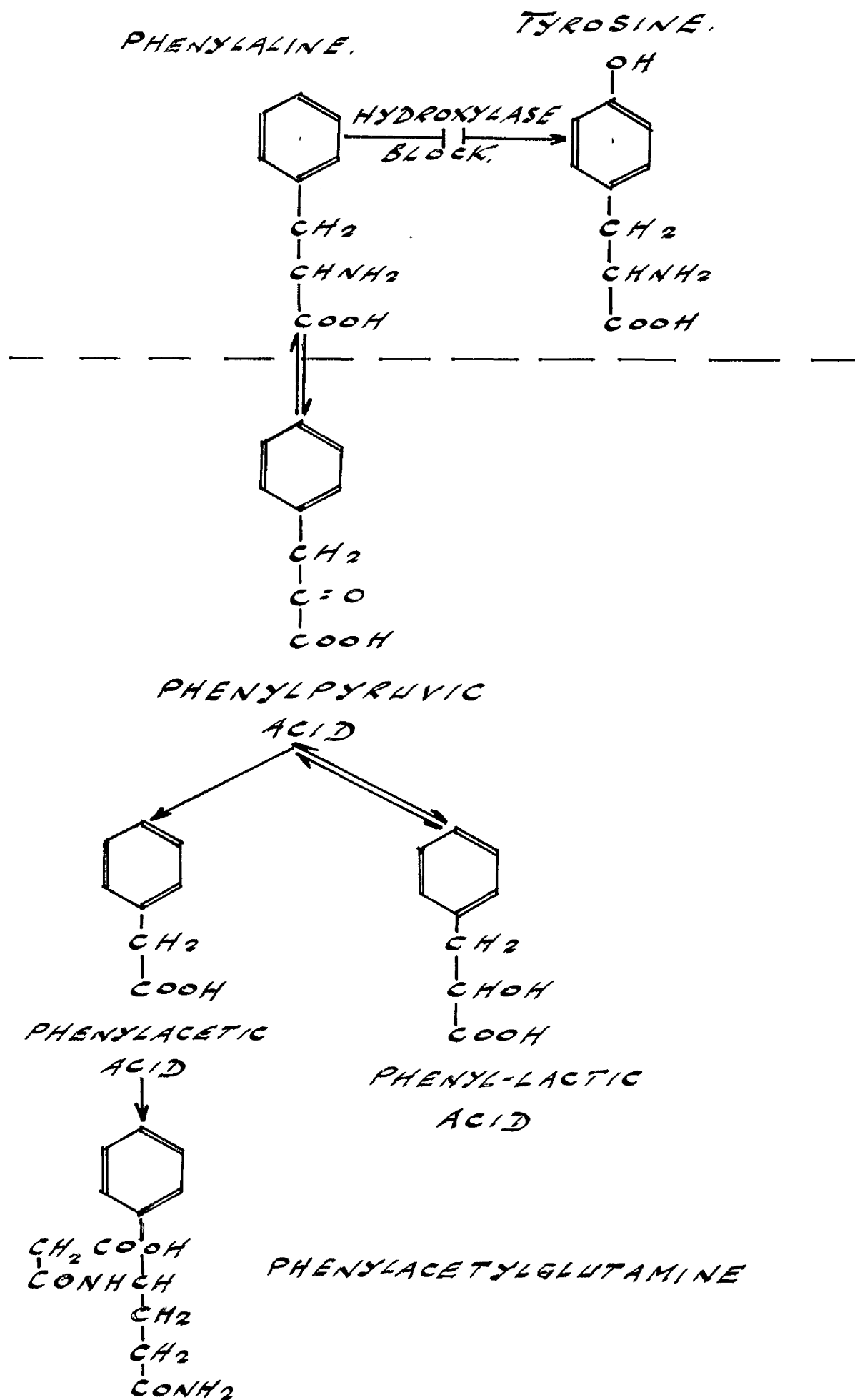
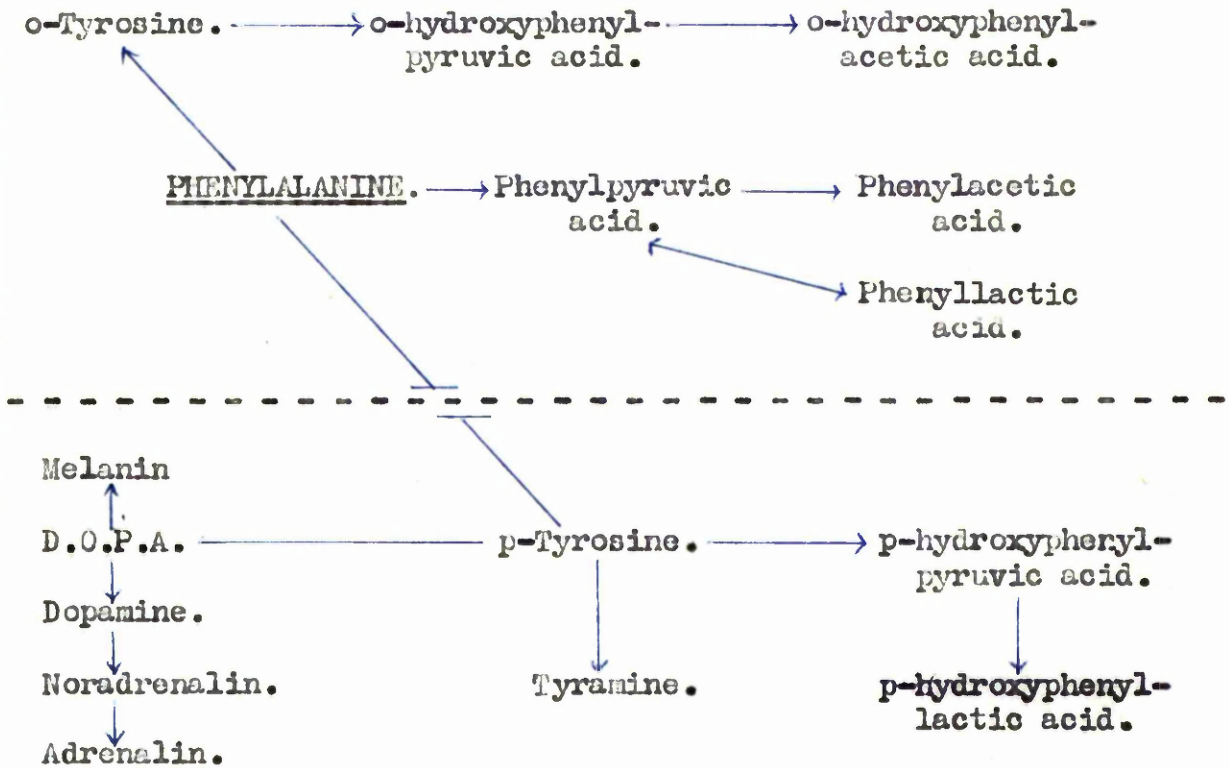


Figure 3. (From Larson, 1964).

measurable amount. In the subsequent degradation process, transamination by phenylalanine transaminase leads to the conversion of large amounts of phenylalanine to phenylpyruvic acid. Such phenylpyruvic acid is subsequently degraded to phenyllactic and phenylacetic acids. Excretion of such metabolites was stated by Udenfriend (1961) to take place via the urine when the serum phenylalanine reached 12 to 15 mg. per cent or higher (figure 3). Weisman et al., (1959) added 2.5 to 7 per cent L-phenylalanine to the diet of weanling rats or infant and adolescent monkeys and duplicated the characteristic aberrations found in the human P.K.U. sufferer, including the high plasma phenylalanine level, phenylketones in the urine and the unusual odour attributable to phenylacetic acid and phenylacetylglutamine.

While the above outline of the metabolic pathway involved in the hydroxylation of phenylalanine to tyrosine is, as stated, at present the currently held view, Harper (1963) detailed several aspects of the problem with particular reference to the ortho-hydroxy derivatives. He stated that normal processes in the metabolism of phenylalanine cause its early conversion to phenolic derivatives in which the hydroxyl group is added to the aromatic ring either in the para- or the ortho- positions. Normally the para- derivative is favoured, so that tyrosine (para-hydroxy-phenylalanine) is the major product of the hydroxylation. However, the finding of small but nonetheless significant amounts of ortho-hydroxy derivatives in the urine of normal subjects, and of very large amounts in the urine of P.K.U. patients (Armstrong and Shaw, 1955), suggested that both types of tyrosine could be formed by normal subjects. The P.K.U. patients are unable to produce the para- derivative, showing that the basic enzyme defect is the lack /

Metabolic Pathways of Phenylalanine.  
(in P.K.U.)



The dotted line denotes the hydroxylase-block in P.K.U.

All metabolites above the dotted line are increased in P.K.U. and all below are deficient.

Figure 4.

of such enzymatic activity for the para-oxidation of phenylalanine. Since this major pathway for the utilisation of phenylalanine is blocked, phenylalanine and some of its metabolites are diverted to the ortho- pathway, normally a very minor route. There is no impaired ability on the part of P.K.U. patients to metabolise ortho-hydroxyphenyl compounds; the excess amounts of those substances in the urine are directly attributable to their greater production in consequence of the underlying inability to produce para-hydroxy derivatives (figure 4).

Kaufman (1961) raised an interesting point with regard to the possibility of there being a free intermediate (e.g., an "activated phenylalanine") in the hydroxylation of phenylalanine to tyrosine, after it had been shown that the conversion required a double enzyme system. He postulated that this hypothetical intermediate could accumulate and, in abnormally high concentrations, behave as a toxic compound. Be that as it may, the hydroxylation reaction has not yet been separated into two steps. While further work may achieve such a separation, there still is no direct evidence that a free intermediate is formed in the reaction. In consequence of this, although the idea of such a toxic intermediate being operative in P.K.U. has a certain attraction, it has not been substantiated by any recent work on the mechanism of the reaction.

An earlier piece of research was that of Dancis and Balis (1955), who showed that, in vitro, L-phenylalanine partially inhibited the action of tyrosinase on tyrosine. They suggested that the competition of the two amino acids for the tyrosinase depended upon the similarity of structure between them. /

Metabolites of Phenylalanine in Plasma and Urine.

|                        | Plasma.<br>(mg. per 100 ml.) |           | Urine<br>(mg. per 100 ml.) |                 |
|------------------------|------------------------------|-----------|----------------------------|-----------------|
|                        | Normal.                      | P.K.U.    | Normal.                    | P.K.U.          |
| Phenylalanine.         | 1 - 4.                       | 7.5 - 7   | 30.                        | 300 -<br>1,000. |
| Phenylpyruvic acid.    |                              | 0.3 - 1.8 |                            | 300 -<br>2,000  |
| Phenyllactic acid.     |                              |           |                            | 290 -<br>550.   |
| Phenylacetic acid.     |                              |           |                            | Increased       |
| Phenylacetylglutamine. |                              |           | 200 -<br>300.              | 2,400.          |

Table 1. (Adapted from Harper, 1963).

Table 1, (adapted from Harper, 1963) shows the relative amounts of phenylalanine and some of its metabolites in the plasma and urine of normal individuals and P.K.U. patients. The excretion of phenylacetic acid in the urine takes place mainly in the form of phenylacetyl-glutamine and it is this substance which is responsible for the malodour in the disease (Jervis, 1954).

Tyrosine derivatives such as melanin and adrenalin are deficient and P.K.U. patients may thus show general diminution of pigmentation and have a low blood level of adrenalin. Figure 5 shows the sites of the various enzyme blocks in:- I. P.K.U.; II. Tyrosinosis; III. Alcaptonuria; IV. Albinism.

Knox (1960), in his masterly review of the subject, made two further points which are worthy of note, to the effect that:-

- 1. all metabolites are normal, although present in abnormal amount;
- 2. the disease, but not the primary defect, is acquired.

He pointed out that before and immediately after birth, all animals have the "phenylketonuric" defect in consequence of phenylalanine hydroxylase only appearing during the biochemical differentiation of the liver following birth; it is the failure to develop phenylalanine hydroxylase activity in P.K.U. which is hereditary.

Although it would appear that the specific metabolic defect involved in the determination of the disease may have been determined, the actuality of the pathogenetic mechanism responsible for the clinical manifestations of the disease is, as yet, only incompletely known. Such an incomplete knowledge raises several speculative queries regarding the pathogenesis of the disease. For example, could increase in the quantity of one or more normal metabolites, as pointed out by Knox, produce the mental retardation /

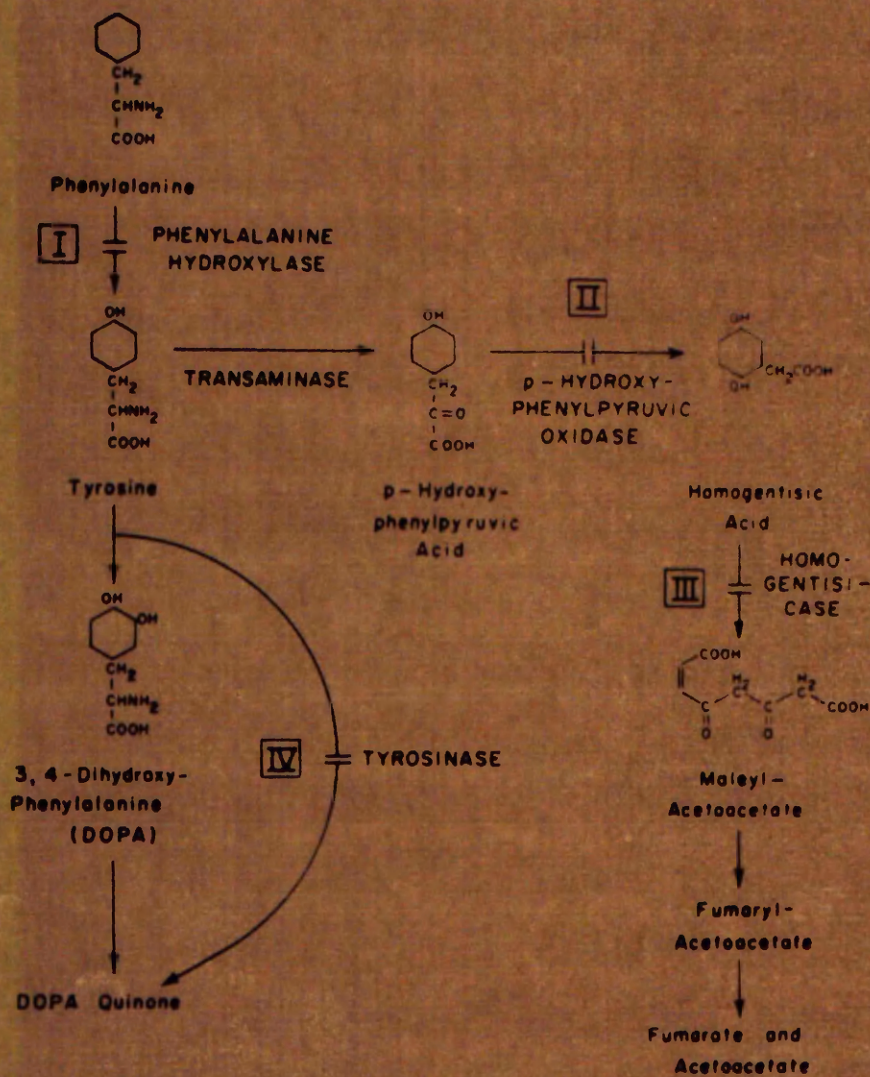


Figure 5.

(Modified from Hsia, 1964).

observed in the majority of P.K.U. patients?

The answer to this, at the present state of our knowledge, would appear to be reservedly affirmative. At a purely empirical level, the therapeutic effects of the early initiation and maintenance of phenylalanine-low dietaries favour the hypothesis that phenylalanine in substantially increased amount thwarts normal mental function. Nevertheless, as has been pointed out by Borek et al., (1950), Armstrong et al., (1958) and by many others, in the majority of cases of P.K.U., phenylalanine levels in serum do not parallel levels of intelligence. P.K.U. patients who display normal or near-normal intelligence, as instanced by Sutherland et al., (1960) and Partington (1962), and who concomitantly possess relatively low levels of serum phenylalanine, have been reported only on very few occasions. Such reports, by their very paucity, would appear tailored to fit the hypothesis that phenylalanine in amounts in excess of physiological levels does cause impairment of brain function. Whatever does happen to the brain would appear to be fairly final after the first year of life. If any conclusions may be drawn from the sparse and mainly negative observations of changed brain-structure in P.K.U., not much happens to the brain before birth. The results of initiation and maintenance of a phenylalanine-low dietary in the early weeks of life in P.K.U. patients indicate that this therapeutic measure, in the vast majority of cases, will keep brain function closely aligned to the normal.

Kenny et al., (1958) suggested that there was strong indirect evidence to suggest the exclusion of toxic action of phenylalanine prior to birth in consequence of the foetus lacking the hydroxylation mechanism which operates in the normal non-P.K.U. /

child. This suggestion, however, can no longer be upheld if Knox's statement (1960), to the effect that phenylalanine hydroxylase only appears for the first time in the biochemical differentiation of the liver subsequent to birth, is correct. The reports on the effects of raised levels of phenylalanine on the foetus, in respect of the subsequent development of mental retardation, are themselves inconsistent. Mabry et al., quoted by Guthrie and Whitney (1964), described three women whose abnormally high blood phenylalanine levels apparently produced mental retardation in all of the 14 children born to them. None of the children were P.K.U. homozygotes, although they were all presumably heterozygotes. Guthrie and Whitney (1964) also cite a case reported by Coffelt, St. Joseph's Hospital, Burbank, California, where a homozygote P.K.U. mother had had 2 children. The second child was shown to have a blood phenylalanine level of 6 mg. per 100 ml. on the third day of life. This had risen to 12 mg. per 100 ml. at the age of six weeks but had fallen to within normal limits at seven weeks. It was then found that the mother had a blood phenylalanine level, on two separate occasions, of 26 mg. per 100 ml. and 33 mg. per 100 ml. and that her first child was retarded. The latter child shows no evidence of mental retardation (Guthrie, 1965).

From the cases quoted by Mabry et al., it would seem that the inference to be drawn is that phenylalanine (and/or possibly its abnormal metabolites) can pass from maternal to foetal circulation and thus produce an abnormal pre-natal brain metabolism causing mental retardation to become evident several months after birth. On the other hand, the case cited by Coffelt seems to indicate that although a foetus may be exposed to an abnormally high phenylalanine level prior to birth, development of mental /

retardation does not necessarily result. Woolf et al. (1961) had already indicated that such was a possibility, by instancing the cases of two sisters, each of whom suffered from homozygous P.K.U., who produced 6 children between them, none of whom showed signs of mental retardation. At the present stage of our knowledge it is, on this particular aspect, not possible to make any dogmatic pronouncement.

Can it be, as suggested by Bickis et al. (1957), that high blood levels of phenylalanine lead to competition with enzymes involved in the metabolism of tyrosine? These investigations reported that a patient suffering from P.K.U. shows both a decrease in total serum tyrosine - from lack of conversion of phenylalanine - and an inability to utilise all that is available, due to the high phenylalanine level prevailing. It is possible that mental retardation results from phenylalanine or its metabolites interfering with other equally-important enzyme systems.

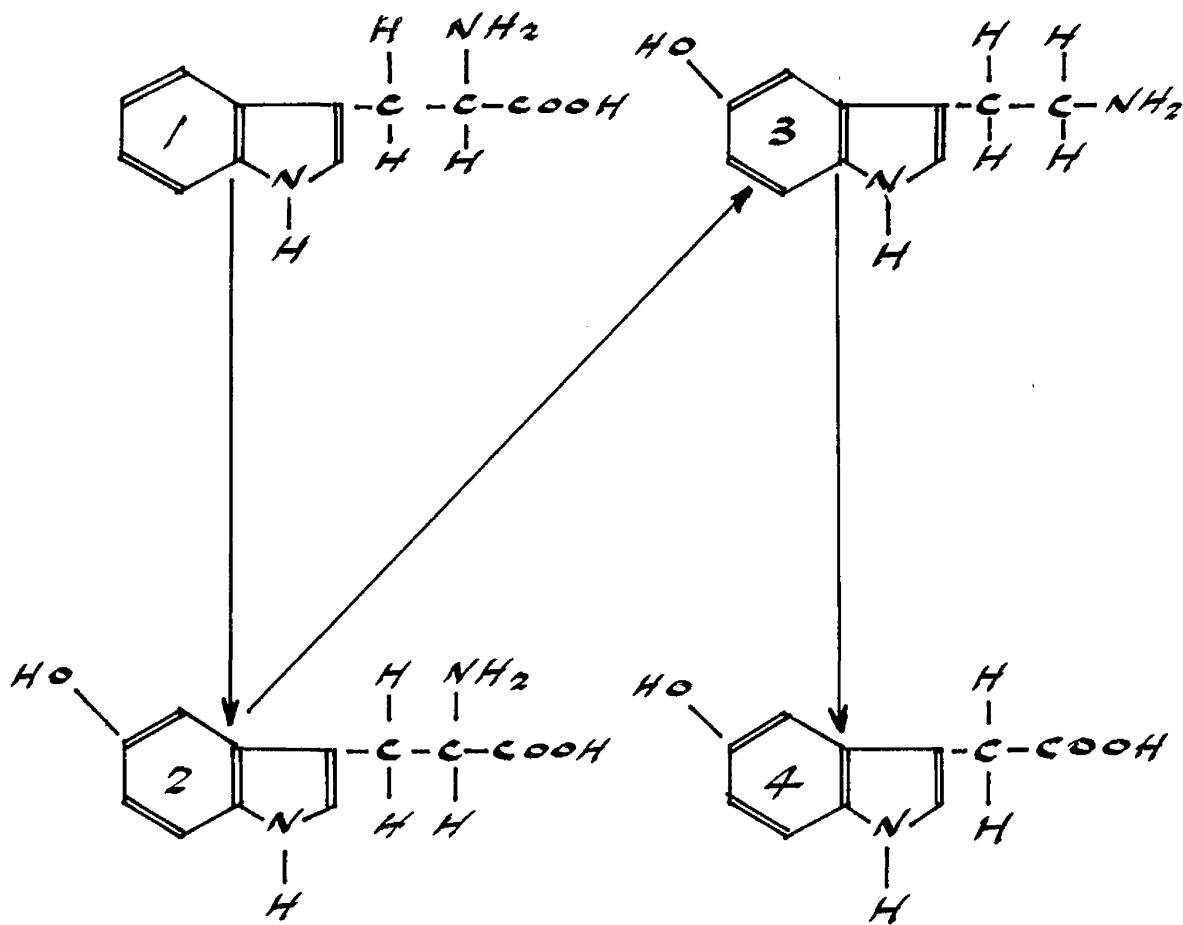
Several other possible mechanisms for the development of the mental retardation in P.K.U. have been postulated, among them that proposed by Udenfriend (1961). He showed that traces of phenylethylamine are normally present in urine. Following upon the administration of mono-amine-oxidase inhibitors, P.K.U. subjects excreted more than 3 mg. phenylethylamine per day whereas normal subjects excreted less than 0.05 mg. per day. The P.K.U. patients who received mono-amine-oxidase inhibitors developed increased tremor, ataxia and ankle clonus, suggesting that phenylethylamine did possess neurotoxic properties. He established that as much as 50 mg. of this amine may be synthesised in P.K.U. each day. Phenylethylamine is pharmacologically active, possessing weak, amphetamine-like properties in small doses and convulsant /

properties in large doses and it can be formed by decarboxylation of phenylalanine in mammalian tissues. Udenfriend et al. (1960) demonstrated the existence of aromatic L-amino oxidase in many tissues and, in the brain, especially in the stem areas. They also demonstrated that the affinity of phenylalanine for the decarboxylase is so low that saturating levels are not reached. It was shown that in the presence of excess phenylalanine the decarboxylation rate was increased and therefore the possibility presents itself that phenylethylamine production takes place at a rate 20 to 60 times that of the normal in P.K.U. brains since there is no evidence that brain levels of phenylethylamine do not correspond with blood and C.S.F. levels. This then may be the "neurotoxic" substance, the existence of which had already been suggested by Armstrong (1957).

Udenfriend (1961), Knox (1960) and Reichle et al. (1961) have demonstrated that P.K.U. patients all have lower levels of serotonin (5-hydroxytryptamine) than normal non-P.K.U. persons. How significant is this fact in relation to the pathogenesis of the mental defect? The possibility does exist that the untoward effect of phenylalanine, if operative in P.K.U., could be intermediated by serotonin. There has been a great deal of speculation on the part which serotonin plays in normal brain-functioning but the only real conclusion which could be drawn from much extensive research, fully documented by Page (1958), was to the effect that meddling with serotonin impairs brain-functioning. It had been demonstrated by Armstrong and Robinson (1954) that there is a disturbance in the metabolism of indole in P.K.U. patients. This was suggested by the finding of the indole derivative, indoleacetic acid, in the urine of such patients and, /

in some patients only, a diminished excretion of 5-hydroxy-indoleacetic acid (figure ). Pare et al. (1957) compared the levels of serotonin in the serum and 5-hydroxyindoleacetic acid in the urine in P.K.U. with the levels in non-P.K.U. control subjects, and found that in P.K.U. there was a marked diminution in both. In 1958, Pare et al. studied the effect of a phenylalanine-low dietary administered to seven children suffering from P.K.U. and were able to demonstrate that there was a significant rise in the mean serum serotonin level in consequence of such a dietary regime. After intravenous 5-hydroxytryptophan injections, four patients showed a lower urinary output of serotonin and 5-hydroxyindoleacetic acid when compared with matched control subjects. These results suggested that 5-hydroxytryptophan decarboxylase might be inhibited by abnormal metabolites of phenylalanine in P.K.U. If such inhibition were to take place then serotonin formation would be diminished and, if the assumption to the effect that serotonin is a transmitter substance at synapses be valid (Page, 1954; Erspaner, 1954; Spector and Willoughby, 1957), this diminished level might thus cause an impairment of cerebral function. In 1959, Pare et al. investigated P.K.U. children who were mentally retarded and also mentally retarded children who were not sufferers from P.K.U. They were able to show that there was a very much higher level of serotonin in the blood of the non-P.K.U. mentally retarded children and were also able to demonstrate a relationship between 5-hydroxyindole levels and intelligence. From this study they concluded that justification had been made for the postulate to the effect that 5-hydroxytryptophan decarboxylase can be inhibited by abnormal metabolites of phenylalanine, and that the rise in the serum level of serotonin and increased urinary output of 5-hydroxyindoleacetic /

One of the pathways of tryptophan metabolism.



1. Tryptophan.
2. 5-hydroxytryptophan.
3. 5-hydroxytryptamine (Serotonin).
4. 5-hydroxyindoleacetic acid.

Figure 6.

(Modified from Larson, 1964).

acid, consequent upon the introduction of a phenylalanine-low dietary in P.K.U., was convincing evidence of such inhibition. The next step was taken by Wang et al. (1961) who observed that rats fed with excess phenylalanine showed diminished levels of serotonin in their brains. They also were able to demonstrate that such diminution in brain-levels of serotonin could be remedied by the administration of an excess of tryptophan. The conclusions of Pare et al. could not altogether exclude the possibility of supplementary action by altered metabolites in the brain in P.K.U. This latter point was stressed by Yuwiler and Loutitt (1961) after presentation of evidence which had initially seemed to show that poor mental performance in rats resulted from the presence of low levels of serotonin. Phenylalanine fed in excess to rats (to increase the brain level of the amino acid) seemed to lead to such rats performing maze and discrimination tests in a poorer fashion than control rats. Nevertheless it was quickly shown that there was no correlation between brain serotonin levels and test results and that administration of such excess phenylalanine did not of itself lead directly to a diminution of brain serotonin. The final conclusion was to the effect that phenylalanine loadings alter a number of metabolic processes in the brain and that low serotonin levels in serum in P.K.U. result, in a subsidiary fashion, from such a general metabolic change. Further support for such a concept was given by Schanberg et al. (1961) who showed that phenylalanine inhibited the uptake of 5-hydroxytryptophan into brain tissue. Further clarification was made by Freedland et al. (1961) and Renson et al. (1961). Freedland et al. showed that phenylalanine hydroxylase could also function to hydroxylate tryptophan and corroboration of this somewhat surprising finding /

54.

was afforded by Renson et al. (1961; 1962) who proved that in an in vitro system utilising liver as the source of the enzyme, phenylalanine is a potent inhibitor of tryptophan hydroxylation and that the maximum rate for hydroxylation of phenylalanine is thirty times that for tryptophan. They suggested that in patients suffering from P.K.U. accumulation of phenylalanine would be sufficient to inhibit hydroxylation of tryptophan in the liver. Were this proven to be so, then previously-noted observations with respect to low levels of serotonin in the serum of P.K.U. patients could be explained satisfactorily. Such a metabolic process could also account for the diminished excretion of 5-hydroxyindoleacetic acid by such patients. In connection with the two latter suggestions, it is again of value to recall that when P.K.U. patients are given a phenylalanine-low dietary, blood serotonin and urinary 5-hydroxyindoleacetic acid levels soon reach normal levels. Wang et al. (1962) investigated the influence of various concentrations of both phenylalanine and tryptophan on the serotonin content of brain and liver. They showed that high concentrations of tryptophan encouraged preferential hydroxylation of this amino acid in the liver but when significant concentrations of phenylalanine were added along with such tryptophan, the hydroxylation of tryptophan was suppressed. It remains yet to be proven whether the high level of phenylalanine in P.K.U. patients acts as an inhibitory factor in tryptophan hydroxylation in such cases. One further fact to emerge from their studies was that serotonin necessary for normal brain function must be formed in that organ itself. Such serotonin formation takes place through decarboxylation of 5-hydroxytryptophan and this substrate, capable of crossing the blood:brain barrier /

is produced by hydroxylation of tryptophan which only takes place in the liver. The serotonin production-and-effect complex, involved as it has been shown to be, may take place in the following sequence:-

lack of phenylalanine hydroxylase → accumulation of phenylalanine in liver → inhibition of tryptophan hydroxylation → diminished production of 5-hydroxytryptophan → lessened amount available for decarboxylation → diminished production of serotonin → ? effect on brain function ?

Practical proof of this suggested line of reasoning will not merely consist of production of experimental evidence to the effect that diminished serotonin levels in brain can be remedied by an excess of tryptophan, as was shown by Wang et al. Only purification of the hydroxylase enzyme will enable definitive experiments to be made regarding the role played by serotonin in normal brain functioning.

The conclusion reached by Yuwiler and Loutitt to the effect that phenylalanine loading altered a number of metabolic processes in the brain received confirmation from Lajtha and Mela (1961). They showed that a rapid exchange of free amino acids took place between plasma and brain even when there was an increase in the concentration of such amino acids. When brain concentration was increased, there was an increase in the rate of exchange of any one amino acid, suggesting the existence of a homeostatic mechanism to preserve equilibrium between the levels of such amino acids in plasma and brain in order, possibly, to prevent the storage of excess amino acids or metabolites in the brain sufficient to cause impairment of structure or function. Following this line of reasoning, the supposition emerges that marked increase of plasma /

phenylalanine above physiological levels may bring about breakdown of the underlying homeostatic mechanism, due to successful competition of this excess phenylalanine for enzyme and transport mechanisms. Interesting although the above hypothesis may be, at the present state of our knowledge such reasoning cannot be validated or disproven.

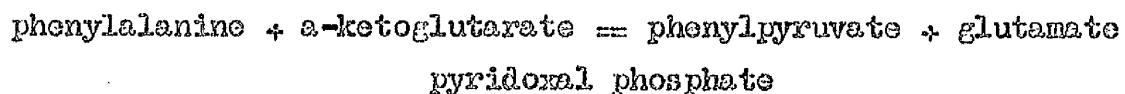
It may be of value at this juncture to examine several of the hypotheses mentioned in the text by referring to figures 5 and 7. Figure 5 shows the normal pathway for the degradation of phenylalanine and tyrosine and figure 7 shows the real and apparent metabolic blocks in P.K.U. The main metabolic routes of phenylalanine, tyrosine and tryptophan are indicated by double arrows.

1. Conversion of phenylalanine to o-tyrosine, o-tyramine  
o-hydroxyphenylacetic acid:- figure 7, B 1, 2 and 3.

It is probable that orthohydroxylation of phenylalanine occurs first to form o-tyrosine and that this is excreted as o-hydroxyphenylacetic acid. An alternative route would be for the o-tyrosine to be converted to o-tyramine, with the reaction being catalyzed by a decarboxylase.

2. Conversion of phenylalanine to phenylpyruvic acid  
through transamination:- figure 7, C 1, 2, 3 and 4.

Through the action of phenylalanine transaminase, phenylalanine is converted to phenylpyruvic acid by the following reaction:-



As Meister et al. (1956) have shown, evidence for the reversibility of this reaction is indicated by the fact that the administration of glutamic acid or glutamate to patients suffering from P.K.U. results in an inhibition of phenylpyruvic acid excretion. In such patients,

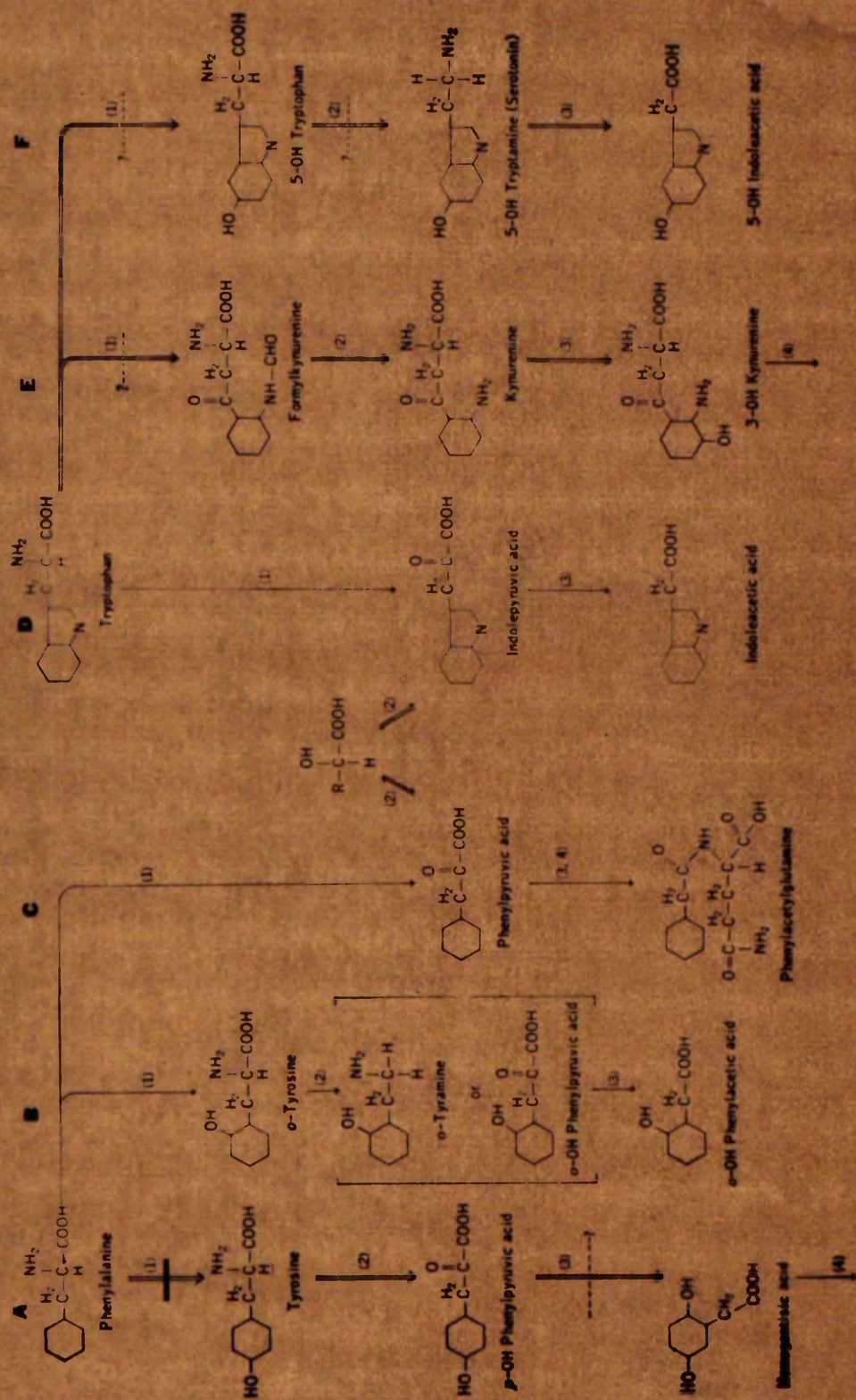


Figure 7. (Modified from Hsia, 1964).

the excretion of phenylpyruvic acid is largely a function of protein intake and, in consequence of active renal tubular secretion, plasma concentrations of this metabolite are low. The observation that phenylalanine transaminase appears in rat liver only after birth may account for the relatively late appearance of phenylpyruvic acid in the urine of P.K.U. patients. Zeller (1943) demonstrated that the formation of phenyllactic acid occurred in consequence of lactic dehydrogenase catalyzing the reversible reduction of alpha-keto acids by DPNH to their lactic acid derivatives. Since the mechanism for the conversion of aromatic alpha-keto acids to the acetic acid derivatives is not known, there is at present no explanation for the conversion of phenylpyruvic acid to phenylacetic acid.

3. Tryptophan transaminase:- figure 7, D.

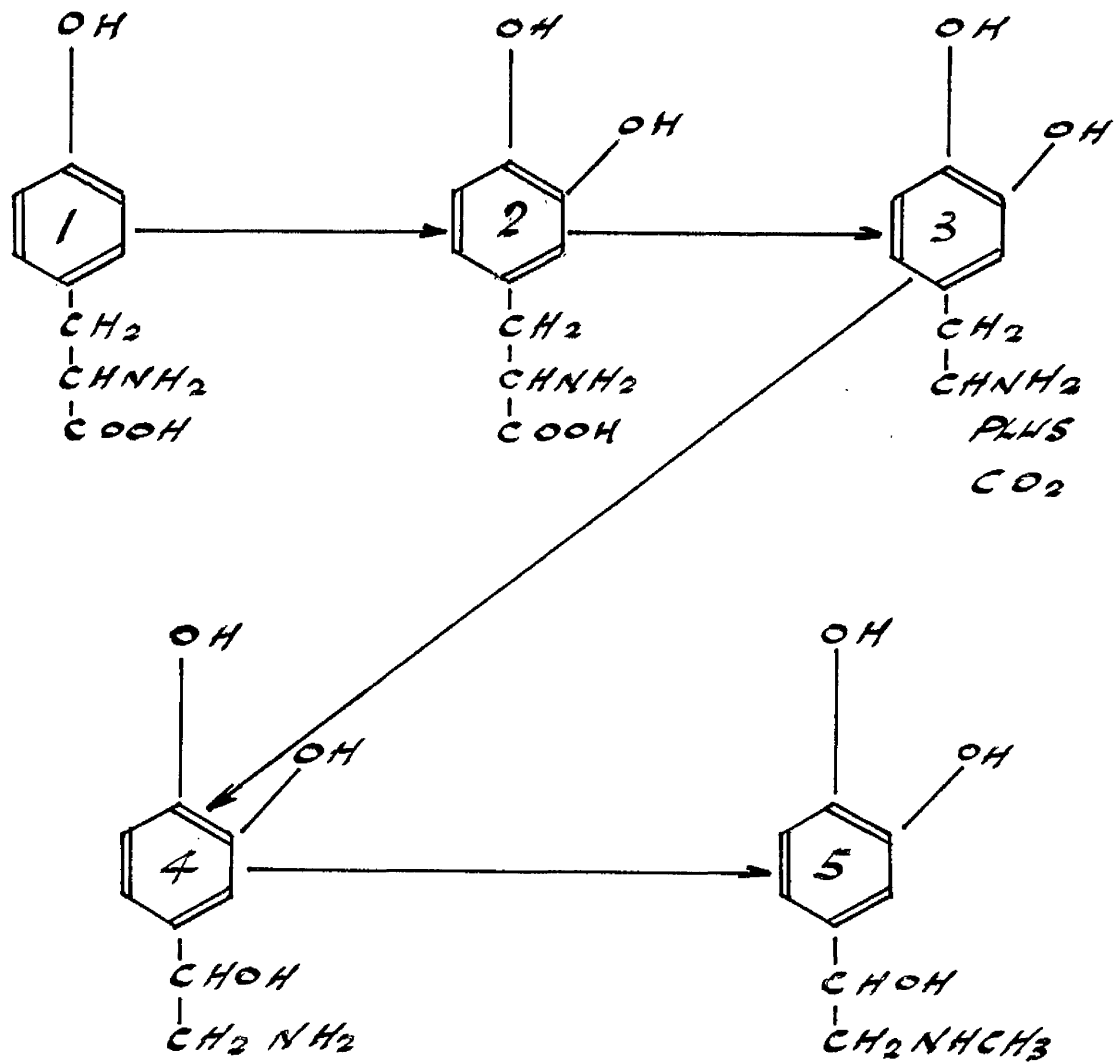
While it is known that tryptophan is converted to indolepyruvic acid and this, in turn, is converted to indoleacetic acid, the mechanism responsible for the transamination has not been fully explained (Armstrong and Robinson, 1954; Hsia and Huang, 1961). Bessman and Tada (1960) have shown that the urinary excretion of indican parallels the blood level of phenylalanine. They also showed that the net conversion of indole to indican, subsequent to indican leading, was less in the case of P.K.U. patients than in control subjects, suggesting that excessive amounts of phenylalanine may cause an inhibition at this step.

4. Effect of phenylalanine upon mammalian tyrosinase:- figure 5.

The formation of melanin involves, firstly, the conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and, secondly, the conversion of DOPA to DOPA-quinone. Tyrosinase catalyzes the /

Probable pathway of adrenalin formation.

(Documenta Geigy Scientific Tables, 5th. Ed.)



1. Tyrosine.
2. 3,4-dihydroxyphenylalanine (DOPA.).
3. 3,4-dihydroxyphenylethylamine (Dopamine).
4. Noradrenalin.
5. Adrenalin.

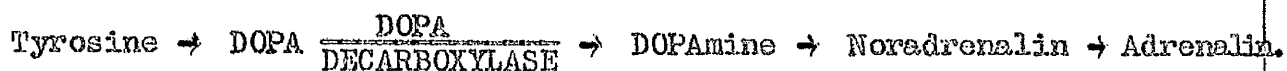
Figure 8.

first step and the second step is markedly accelerated by this enzyme but it can also occur nonenzymatically (block IV). Dancis and Balis (1955) and Miyamoto and Fitzpatrick (1957) have shown that L-phenylalanine acts as a competitive inhibitor of tyrosine-tyrosinase activity at the melanocyte level and that this is the reason for the comparatively common occurrence of blonde hair and fair skin in P.K.U. patients rather than the usually-accepted mechanism of failure of hydroxylation of phenylalanine leading to a reduction in blood tyrosine with consequent diminution of tyrosine derivatives. It is a matter of general observation that perseverance with a phenylalanine-low dietary will, in the majority of cases, lead to a gradual darkening in the colour of the hair.

5. Effect of phenylalanine upon DOPA decarboxylase:-

figure 8.

It has been shown that the primary pathway of adrenalin formation is as follows:-



Cawte (1956) and Knox and Hsia (1957) pointed to the possibility of there being a disturbance in the metabolism of adrenalin in P.K.U. patients by demonstrating an increased rise in blood pressure following upon the administration of an intravenous dose of adrenalin when compared with the rise in normal subjects. Fellman (1956) and Boylen and Quastel (1961) have shown that the derivatives of phenylalanine inhibit DOPA decarboxylase in vitro and Nadler and Hsia (1961) have shown that there is a decrease in the levels of noreadrenalin and adrenalin in the plasma and of DOPAmine, noreadrenalin and adrenalin in the urine of P.K.U. children. /

6. Effect of phenylalanine upon 5-hydroxytryptophan decarboxylase.

The normal pathway for the degradation of tryptophan is shown in figure 7 pathway F.

Notwithstanding the multiplicity of alternative pathogenetic mechanisms postulated as being responsible for the development of the mental retardation in P.K.U., at the present stage in our understanding of the various biochemical processes operative in the disease, it is reasonable - and indeed justifiable, from a practical standpoint - to assume that the mental retardation arises as a direct consequence of the blockage of the hydroxylation of phenylalanine. An alternative and not altogether insupportable theory would suggest that the P.K.U. gene may produce an enzyme-inhibitor which, in turn, would cause the initiation of sequences of events giving rise to disturbances in processes of synthesis of importance to proper functioning of the brain. Indeed, Knox (1961) has founded a working hypothesis based upon such induced adaptive enzyme changes, suggesting that the abnormal chemical environment in the brain cells of P.K.U. infants produces an abnormal pattern of enzymic maturation, such adaptive changes thereafter becoming irreversible and capable of being produced by several agents. Such a hypothesis, however, rests substantially on what Knox implies by "normal biochemical maturation of the brain". Many painstaking studies on the methods of action of microsomal and nonmicrosomal enzymes will be necessary before the very edge of this particular curtain may be lifted.

All-in-all, there can be no doubt that the majority of observed facts support the concept of P.K.U. being primarily a genetic disorder with mental impairment resulting from one primary /

enzymatic block, irrespective of how such impairment may, in actual fact, be determined. The number and complexity of hypotheses with regard to one aspect or another of the biochemistry, metabolism and pathogenesis of P.K.U. mentioned in this chapter, indicates the amount of investigation which still remains to be undertaken.

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## Pathology

In this chapter it is proposed that primarily only the pathology of the central nervous system in P.K.U. will be discussed.

Alvord et al. (1950) examined the C.N.S. of five cases who had clinical manifestations of P.K.U. A marked lack of myelination involving principally the optic, cortico-spinal and cortico-ponto-cerebellar tracts in the central and peripheral nervous systems was demonstrated in siblings of 16 months and 5 years. In one adult case a defect in myelination was noted in the optic chiasma but two other adults showed no such myelination-defect. Other abnormalities, including gliosis and increase in perivascular fat of the cerebral vessels were present in all five cases. Alvord et al. raised the question as to whether-or-not the myelination-defect could have been, in part at least, responsible for the mental deficiency which had been present in all five cases. Poser and Van Bogaert (1958) published comprehensive findings and deductions regarding the pathology of the brain of an 18 year old P.K.U. patient. The principal finding was the presence of "multiple areas of altered myelination associated with variable degrees of fibrillary gliosis", and, taking their own case and comparing their findings with those of Alvord et al., concluded that the pathogenetic defect in P.K.U. was possibly "a metabolically-determined disturbance of the glial cell - myelin sheath relationship". Furthermore, they stated that "neuropathologic observations indicate that this disease should be classified with the leucodystrophies and the cerebral lipidoses in the general group of dysmyelinating diseases". They thus took their stand in favour of the disturbance of myelination being in the nature of a defect in the laying-down and not the result of a /

process of demyelination. Crome and Pare (1960) published a review of the literature and of their own pathological findings in four cases. Their main findings were those of micrencephaly and fibrous gliosis of the white matter in all four cases but slight pallor of myelin staining was also demonstrated in two. They stated that, in 20 cases reviewed, the most consistent finding was of reduction in brain weight, in many cases accompanied by ventricular dilatation. In many of the smaller brains there was a reduction in the total volume of the white matter accompanied by a diffuse fibrous gliosis. They also reported that older patients did actually show widespread demyelination. No very firm conclusions were drawn by the authors, but, after stating "entire fibres in the white matter are removed" and that "there is no evidence as to whether this precedes myelination and explains the lack of myelin or whether the function of the oligodendroglial cells is interfered with and the resulting non-myelinated or poorly-myelinated fibres later destroyed", they agreed that either of the above possibilities corresponded with the observation that most of the damage to the central nervous system occurs in the earliest months of life. Their summing-up was to the effect that the observed defects in myelination might result from degeneration of some neurones taking place early in life, thus resulting in their showing signs of poor myelination; other neurones dying later in life and thus showing actual demyelination. It thus appeared that both the processes of dysmyelination and demyelination may be operative. Crome (1962) noted that reports of Schilder's disease (leucodystrophy) occurring in association with four cases of P.K.U. had been reported. From this he stated, "leucodystrophy occurs in conjunction with phenylketonuria in a sufficiently large number of /

cases to exclude the possibility of such an occurrence being of a fortuitous nature". In another publication in 1962, Crome et al. subjected the white matter of the brains of P.K.U. patients to chemical analysis and compared the results with those from age-matched controls. They found a reduction of total solids content in P.K.U. brains and showed the percentage of the solids represented by cerebrosides and cholesterol to be less than in normal individuals of the same age. Cholesterol esters were not found except in parts of the brain of one patient with leucodystrophy. In this publication it was suggested that the laying down of myelin appeared to be hindered in P.K.U., but that gradual active demyelination could not be excluded. Thus Crome et al. went on record as protagonists of combined processes of dysmyelination and demyelination being operative in P.K.U. In 1963 however, Crome suggested that Schilder's disease or, as it is now called in preference, non-inflammatory sudanophilic leucodystrophy, may be one of the reaction patterns of the brain and, as such, common to a number of pathogenetic processes. Should this prove to be the case, earlier views with regard to the causal relationship between P.K.U. and this pathological finding are not necessarily valid. In this particular paper, Crome also noted that he had not found any structural changes in the oligodendroglial cells in the brains examined in P.K.U.

Lesions outwith the central nervous system have occasionally been reported. These include pituitary and testicular hypoplasia, fatty degeneration of the liver and absence of hypophyseal chromophilic elements. Such reports would appear to be in the nature of naturally-occurring chance findings and not in direct consequence of the pathogenetic and pathologic processes operative /

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in P.K.U. There can be no doubt that the opportunities for investigation of the pathology of P.K.U. have not been at all numerous, if the published articles are an indication. On so few cases, so little of a dogmatic and factual nature can be pronounced. The belief of Poser and Van Bogaert and of Crome, to the effect that dysmyelination is possibly of more importance than demyelination, remains to be proved and the question as to whether the alteration in myelination is responsible for the mental retardation, or merely a further manifestation of the total picture of P.K.U., remains unsettled. Conclusive pathological proof would necessitate fulfillment of the following postulates:-

1. that the central nervous system changes must be caused by P.K.U.:
2. that the central nervous system changes must be shown to be the obvious cause of the mental retardation associated with this particular upset.

At present, convincing pathological proof is lacking.

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### Clinical Findings.

It must be stated that there is no comprehensively infallible picture of P.K.U. as a clinical entity. The signs and symptoms of the disease may be quite diverse and, although a patient-conformance picture of the blonde haired, blue eyed, mentally retarded child is commonly held, adherence on the part of the clinician to such a mental impression will frequently allow the non-conforming infant or child to pass unsuspected. It is true that the majority of homozygote cases exhibit fair hair, fresh complexions and blue eyes. It is equally true that many cases have brown hair, sallow complexions and brown eyes. The only absolute truth in the disease is the demonstration of the existence of an elevated level of serum phenylalanine. This, then, is the criterion to be adopted and none other.

Partington (1961) noted the Protean manifestations of the disease as they may present in the first year of life as follows:-

"suspected or actual mental retardation; a retarded or P.K.U. sib; infantile eczema or other non-specific inflammatory skin lesions including diaper rash; seizures; a complaint on the part of the parents that the child or its urine has a peculiar smell, even if the physician cannot appreciate it; vomiting in the early weeks of life, including feeding problems, colic and possibly (symptoms suggestive of) pyloric stenosis; irritability".

He pointed out that if the child presenting with any of the above did happen to be blonde and blue eyed, the suspicion should be increased but that presence of adequate pigmentation must never allow the possibility of the existence of the disease to be summarily dismissed. He also found, in the early history of 36 /

patients, that more than 50 per cent had suffered from symptoms of one sort or another in the early weeks of life which had been noted by the parents long before they had suspected the existence of mental defect. He categorised these symptoms as follows:-

1. vomiting (17 patients).
2. seizures (13 patients).
3. irritability (12 patients).
4. eczema (6 patients).

Knox and Hsia (1957) stated that the commonest neurological finding was restlessness. Neurological signs are apparent in the majority of P.K.U. patients and are predominantly extra-pyramidal. They consist of tremors, ataxia, muscular hypertonicity, athetoid movements and exaggeration of deep reflexes. Paine (1957) reported upon a series of 109 untreated patients. He pointed out that approximately one-half showed mild to marked microcephaly and that, especially among those of low intelligence, hand posturing was a very striking characteristic. The purposeless hand movements include rhythmic pill-rolling, irregular tic-like motions, aimless to-and-fro finger movements and frequent habitual fiddling of the fingers held close before the eyes. These movements may be accompanied by a rhythmic rocking back and forth lasting for several hours. Such extra-pyramidal signs are presumably related to damage of developing pathways within the C.N.S. Microcephaly was also mentioned by Crome (1962) as occurring in several cases.

The question of the colour of the hair was studied by Cowie and Penrose (1951) who showed, by reflectograms, that there is a significant lightening of the hair colour of P.K.U. children compared with their unaffected sibs. This matter of hair colour is possibly most evident in the case of Japanese sufferers from /

the disease. All Japanese children normally are born with, and retain, black hair colour. Shizume and Naruse (1958) showed that Japanese P.K.U. children have brown hair, lighter skin and lighter iris colour than non-P.K.U. Japanese children. This lighter colouration, in the case of the untreated individuals, persists into adult life.

Knox and Hsia (1957) found that many P.K.U. patients showed some form of skin disease varying from a mild eruption to a severe eczematoid lesion or the presence of a rough, scaly skin of the ichthyotic type. They were unable to state whether such epidermal changes were the direct result of the metabolic error or resulted from trauma to an epidermis essentially normal but hypersensitive in consequence of the lack of melanin. Fleisher and Zeligman (1960) studied 23 P.K.U. patients over a period of 8 years and demonstrated atopic dermatitis in three. In investigation of intradermal test-responses, they demonstrated 10 positive in 21 P.K.U. subjects and only 4 positive in 21 controls.

Little (1962) reported on two cases in which the presumptive diagnosis of P.K.U. had been made by himself, an otolaryngologist. Both children had been presented on account of supposed hearing difficulties but he was unable to find any objective evidence for the existence of the hearing difficulties. This led him to suspect the development of mental retardation as being the responsible condition for the difficulties of the children, and subsequent demonstration of an elevated serum phenylalanine confirmed the diagnosis of P.K.U. In consequence of this experience, he advocated the routine screening for P.K.U. for all children under the age of five suspected of deafness. There is also the possibility of speech difficulty being the presenting feature of the

disease. Diedrich and Poser (1960) discussed two sibs with congenital aphasia, later diagnosed as P.K.U. sufferers. On a phenylalanine-low dietary maintained during three years, there was marked improvement in language as well as functional mentation. Since such a dietary was not commenced till the children were 4 and 3 years respectively, it was suggested by them that speech therapists should be made aware of P.K.U. as a possibility in the case of delay in language development in children who might otherwise appear normal.

Feinberg and Fisch (1962) presented radiological evidence of unusual bone change in growing long bones of P.K.U. children. Those changes were initially noticed in the cases of children who had been placed on phenylalanine-low dietaries, and consisted of "calcified spicules of cartilage projecting into the epiphyseal cartilage from the zone of the distal metaphyses". Such spicules were shown to persist during growth, but "the original portions were simultaneously incorporated in the osseous metaphyses". Four older patients who had not been on dietary restriction displayed "moderately positive findings". One child showed the change only after the age of 4 when he was placed on a restricted diet. Such findings, interesting though they may be, cannot truly be ascribed to the presence of P.K.U. per se. More than likely they result from general amino acid deficiencies rather than specific deficiencies, and further radiological investigations will be necessary together with concomitant biochemical studies before this particular aspect of the P.K.U. problem can be clarified.

Epileptiform seizures occur with increased frequency in P.K.U. In sufferers from the disease, E.E.G. studies have been made during sleeping and waking states. Fois et al. (1955) demonstrated that /

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waking activity is usually abnormal and, in their study of 19 patients, showed that such activity was of high voltage, slightly fast, slightly slow, or a mixture of fast and slow, with seizure discharges of the petit mal type occurring quite commonly. Eleven patients showed seizure activity in the waking state and 18 showed it in the sleeping state. Fois et al. suggested that, in P.K.U., there was a profound disturbance of both cortical and thalamic function. Low et al. (1957) confirmed, from their own observations, that the majority of P.K.U. patients showed E.E.G. abnormalities if untreated. Their findings suggested epileptiform seizures as being more common as an infantile manifestation of the disease, such cases showing hypsarhythmia and multiple seizure foci. As these children matured, Low et al. showed that tonic-clonic convulsive attacks frequently developed, accompanied by E.E.G. changes showing focal or generalised spike discharges. In the series of 23 cases investigated, they stated "7 had spike-and-wave complexes similar to those evident in petit mal epilepsy" and furthermore, that 8 patients placed on a phenylalanine-restricted diet showed improvement or normalisation of the E.E.G. with the passing of time, while in 3 out of 5 cases with clinical seizures, such seizures ceased to occur. Grumer (1962) confirmed Low's findings with regard to the infrequency of epileptiform seizures in adults. Herrlin (1962) published a clinical and E.E.G. study of a pair of monozygotic P.K.U. twins. Both had shown signs of mental retardation and epilepsy simultaneously at the age of a few months, but at the age of two and one-half years, their oligophrenia, epilepsy and E.E.G. findings showed appreciable differences. Pointing to the P.K.U. as being the only known common factor in each case, he stated "it appears that their brain damages differ in type and degree of severity, /

although the metabolic disturbances could be expected to be equally pronounced in both and the reaction norms of their brain tissue the same, since they are genetically identical". While it can be stated that E.E.G. changes are evident in the majority of P.K.U. patients whether or not they exhibit epileptiform seizures, there is no constantly-appearing pathognomonic feature in the E.E.G. It would appear that what differences in patterns are seen depend primarily upon random differences in the extent and localisation of the causative cerebral lesions.

In spite of the various clinical findings so-far mentioned, there is no disputing the fact that mental retardation is the most significant and consistent finding in P.K.U. From what has been stated, both in this and previous chapters, such retardation has been shown not to be evident in the earliest months of life but to become manifest, in the untreated case, after the first months of life have passed. Hsia (1960) has stated that the majority of untreated cases have a low level of intelligence with I.Q.'s. of 30 or less and, although in general agreement with this statement, Knox (1960) has pointed out that a minority show an I.Q. in the range of 60 to 80 and may be educable. Knox and Hsia (1957) had stated, in more general terms, that "the majority of patients with P.K.U. are idiots" and "perhaps one-half of them never learn to talk and one-third of them never learn to walk". They also pointed to the fact that a small proportion can show borderline intellectual development but, as was the case with Knox (1960), could point to no obvious genetic explanation for such differences in intellectual capacity. This matter will be considered further in a later chapter. /

The significant clinical manifestations of the disease are only apparent in the homozygote. Although it has been suggested by Knox (1960) that there may be a higher incidence of mental disorder among heterozygotes, this has not yet been proven conclusively. In addition, although the heterozygote may possibly have a higher fasting serum phenylalanine level than the normal person, it has never been shown that other clinical features of P.K.U. can occur in conjunction with the heterozygote state.

In general, signs and symptoms of P.K.U. may be divided into three major groups:-

1. mental manifestations: mainly retardation but also embracing restlessness and otherwise inexplicable irritability;
2. neurological abnormalities: including, especially in infants and young children, epileptiform seizures; muscular hypertonicity; motor impairment; microcephaly;
3. extraneural manifestations: including defect in pigmentation; skin lesions; malodour from the urine or person; vomiting (in infants).

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## Psychological Aspects of P.K.U.

In 1934, Fölling stated "the study of phenylpyruvic amentia may throw light on the whole problem of mental deficiency". In the years which have intervened, no clear understanding has as yet been reached between the relationship of the biochemical abnormality and the deficiency in intellectual function usually reflected in severe mental retardation. No matter what the true pathogenesis of the amentia may be, there can be no doubt that the disease interferes with psychological processes necessary for normal development of cortical activity. While the untreated case, in the majority of occasions will quickly progress to irreversible mental retardation, the treated case - assuming diagnosis to have been made sufficiently early - should develop normal or average cerebration. In the event of diagnosis not having been made before the onset of processes of mental deterioration, introduction and maintenance of proper treatment should arrest such deterioration in developmental or intelligence levels. Thus, psychological aspects of the disease will primarily be concerned with the observation, measurement and recording of developmental, intellectual and behavioural facets of the total personality of the individual in relation to the beneficial effects of dietary treatment.

Among the most interesting of the many reports on the psychological aspects of the disease was that presented by Koch (1964). In his report he dealt with 31 patients from 23 families. The ages at which diagnosis had been made ranged from 5 days to 8 years, with the average age of diagnosis being 2 years and 4 months. Ethnic backgrounds were predominantly Irish, English, Scottish and German. All cases displayed an elevated serum /

DQ/IQ DISTRIBUTION AT THE TIME OF PKU DIAGNOSIS

N=31

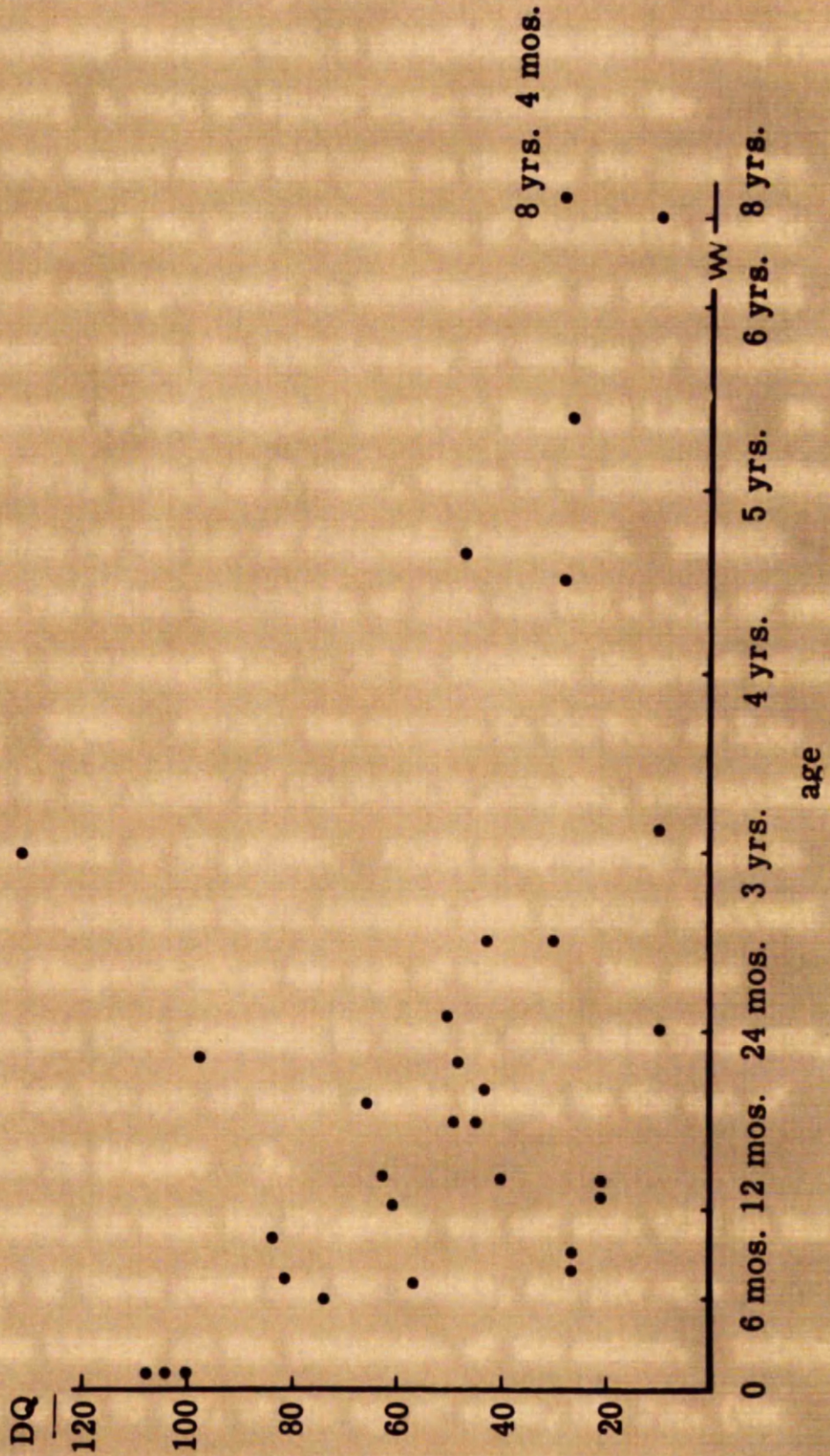


Figure 9.  
(Koch, 1964)

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phenylalanine ranging from 13 to 64 mg. per 100 ml. when such was estimated by the McCamen and Robins fluorimetric method. 24 of the 31 had been adequately treated with a phenylalanine-low dietary for more than one year; 4 were no longer being treated at the publication of the report and 3 had been on therapy for less than one year. In spite of the late average age of diagnosis, nearly every child showed an improvement in developmental or behavioural progress. Figure 9 shows the relationship of the age of diagnosis to the developmental or intelligence quotient (D.Q. or I.Q.). It should be noted that, except in 2 cases, there is a gradual decline in the D.Q. or I.Q. as the age of diagnosis increases. Infants diagnosed during the first six months of life all had a D.Q. of over 75, compared with an average D.Q. of 35 in children diagnosed after the age of two. Table 2 reveals the changes in the D.Q. or I.Q. ranging from +4 to +43 points in the treated patients, corresponding to an average increase of 17 points. Again a noteworthy fact is the intellectual improvement which occurred with dietary treatment even in children diagnosed after the age of three. The most spectacular gain was that occurring in case 8, who, when diagnosed, had a D.Q. of 37 at the age of eight months which, by the age of five, had reached 79 (figure 10).

Another child, in whom the diagnosis was not made till he had attained the age of three years and two months, was put on a phenylalanine-low dietary at the insistence of the mother. Initial I.Q. was stated to be 10 and, at the age of seven, his I.Q. (the then most-recent) was estimated to be 53. At this period he was in a school for the educable mentally retarded, had developed language ability and behaved well. Another five-year old boy (case 29) was put on dietary therapy because of severe uncontrollable/

DQ CHANGES IN 31 PKU CHILDREN

WITH OR WITHOUT TREATMENT

| Case No. | Age Diagnosed | Initial DQ/IQ | Dietary Control* | Latest DQ/IQ | DQ/IQ Change | Diet Duration | Present Age |
|----------|---------------|---------------|------------------|--------------|--------------|---------------|-------------|
| 1        | Birth         | 104           | Excellent        | 100          | -4           | 1-1           | 1-1         |
| 2        | Birth         | 100           | Excellent        | 94           | -6           | 1-9           | 1-9         |
| 3        | 0-1           | 108           | Excellent        | 108          | -            | 0-8           | 0-8         |
| 4        | 0-6           | 75            | Good             | 83           | +8           | 1-0           | 1-6         |
| 5        | 0-7           | 57            | -                | -            | -            | 0-4           | 0-11        |
| 6        | 0-7           | 81            | Excellent        | 74           | -7           | 1-2           | 1-9         |
| 7        | 0-7           | 37            | Excellent        | 73           | +36          | 1-4           | 1-11        |
| 8        | 0-8           | 37            | Excellent        | 75           | +38          | 4-3           | 4-11        |
| 9        | 0-10          | 84            | Excellent        | 101          | +17          | 1-5           | 2-3         |
| 10       | 0-12          | 61            | Good             | 77           | +16          | 1-8           | 2-8         |
| 11       | 1-1           | 31            | Good             | 52           | +21          | 2-7           | 3-8         |
| 12       | 1-2           | 62            | Good             | 73           | +11          | 3-10          | 5-0         |
| 13       | 1-2           | 40            | Excellent        | 68           | +28          | 6-2           | 7-4         |
| 14       | 1-2           | 30            | Poor             | 29           | -1           | 3-6           | 4-8         |
| 15       | 1-6           | 49            | Good             | 86           | +37          | 1-5           | 2-11        |
| 16       | 1-6           | 46            | Good             | 62           | +16          | 1-9           | 3-3         |
| 17       | 1-7           | 66            | Excellent        | 72           | +6           | 1-0           | 2-7         |
| 18       | 1-9           | 45            | -                | -            | -            | 0-1           | 1-10        |
| 19       | 1-10          | 48            | Good             | 59           | +11          | 1-10          | 3-8         |

Table 2.

(Koch, 1964)

| Case No. | Age Diagnosed | Initial DQ/IQ | Dietary Control*    | Latest DQ/IQ | DQ/IQ Change | Diet Duration | Present Age |
|----------|---------------|---------------|---------------------|--------------|--------------|---------------|-------------|
| 20       | 1-10          | 98            | Excellent           | 102          | +4           | 2-3           | 4-1         |
| 21       | 2-0           | NM**          | Poor<br>(discont'd) | NM**         | -            | 1-6           | 10-0        |
| 22       | 2-1           | 50            | Excellent           | 67           | +16          | 2-8           | 4-9         |
| 23       | 2-6           | 43            | Good                | 76           | +33          | 3-11          | 6-5         |
| 24       | 2-6           | 30            | Poor                | 40           | +10          | 3-4           | 5-10        |
| 25       | 3-0           | 132           | None                | 124          | -8           | -             | 7-0         |
| 26       | 3-2           | 10            | Good                | 53           | +43          | 3-9           | 7-9         |
| 27       | 4-6           | 29            | Poor                | 28           | -1           | 2-1           | 6-7         |
| 28       | 4-8           | 46            | -                   | -            | -            | 0-1           | 4-9         |
| 29       | 5-5           | 26            | Good                | 37           | +11          | 2-7           | 8-0         |
| 30       | 8-0           | NM**          | None                | NM**         | -            | -             | 15-10       |
| 31       | 8-4           | 27            | Poor<br>(discont'd) | 10           | -17          | 1-0           | 10-4        |

\* Excellent control indicates serum phenylalanine content less than 6 mg.%.  
 Good control indicates 6-12 mg.%.  
 Poor control, serum levels above 12 mg.%.

\*\* NM - Abbreviation for not measurable.

DQ/IQ DATA IN CHILD WITH PHENYLKETONURIA (PKU)

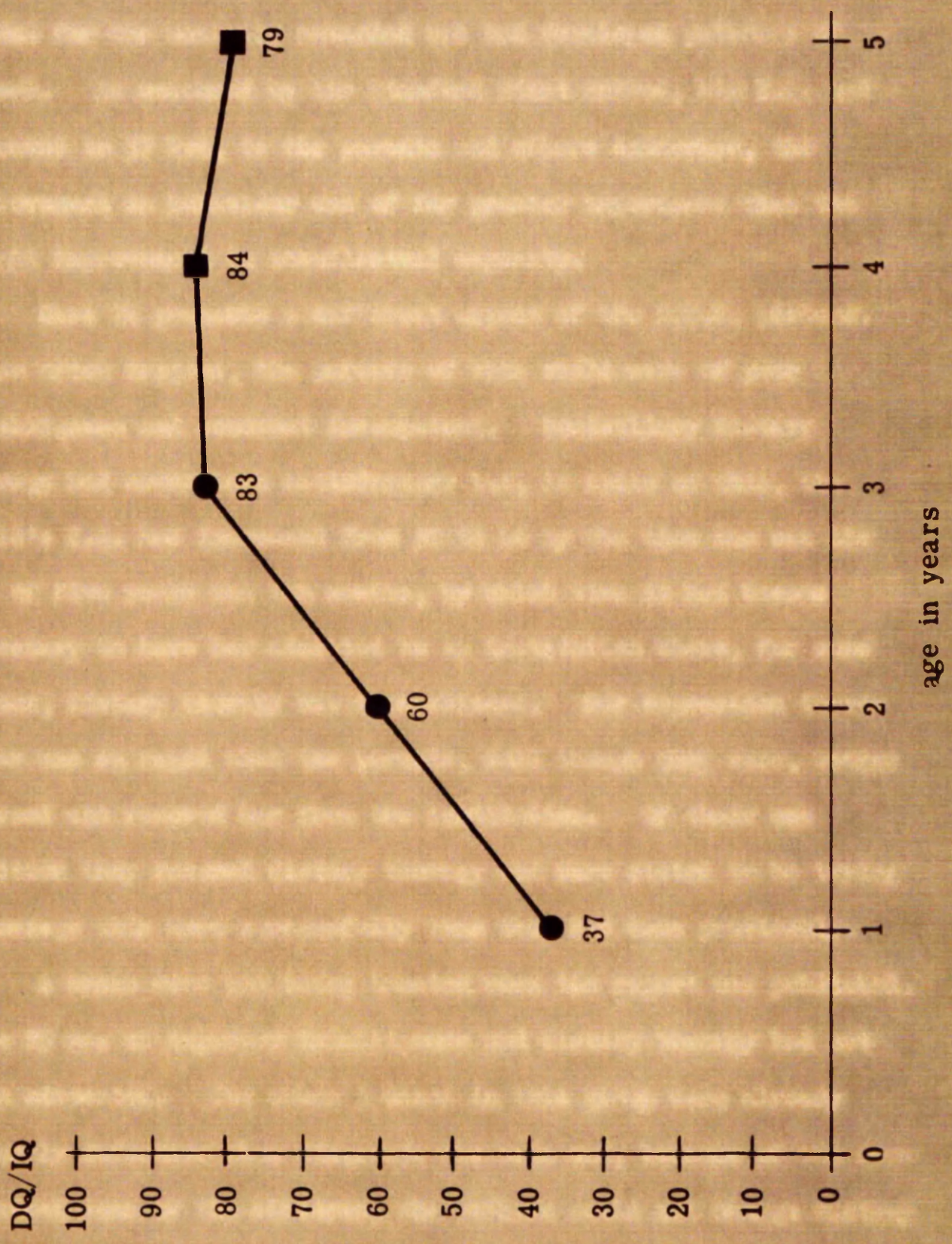


Figure 10.  
(Koch, 1964).

behaviour; I.Q. at that time was 26. With continuation of the dietary therapy he calmed down remarkably, was able to attend a school for the mentally retarded and had developed patterns of speech. As measured by the Stanford Binet method, his I.Q. had improved to 37 but, to the parents and others concerned, the improvement in behaviour was of far greater importance than the slight improvement in intellectual capacity.

Case 24 presents a different aspect of the P.K.U. picture. As so often happens, the parents of this child were of poor intelligence and somewhat incompetent. Domiciliary dietary therapy had never been satisfactory but, in spite of this, the child's I.Q. had increased from 30 to 40 by the time she was the age of five years and ten months, by which time it had become necessary to have her admitted to an institution where, for some reason, dietary therapy was discontinued. Her regression thereafter was so rapid that the parents, dull though they were, became alarmed and removed her from the institution. Of their own volition they resumed dietary therapy and were stated to be trying to manage this more successfully than they had done previously.

4 cases were not being treated when the report was published. Case 25 was of superior intelligence and dietary therapy had not been advocated. The other 3 cases in this group were already severely defective when diagnosis was made and, because of the more advanced ages of the children, the mothers could not manage dietary supervision. On publication of the report, 2 of the 3 cases had been admitted to institutions. From Koch's findings it would appear that, if at all possible, there is justification for initiating and maintaining dietary measures in all mentally subnormal P.K.U. children. There would appear to be no doubt that the /

sooner such dietary therapy is initiated, the more effective it will prove to be and the greater will be the likelihood of prevention of further intellectual deterioration. Centerwall et al. (1960) had already stated this but had not been as optimistic as Koch, making the dogmatic pronouncement that "an infant whose treatment is started at the age of one month may be expected to develop his full potential. When treatment is started at two months, mentality will probably be normal but may lag 10 or more points behind the expected I.Q. level of the child. In most cases when the treatment is delayed until the second half of the first year of life, some retardation will result. Those who are diagnosed past one year of age, or later in life, and then placed on diet may show little significant improvement in I.Q. and are often moderately to severely mentally retarded".

Wright et al. (1958) had reviewed 362 cases recorded in the literature and had shown that:-

- 63 per cent had an I.Q. between 1 and 20;
- 32 per cent had an I.Q. between 21 and 50;
- 4 per cent had an I.Q. between 51 and 70;
- 1 per cent had an I.Q. above 71.

In another review published in 1957, Paine had shown that, of 106 cases:-

- 70 per cent had an I.Q. below 20;
- 84 per cent had an I.Q. below 30;
- 93 per cent had an I.Q. below 40;
- 98.1 per cent had an I.Q. below 50.

From those studies it can be seen that the majority of P.K.U. patients fall within the category of 'moderate to severe' mental retardation. /

Fishler (1964) investigated the levels of intellectual functioning of all the parents, together with the normal sibs, in each of 23 families with a child suffering from P.K.U. The results are presented in table 3. From this table it becomes evident that the parents and sibs of the P.K.U. children, in this particular study, were of normal intelligence although 4 of the mothers and 1 of the sibs were in the borderline range of I.Q. testing. It is of interest to note that the average I.Q. of the fathers compared favourably with those of the sons and fell within the bright, normal range. The comparison between the I.Q.'s of the mothers and daughters was also close but at a somewhat lower level.

Although there is no doubt that the majority of untreated P.K.U. children become irreversibly mentally retarded, there is equally no doubt that a small minority do not develop intellectual deterioration in spite of the fact of their never receiving treatment. Why this should be so is not at all clear, but this fact should never allow an infancy-diagnosed case of P.K.U. to be deprived of dietary treatment. If such atypical cases be considered in relation to the question of intelligence levels in general in P.K.U., there can be no doubt that such cases may occur for a variety of reasons. When the distribution of intelligence among all P.K.U. patients is considered, it is to be expected that 1 to 2 per cent should have a level in excess of 60. The description of 25 such individuals among about 1,000 known P.K.U. patients (2.5 per cent), as mentioned by Hsia (1964), is probably not excessive. The possibility also exists that such cases represent a 'forme fruste' of P.K.U., with those patients lying in a region between true homozygosity and true heterozygosity, where such are expressed in terms of biochemical and mental findings. Such patients would /

DISTRIBUTION OF IQ LEVELS IN PARENTS  
AND UNAFFECTED SIBLINGS OF PKU PATIENTS

| <u>Fathers</u> (N = 22*) | <u>Mothers</u> (N = 23)   |
|--------------------------|---------------------------|
| Range: IQ 89 - 130       | Range: IQ 72 - 130        |
| Mean: IQ 112.4           | Mean: IQ 103.2            |
| <u>Sons</u> (N = 11)     | <u>Daughters</u> (N = 10) |
| Range: IQ 94 - 126       | Range: IQ 78** - 128      |
| Mean: IQ 113.2           | Mean: IQ 102.6            |

\* One father is separated and not available for testing.

\*\* Sibling with IQ 78 has parents with following IQ's:  
Father: IQ 97 - Mother: IQ 72.

Table 3.  
(Fishler, 1964)

then represent the existence of another allele for the abnormal gene or reflect the influence of modifying genes. This matter will be considered in the chapter dealing with Heredity and Prevention. The following case-reports typify the intellectually-aberrent P.K.U. sufferer as distinct from the usual P.K.U. ament.

Ksia et al. (1957) reported the discovery of an educable P.K.U. homozygote during a survey for heterozygote-determination among sibs of known cases. The patient was aged thirteen and a pupil in the 6th. grade of an ordinary school. She had an I.Q. of 78 (Wechsler) and a mental age of nine years and seven months as measured by the Stanford-Binet method. A careful evaluation of her school record showed that she was not performing to the level of her class and had made the remark, "Why am I so dumb when everyone else is smart"? The other 2 patients discovered were siblings aged nine and twelve years who were doing entirely normal work in the 3rd. and 6th. grades in school. Their serum phenylalanine levels were 26.6 and 24.3 mg. per 100 ml. and their urine phenylpyruvic acid tests were strongly positive. Also in 1957, Coates et al. reported on the occurrence of P.K.U. in a child suffering concomitantly from Gower's muscular dystrophy. There was apparently no possibility of connection between the two diseases, such an occurrence being a random happening. At the age of six years and six months, the child had an I.Q. of 103. In this particular case, dietary therapy was instituted but only maintained for a period of three months, the child having shown no gain in intellectual capacity on reassessment at the expiry of this period. After the lapse of two years there was found to have been a significant intellectual deterioration, I.Q. now being - rather unfortunately - 85. Leonard and McGuire (1959) reported on a case with borderline intelligence who /

nevertheless had a high ability for rote memory. They suggested that continued environmental security might, of itself, bring about increase in mental age. Caudle (1960) reported on an untreated P.K.U. homozygote child who, at the age of two years and nine months, had an I.Q. of 134 and Sutherland et al. (1960) on two cases of untreated P.K.U., one with normal and the other with near-normal intelligence.

Tischler et al. (1961) reported on a very interesting family of three siblings, all male. The eldest was a known P.K.U. homozygote with marked mental retardation, the second-born was also a P.K.U. homozygote but with less-marked mental retardation, while the youngest showed no sign of the disorder and was of average mentality. Notwithstanding the differences in degree of intellectual attainment of the brothers, all exhibited similar E.E.G. patterns, with the youngest brother showing the best regulated. The oldest child was placed on a phenylalanine-restricted diet with some subsequent improvement in I.Q., behaviour and E.E.G. pattern.

The association of homozygous P.K.U. with average or near-average intelligence but with a concomitant behaviour disorder has been a frequent subject of comment. Among various reports, those of Allen and Gibson (1961) and Tapia (1961) will be mentioned. Allen and Gibson's patient was a twelve-year old boy who, possibly because of environmental factors, showed an improvement in the behaviour disorder over a two-year period and in whom initiation and maintenance of a phenylalanine-low dietary over a further period of seven months produced no further improvement. Tapia's report concerned an eight-year old boy who had an I.Q. of 91 and whose behaviour disorder manifested itself primarily by "nervousness", /

motor and speech difficulties, In this case, improvement in the behaviour disorder had taken place after only six months on a phenylalanine-restricted dietary. The child's teacher remarked that there had been sudden improvement in his reading and writing towards the latter part of this period. In cases such as those instanced above, it cannot be stated dogmatically how much the improvement is due to environmental factors and how much to the dietary therapy. The possibility of relationship between the levels of serum phenylalanine and the degree of intellectual capacity in P.K.U. was investigated by Partington in 1961. His findings with regard to the levels of intelligence in such patients were very similar to those previously published and showed that, for 75 untreated patients over the age of two, over 90 per cent had an I.Q. of 60 or less. He was unable to establish any relationship between serum phenylalanine levels and I.Q. except - somewhat tenuously - in the cases of three patients, each of whom had an I.Q. or more than 60 and whose serum phenylalanine levels were less than 25 mg. per 100 ml. He was also able to demonstrate in this paper that mental retardation in the sib of a known P.K.U. patient is not necessarily due to P.K.U. From his observations, he concluded that the full benefits to be obtained from a phenylalanine-restricted dietary, especially in the case of children whose disease had not been diagnosed in early infancy, would not be apparent until a sufficiently large number of such patients had been treated dietetically over a very long period. Knox (1960) also investigated the psychological aspects of P.K.U. and stated "there is a loss of 2 points of I.Q. per week of delay in initiating therapy". The significance of such a statement from an authority of his stature cannot be summarily dismissed. /

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As has already been stated, although mental retardation occurs in nearly all persons affected by untreated P.K.U., there can be great variety in its clinical picture. The mentally retarded P.K.U. child is typically hyperactive and behaviour is characterised by unpredictable and erratic activity. Excessive rocking movements, arm waving, grinding of teeth and general overall purposeless behaviour often suggest autism or childhood schizophrenia. Where mental retardation is severe, there is significant limitation in verbal communication and vocalisation may be unintelligible. Frequently there may be aggravation of the bizarre displays of motor activity, poor co-ordination and difficulties in visual-perceptual areas, by superimposition of convulsive disorders of the petit mal type. Untreated children are irritable, easily distractible and, in general, show an extremely induced frustration. They do not readily integrate, either with their own family circle or with society in general. Emotionally and socially they present a picture of very immature and overly-dependent youngsters. The desirability of continued environmental security, stressed by so many workers, is not isolated from the maintenance of such continued environmental security in other groups of mentally retarded children. In this respect, the three cardinal "R's" - Routine, Repetition and Relaxation - used with mentally retarded children in general, are appropriate in helping retarded P.K.U. children attain a more satisfactory environmental adjustment.

The early diagnosis of the disease and the initiation and maintenance of a phenylalanine-low dietary are all essential if normal or near-normal mental development is to be attained. Regardless of when childhood treatment is initiated, although intellectual gain may be minimal, improvement in behaviour is /

almost always manifest. To the perpetually-harassed mother of such a child, improvement of this sort may mean the crossing of the line dividing impossibility from possibility insofar as the day-to-day management of the family is concerned. In the successful management of a P.K.U. patient, particularly if a child, periodic psychological assessment is mandatory, even although there is not, as yet, complete understanding of the dynamics involved. The degree of mental retardation and the subsequent improvement derived from initiation and maintenance of a phenylalanine-low dietary combine to shift the emphasis more on to the physical management of the child rather than on to any attempted rehabilitation in terms of personality change to be brought about by conventional psychotherapy to enhance emotional maturation and stability in such children.

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## Methods of Detection and Diagnosis.

Despite the fact that signs and symptoms of the disease are well-known and documented, the diagnosis of phenylketonuria can be made with certainty only on the basis of certain laboratory findings. Prior to discussion of diagnostic laboratory procedures, several requirements merit consideration. For example, in large-scale screening of a population the primary requirements should be cheapness, speed of performance and simplicity. In such circumstances an absolute degree of accuracy is not essential, the reason for this being that such methods must, of necessity, be qualitative and will tend to discover false positive results. The selection of those members of the community under investigation who, by demonstration of presumptive positive results, may possibly be sufferers from the disease, is more important than their exclusion by initial adoption of methods of screening which may tend to allow truly positive cases to pass undetected. Again, with regard to the testing of individuals, is the patient a suspected homozygote or heterozygote? If the former, a comparatively simple diagnostic procedure will suffice; if the latter, load-testing and other complex procedures must be undertaken.

In the case of detection of the homozygote, two categories of tests are in general use:-

1. urine testing for the presence of phenylpyruvic acid by means of ferric chloride solution, "PHENISTIX", 2,4-dinitrophenylhydrazine, chromatography;
2. blood testing for the presence of phenylalanine by means of biochemical or microbiological methods. /

Ferric chloride testing of urine has long held pride of place as the method of detection employed in diagnosing P.K.U. First used by Fölling in 1934, surveys utilising this method have been operated till the present day. Centerwall (1957), - whose report was the first to deal with its large-scale application - Gibbs and Woolf (1959), Allen (1960), Centerwall et al. (1960), Farquhar et al. (1962), Lund and Wamberg (1964) and Larson (1964) were among those to publish reports of surveys conducted by means of ferric chloride. Several methods of performing the test exist, the simplest consisting in the dropping of a few drops of a 10 per cent ferric chloride solution onto a diaper previously wetted with an infant's urine. When this method is used, it is advisable that the diaper be recently wetted. Should this not prove feasible, it is possible for testing to be made on the diaper after the urine has dried but, as Centerwall et al. (1960) have pointed out, this leads to a diminution in the effectivity of the test. A positive result is reported when a medium-dark blue-green colour develops immediately and fades generally in less than 30 seconds. The fading starts at the centre of the spot of colour and the green-rimmed periphery is last to disappear. Farquhar et al. (1962), in the course of their Edinburgh survey, eventually preferred to use "KLEENEX" tissues which had been impregnated with the infant's urine rather than the diaper itself and to use the cork stopper of the ferric chloride solution bottle as a "stamp". They found increased reliability where the "KLEENEX" tissue had not been allowed to dry, and suggested "the tests should be carried out by an experienced person with normal colour vision, preferably at a central point such as the local health department". There is no doubt that all ferric chloride methods are liable to different interpretations of colour /

values, especially where slight differences in hue present themselves. Such differences in interpretation are most evident in the case of "PHENISTIX" test-strips, which will be dealt with later in this section.

The second method utilising ferric chloride solution is to add a few drops of a 5 to 10 per cent aqueous solution to a small amount of urine in a test-tube. The same blue-green colour develops as in the diaper or "Kleenex" test but is more quickly apparent and also fades more rapidly with 10 per cent solution than with 5 per cent (Centerwall et al., 1960). It is immediately obvious that, in the case of infants who are not yet toilet-trained, difficulty may be experienced in collecting the specimen of urine. Lund and Wanberg (1964) showed that with the use of increasing amounts of urine it became necessary to use more and stronger amounts of ferric chloride solutions in order to obtain a positive reaction. They also pointed out that excess of ferric chloride will cause a positive reaction to become apparently negative in a very short time. Larson (1964) pointed out that where the ferric chloride solution method was used and the urine contained excess phosphate, a greyish precipitate would form before the development of the blue-green colour. To obviate this, he suggested the prior addition of a few drops of dilute hydrochloric acid to precipitate such phosphate and thus minimise the danger of missing a weak positive test. Centerwall and Centerwall (1961) did not feel this to be necessary, pointing out that when only a small amount of urine (1 ml.) is used in the performance of the test, the acidity of 10 per cent ferric chloride solution itself (pH 1.8) is such that phosphates are dissolved. /

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The third method of employing ferric chloride is in the filter-paper technique (Berry et al., 1958; Centerwall et al., 1960). In this method a piece of filter-paper is impregnated with urine and allowed to dry, being subjected to the action of ferric chloride solution at some subsequently-convenient place and time. The reactions produced, in the main, are similar to those produced by the other variants of the ferric chloride technique. The advantage of this particular method lies in the fact that such filter-paper strips can be mailed to a central screening depot for examination. There is a disadvantage however, in that there is also a diminution in the effectivity of the test with the passing of time and false negative reactions may result. Thus, as Centerwall et al. (1960) pointed out, one such negative test cannot exclude the presence of P.K.U.

The fourth method is by the use of "PHENISTIX" test-strips. These strips, manufactured by the Ames Company, Nuffield House, London, W.1., are of strong filter-paper impregnated with a buffered ferric salt. The end of the strip is dipped into fresh urine or pressed between the folds of a diaper. Colour change, similar to other ferric chloride evaluations, is then compared against a colour chart incorporated in the label of the containing bottle, in order that a rough estimation of the amount of phenylpyruvic acid present may be obtained.

All the ferric chloride methods of detection may allow false-negative reactions, in consequence of which the sufferer from the disease will not be detected. Such false-negative reactions result from:-

1. the infant suffering from homozygous P.K.U. not yet excreting phenylpyruvic acid at the time of the screening. As has/

been stated previously (Armstrong and Binkley, 1956; Armstrong et al 1961), phenylpyruvic acid may not appear in an infant's urine till the age of six weeks. In this connection, the routine screening of infants' urine by Local Authority Health Visitors shortly after a mother has returned home from confinement in hospital is of little value unless the testing is delayed till after the child has reached the age of six weeks. Should this delay be allowed to elapse, in the case of an infant who shows a positive urine test and is then subsequently followed-up and proven biochemically, much valuable time will have been lost, time which may prove to have been of vital importance insofar as preservation of the child's intellect is concerned. The reasons for the appearance of phenylpyruvic acid in the urine have been dealt with in the chapter dealing with Metabolic Aspects. While it may be a generalisation that a blood level of phenylalanine of 12 to 15 mg. per 100 ml. is the minimum which will allow of the ready detection of urinary phenylpyruvic acid, Farquhar (1965) has shown that it is not absolute, having reported on the case of a child who consistently displayed a positive ferric chloride test for the presence of urinary phenylpyruvic acid and in whom the blood phenylalanine level was never shown to rise above 7.5 mg. per 100 ml. It is of interest to consider the opposite to this state of affairs. Guthrie (1964) quoted MacCreedy as having failed to demonstrate the presence of urinary phenylpyruvic acid in the cases of 4 proven cases of P.K.U. Since then, further instances have been recorded (Guthrie, 1965) and attributed to hyperefficiency of reabsorption by the renal tubule in some cases of the disease.

2. delay in processing filter-paper specimens gives rise to effectivity-diminution of the test (Centerwall et al., 1960). This/

happens when processing of filter-paper or "KLEENEX" specimens is delayed after arrival at central processing depots.

As is the case with false-negative reactions, all the ferric chloride methods of detection can cause the development of false-positive reactions. Such false-positive reactions, with one exception, are caused by the presence in the urine of substances reactive with ferric chloride which are the result of disease or the ingestion of drugs. The exception is the positive reaction which occurs in about 1 in 160 full-term neonates, due to immaturity of the tyrosine degradative pathway with consequent excretion of parahydroxyphenylpyruvic acid (Gibbs and Woolf, 1959). This is a transient and harmless condition not to be confused with P.K.U. Centerwall et al. (1960) listed some of the substances giving rise to such false-positive reactions as:-

1. salicylates - giving a blue-purple colour;
2. bile, homogentisic acid (in alcaptonuria), catecholamines (in pheochromocytoma) - giving a green colour (except that no change of colour is produced by homogentisic acid on "PHENISTIX");
3. maple syrup urine disease, oasthouse urine disease, histidinaemia - giving a green colour;
4. melanin (in malignant melanoma) - giving a grey colour;
5. diacetic acid (in acidosis) - giving a brown colour;
6. chlorpromazine and prochlorperazine - giving a light violet and purple colour respectively.

Centerwall and Centerwall (1961) stated that the "PHENISTIX" method was the most accurate and gave the fewest false-positive results. This is in keeping with the claims of the manufacturers but was not borne out by Gibbs and Woolf (1959) who could find no significant /

differences in the urinary phenylpyruvic acid levels detectable by any of the ferric chloride methods, one versus another. It should be noted that the colours produced by such reactive substances in the production of false-positive results are, with the exception of that produced by homogentisic acid, fairly stable and may persist for days. The colour produced by homogentisic acid is fleeting and lasts for a very few seconds.

Many large-scale screenings, particularly of infants, have been conducted by ferric chloride methods. All buttressed Centerwall's plea (1957) that such testing should be carried out at well-baby examinations in earliest infancy. Nonetheless, useful as they have been, their P.K.U. homozygote detection-rate can be bettered considerably by other techniques.

Centerwall et al. (1960) reported on the effectivity of urine testing with 2,4-dinitrophenylhydrazine, first utilised by Fellman (1956). Fellman's original paper dealt with the inadequacy of only employing ferric chloride solutions in screening programmes, having discovered in institutional screening, that chlorpromazine ingestion produced false-positive results. Centerwall et al. showed that this test was basically more reliable and sensitive than the ferric chloride test, giving false-negative reactions in less than 1 per cent of testings when used on known untreated cases of P.K.U. Of the substances mentioned previously which give a false-positive reaction with ferric chloride, only diacetic acid and the urine of maple syrup urine disease give positive reactions with this substance as does parahydroxyphenylpyruvic acid (Gibbs and Woolf, 1959). The reagent is prepared by adding 4 gm. of 2,4-dinitrophenylhydrazine to 1 litre of one normal hydrochloric acid. This mixture is heated in a water bath overnight to make a supersaturated,

approximately 0.3 per cent solution of 2,4-dinitrophenylhydrazine. The supernatant clear-yellow solution is filtered off and stored in a dark glass bottle. The test is performed in the following manner:-  $\frac{1}{2}$  - 1 ml. urine is placed in a test-tube. To this is added an equal amount of 2,4-dinitrophenylhydrazine as prepared above. Immediately after addition of this solution, the mixture is a pale yellow-orange solution and it will remain thus if the test is negative. The test is positive when, in the course of 1 to 5 minutes, the solution gradually becomes an opaque, bright yellow colour. This is a permanent reaction and will remain essentially unchanged if observed hours or even a day later. There is a possibility of confusion in interpreting test results if the urine is initially cloudy. When in doubt, the tester should duplicate the test and compare the opacities of the older and the fresher mixtures and should cross-check with ferric chloride testing in one of its varieties. Although this test is cheap, it is still about five times as costly as ferric chloride solution in equivalent amounts.

Berry (1959) described procedures of chromatography for testing of urine for phenylpyruvic acid, phenylalanine and ortho-hydroxyphenylacetic acid in patients suffering from P.K.U. and other metabolic disorders. The method for demonstration of phenylpyruvic acid and phenylalanine in urine consisted basically of ascending one-dimensional chromatography overnight, utilising n-butanol : acetic acid : water :: 1,000:33:1,000 as the solvent and, after drying, developing either with 0.3 per cent ninhydrin in 95 per cent ethanol for the demonstration of phenylalanine or 5 per cent aqueous ferric chloride to demonstrate phenylpyruvic acid. The method advocated for the demonstration of orthohydroxyphenylacetic acid /

was advanced by Armstrong et al. (1956), Veal (1961) and finalised by Woolfe (1963) as follows:-

"A sheet of Whatman No. 52 filter paper 10" square with corner holes punched to pattern A has 10 rectangular holes, 2 cm. X 1 cm., cut out so that the centres of the holes are 2 cm. apart and 3.5 cm. from one edge. From each urine-impregnated filter-paper, a rectangular strip, 3 cm. X 6 mm., is cut, the serial number being first written on the strip in pencil. The strip is fixed across the hole, using 2 Rexel No. 25 staples and a Rexel "Nipper" stapler. These tinned steel staples do not interfere with the running of development of the sheet - copper staples are inadvisable. The sheet is run overnight in isopropanol - 0.880 ammonia - water (8:1:1), taken out and dried in air. It is then sprayed with one of the reagents for phenols, viz., diazotised sulphanilamide reagent as described by Block, Durrum and Zweig (A Manual of Paper Chromatography and Paper Electrophoresis, 2nd. edn., Academic Press, p.132:1958) or a slightly simpler reagent, Brentamine Fast Red GG (Imperial Chemical Industries, Ltd.) with sodium acetate and sodium carbonate (Bloxam, Day, Gibbs and Woolf: Biochem. J., 77:320:1960). The finding of orthohydroxyphenylacetic acid, giving an orange spot of characteristic Rf with diazotised sulphanilamide or a purple spot with the Brentamine Red GG, is diagnostic for P.K.U. Occult cases are also detectable by this test".

Performance of this method was found to be time-consuming and frustrating and, as an alternative, adoption of the technique employed in chromatography for the demonstration of blood phenylalanine was undertaken. One-half inch diameter circular pieces of Whatman 3MM filter paper were punched from a whole sheet, using a commercial hand punch. The circles were separated by 4. cm. and /

and were punched 2 cm. from the bottom of the sheet. From urine-impregnated paper of similar grade,  $\frac{1}{2}$ " discs were punched and fitted carefully into the corresponding holes in the base of the complete large sheet. It was calculated that the area of the circle (154 sq.mm. approx.) would contain sufficient urine for the demonstration of the orthohydroxyphenylacetic acid and this was found to be so. After overnight running in the solvent advocated, a mixture of equal parts of an aqueous 0.5 per cent Brentamine Fast Red GG solution and 50 per cent (W/V) aqueous sodium acetate solution was sprayed on the paper, followed, one minute later, by spraying with a half-saturated aqueous solution of sodium carbonate. The orthohydroxyphenylacetic acid appeared as a very marked purple spot with an Rf of 0.89. The significance of the method will be discussed subsequently.

Biochemical methods for the determination of serum phenylalanine are the most widely-used where quantitative results are desired and are mandatory where any screening method demonstrates the existence of a "presumptive positive" P.K.U. With the gradual development of awareness in the efficiency of the modern microbiological methods, the possibility exists that such biochemical methods may be ousted in the future insofar as the routine check on blood levels of phenylalanine during dietary therapy is concerned. Nevertheless, biochemical evaluation allows of a truly-positive diagnosis being made very early in life. Guest et al. (1962) have, with such methods, demonstrated the existence of homozygous P.K.U. at 3 to 5 days after birth, thus allowing of the earliest possible introduction of a phenylalanine-low dietary. When such biochemical methods are properly conducted, the chance of a false-negative result is negligible. Newly-born sibs of known P.K.U. children /

120.  
should be tested by such methods, as Guest et al. have suggested, 3 to 5 days after birth. In this particular paper it was also suggested that an infant, suspected on such grounds of having the disease and who showed a level of phenylalanine within the normal range of 1 to 4 mg. per 100 ml. blood in the first week, should be retested at two, three and six weeks. Where the initial level in the first week was elevated above 4 mg. per 100 ml. blood, retesting should be performed immediately. Guest et al. stated that a serum phenylalanine level which rises above 15 mg. per 100 ml. by the third week of life confirms the diagnosis of P.K.U.

The earliest, but now somewhat outmoded, biochemical test to detect phenylalanine was the Kapeller-Adler reaction, first described by Kapeller-Adler (1932). In this procedure, nitration of phenylalanine is followed by photometric determination of the nitrated product with hydroxylamine. Armstrong and Tyler (1955) introduced a modification of the method which utilised trichloroacetic acid to precipitate out macromolecules before testing for phenylalanine. Utilising this method, normal levels in blood were found to be 3 to 5 mg. per 100 ml. The major disadvantage of Armstrong and Tyler's method lay in the fact that 10 ml. of serum were required and difficulty was frequently experienced in obtaining complete removal of the trichloroacetic acid prior to photometric examination. Henry et al. (1957) modified the technique still further by using picric acid in place of trichloroacetic acid and by using an ion-exchange resin to isolate phenylalanine before conducting the standard Kapeller-Adler reaction. Although this method only necessitated use of 1 to 2 ml. serum, accuracy was difficult to achieve, especially in the lower ranges. Henry et al. stated "it is probable that the accuracy of the technique is low in /

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the case of normal sera, and undoubtedly the precision is poor because the absorbances obtained with normal levels are in a region of high photometric error. Fortunately, however, these facts do not negate the value of analysis for the diagnosis of phenylpyruvic oligophrenia, in which the serum levels are about 15 mg. per 100 ml. or higher!

Another purely biochemical technique for the estimation of serum phenylalanine, and one which was till recently the most accurate, is ion-exchange chromatography. Moore and Stein (1951) demonstrated the possibility of separation of amino acids from mixtures by the use of such ion-exchange resins. In 1954 they described the use of such methods to determine plasma amino acids and detailed the necessary techniques (Stein and Moore, 1954; Moore and Stein, 1954; Moore and Stein, 1954). The basic method consists of using Dowex 50-X4 resin (Dow Chemical Co.) and eluting with sodium acetate/citrate buffers of gradually rising pH (3.1 to 5.1), utilising ionic strength to separate the amino acids. Photometric determination of the effluent fractions is subsequently made, using a modified ninhydrin reagent. No doubt attaches to the extreme sensitivity of the method, in respect of both qualitative and quantitative determinations, but its complexity and tediousness lessen its value other than as a research tool.

The most widely-used biochemical technique at the present time is the enzymatic spectrophotometric method of LaDu (LaDu and Michael, 1960). In this method, L-phenylalanine in the blood is first oxidised to phenylpyruvic acid by means of L-amino acid oxidase in snake venom. Such oxidation generates an enol-borate phenylpyruvic acid complex and the degree of absorption of this complex is measured spectrophotometrically. The procedure is less complex /

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than many others and gives accurate results.

The latest and possibly the most precise of the biochemical methods - although not yet widely adopted - is that devised by McCaman and Robins (1962). This method requires as little as 5 microlitres of serum whereas the latest modification of the LaDu method requires 100 microlitres (LaDu et al., 1963). Fluorimetric determination of phenylalanine content of serum by this method depends on the enhancement of fluorescence of a phenylalanine-ninhydrin reaction product, by leucylalanine, one of the peptides. Plasma is combined initially with trichloroacetic acid, the resultant precipitate (containing macromolecules) is discarded and the supernatant combined with ninhydrin and L-leucyl-L-alanine in a buffered medium. After appropriate incubation and dilution, the resultant solution is read in a fluorimeter. The basis of the technique rests in the fact that although some fluorescence is developed by the combination of phenylalanine and ninhydrin, the addition of a predetermined amount of L-leucyl-L-alanine increases the fluorescence by a known factor and it then becomes readily possible to calculate the amount of phenylalanine present in the initial specimen. The technique - apart from the initial expense of the particular fluorimeter specified - has many advantages over other methods. It requires only 5 to 10 microlitres of serum, is simple, direct and relatively rapid in performance, allowing the assay of 50 serum specimens in three hours (McCaman and Robins, 1962).

The use of paper chromatography to detect elevation of serum phenylalanine has also found favour as a biochemical method. Berry (1957), Fischer et al. (1960), Berry (1962) and Hudson et al. (1963) have all published reports on its efficacy. Berry, and Fischer /

15.  
et al., utilised semi-quantitative methods involving reading of the ninhydrin-developed chromatograms either visually or with a densimeter. Neither suggested that such techniques should supplant more accurate biochemical techniques but would have a use in following patients during treatment or where extreme accuracy was not required. Hudson's variation involves elution of the phenylalanine from the chromatogram followed by its colorimetric determination, and possibly possesses greater accuracy than the other methods. A full description of the chromatography methods used as corroboration in the performance of the Guthrie Microbial Inhibition Assay will be described fully in the chapter dealing with the Guthrie technique. Chromatography is simple to perform, relatively cheap and requires small amounts of blood or serum, e.g., 0.05 ml. It still remains, nevertheless, a technique not fully suited to large scale investigations and will continue thus until some suitable method of automation is devised.

Regarding the microbiological methods for the estimation of serum or blood phenylalanine, the oldest is that based on the work of Shankman (1943) who noted that the "essentiality" of an amino acid could be determined by attempted culture of suitable micro-organisms in a culture medium lacking one or more such amino acids. In his work he employed Lactobacillus arabinosus, A.T.C.C. 8014. Hier et al. (1945) showed that it was feasible, by utilisation of several strains of bacteria, to label numerous amino acids by prior determination of the amino acid requirements of such bacteria. By this method they were able to test the amino acid content of blood plasma. For the detection of phenylalanine they used Lactobacillus casei, A.T.C.C. 7469 and concluded that the microbiological /

technique provided a "rapid, convenient and accurate procedure" for measuring amino acids quantitatively. From this work, Steel et al. (1949) developed a dehydrated growth medium for the assay of L- and DL- phenylalanine. This medium is free from phenylalanine but contains all other growth factors and amino acids necessary for the growth of Leuconostoc mesenteroides, A.T.C.C. 8042, in the microbiological assay of phenylalanine. The addition of L- or DL- phenylalanine, in specified increased concentrations, gives a growth response by the test organism which may be measured turbidimetrically or acidimetrically. This method, when properly conducted, will give an accuracy of  $\pm 2.5$  microgrammes phenylalanine per 1 ml. The growth medium and the micro-organisms are marketed commercially by the Difco Labs., Detroit, Michigan, U.S.A. and the assay method is excellent for follow-up or individual cases under treatment although too complex for routine use. Since the method has been used for the checking of the Guthrie blood levels in several cases, it will be given in detail. The word "BACTO" is a registered trade mark of the Difco Laboratories.

Stock cultures of the test organism are prepared by stab inoculation into tubes of prepared Bacto-Micro Assay Culture Agar. After 24 hours incubation at 35 - 37 degrees Centigrade the tubes are stored in the refrigerator. The transplants are made at monthly intervals. The inoculum for assay is prepared by sub-culturing the test organism into 10 ml. Bacto-Micro Inoculum Broth. After 16 to 24 hours incubation at 35 to 37 degrees Centigrade, the cells are centrifuged under aseptic conditions and the supernatant liquid discarded. The cells are resuspended in 10 ml. sterile 0.85 per cent sodium chloride solution. One drop of the latter suspension is used to inoculate each of the assay tubes (10 ml.). /

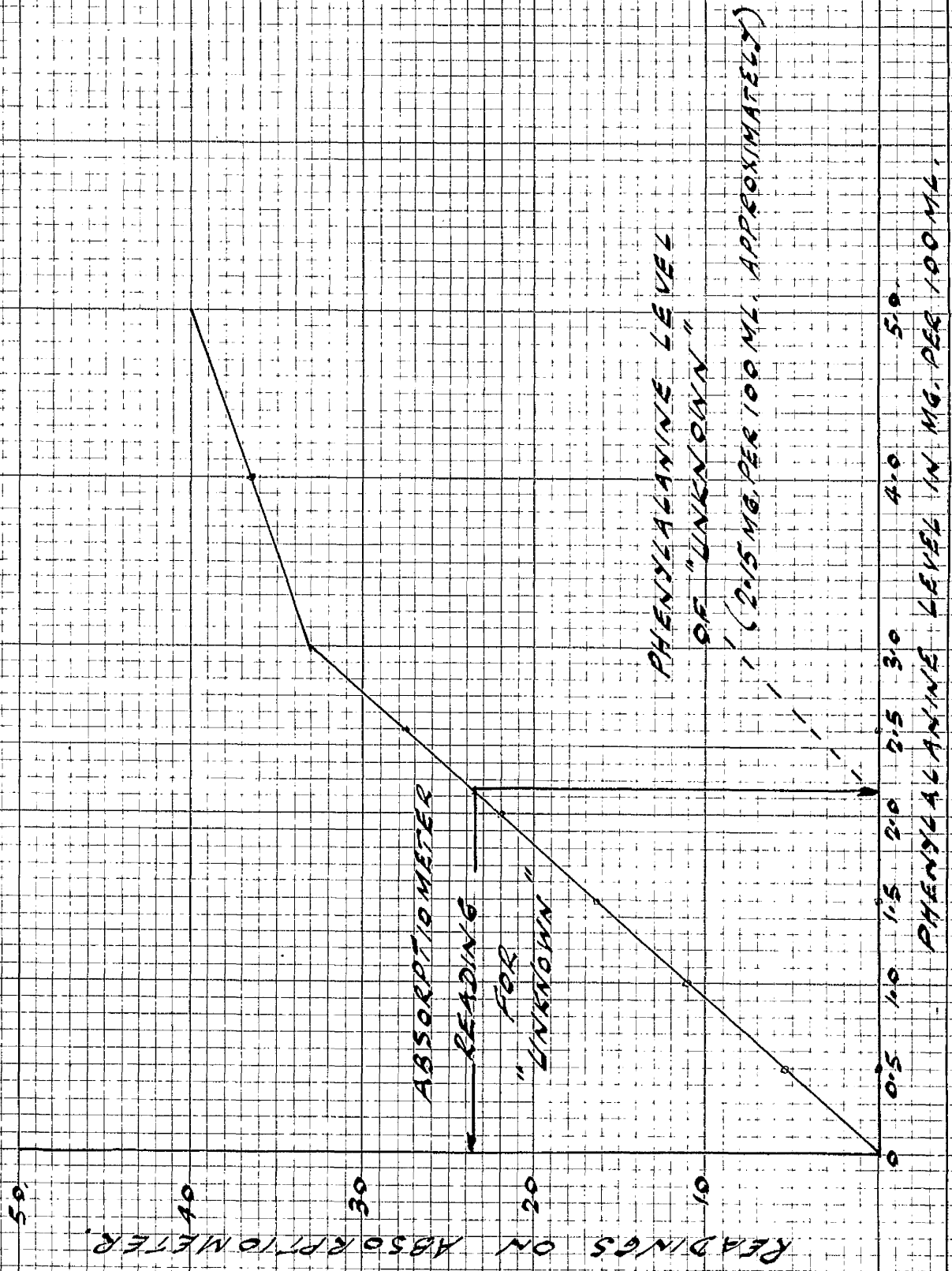


Figure 11.

It is essential that a standard curve be prepared each time the assay is performed, since conditions of autoclaving, temperature and duration of incubation, etc., cannot be accurately duplicated on each occasion. A standard curve is obtained by using L-phenylalanine at levels of 0, 5, 10, 15, 20, 25, 30, 40 and 50 microgrammes per 10 ml. assay tube. Turbidimetric readings are made after 16 to 20 hours incubation at 35 to 37 degrees Centigrade. The concentrations of phenylalanine required for the construction of the standard curves may be prepared by dissolving 1.0 g. of L-phenylalanine in 1,000 ml. of distilled water, giving a stock solution of 1,000 microgrammes phenylalanine per 1 ml. Dilute the stock solution by adding 1 ml. to 99 ml. distilled water to make a standard solution containing 10 microgrammes phenylalanine per 1 ml. Use 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 ml. per tube. When stored under toluene, the stock solution of phenylalanine remains stable for two months at 2 to 6 degrees Centigrade. To rehydrate the medium, suspend 10.5 g. Bacto-Phenylalanine Assay Medium in 100 ml. distilled water and heat to boiling for 2 to 3 minutes. The slight precipitate which forms should be evenly distributed by shaking. 5 ml. of the medium are added to each tube in the preparation of the tubes for the standard curve and to each tube containing material under assay. For the assay, each tube must contain 5 ml. of rehydrated medium, increasing amounts of the standard or unknown, and sufficient distilled water to give a total volume of 10 ml. per tube. The tubes are autoclaved for 10 minutes at 15 lbs. pressure (121 degrees Centigrade). Over-sterilisation of the medium will give unsatisfactory results. A typical curve obtained by this method is shown in figure 11 with insert information with regard to the method of calculating /

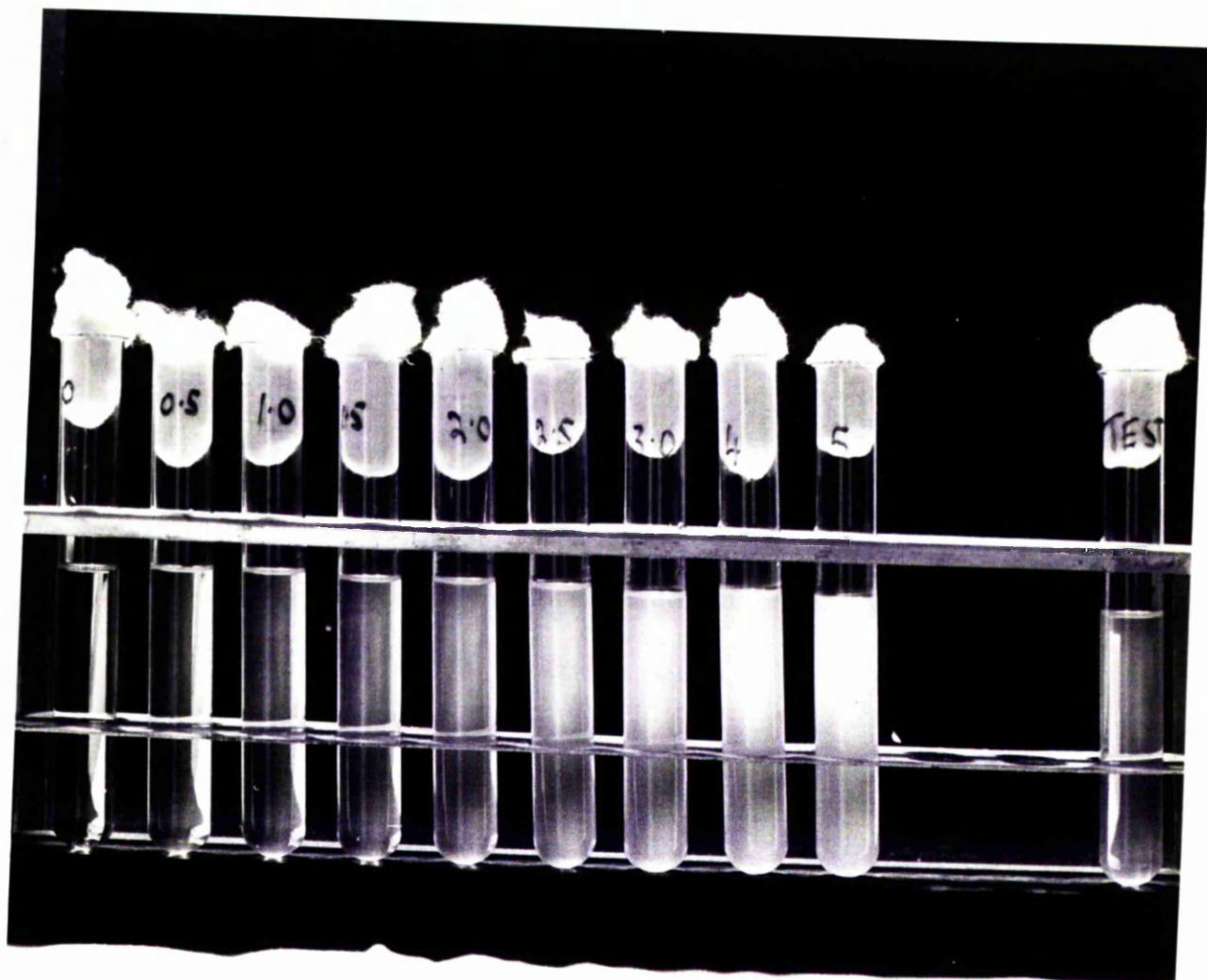


Plate 1.

the amount of the unknown. Plate 1 shows the assay tube-range.

A combined biological/biochemical method is that of Udenfriend and Cooper (Udenfriend and Bessman, 1953; Udenfriend and Cooper, 1953). In this procedure, phenylalanine in plasma is decarboxylated to phenylethylamine through utilisation of Streptococcus faecalis decarboxylase. Determination of the phenylethylamine is then made by means of the methyl orange procedure for organic bases. One mole of the amine is formed for each mole of the phenylalanine decarboxylated so that determination of the amine level is a direct reflection of the phenylalanine level. The method is very accurate but necessitates prior procedures of complexity and tediousness to prepare the bacterial extract before the test can itself be conducted.

The sole remaining method of homozygote detection awaiting discussion is that devised by Guthrie (1961) which will be discussed fully in the following chapter. Before discussing this particular matter, the question of detection of the heterozygote for the disease will be examined.

No simple method exists for the detection of the person heterozygous for P.K.U. From the standpoints of public health, epidemiology of P.K.U. and marriage counselling, such a simple method of detection would prove a boon. Methods of detection in vogue at the present day are primarily only of value as research tools or where a relative of a known P.K.U. patient requires investigation. Penrose (1946) discussed the matter of heterozygote detection and concluded, that for all practical purposes, it was not readily possible to identify the heterozygote state. There is no significant change in the position at the present day. The mainstay in studies designed to detect such heterozygotes has been that of /

phenylalanine loading. Hsia et al. (1956) were the first to investigate such loading studies and, as has already been mentioned in the chapter dealing with Biochemistry and Metabolic Aspects, in consequence of their findings, postulated that Pauling's concept of molecular disease could be extended to P.K.U. It is felt that elaboration of this aspect of the question should now be made. They stated "An extension of this concept of molecular disease leads to the prediction that in hereditary diseases associated with the loss of activity of an enzyme, the asymptomatic heterozygotes should be identifiable by having less of the enzyme normally present and primarily involved in the disease". They suggested, from the mode of inheritance of the disease, that the fraction of enzyme arising from the abnormal gene in the heterozygote should be defective. In the loading studies on relatives of known P.K.U. sufferers and normal controls who had no known connection with anyone suffering from the disease, they used 0.1 g. per kilo body weight. They showed that the relatives of the P.K.U. homozygotes displayed a diminished ability to metabolise the phenylalanine load but only as a group, there being wide divergence in individual levels when comparing such individuals against the control subjects. They stated "The most probable interpretation of the results is that the heterozygotes as a group have significantly less than the normal phenylalanine-oxidising enzyme activity. While the results in a single individual must be interpreted with caution, the abnormal values found in a group of known heterozygotes establish the decreased ability to metabolise phenylalanine as the phenotypic expression of a single P.K.U. gene. This finding provides confirmation for the view that lack of the phenylalanine-oxidising enzyme is the primary lesion in P.K.U. Finally, the presence of /

80.

part of the enzyme in an inactive form in the heterozygote with one abnormal gene, and nearly all the enzyme inactive in the homozygote with two abnormal genes, extends the concept of hereditary molecular diseases, based till now on the abnormal haemoglobins, to those disorders called by Garrod, 'inborn errors of metabolism'. The samples for the determination of phenylalanine were taken at 1, 2 and 4 hours after the load had been administered and it was found that, on the average, heterozygotes had plasma levels twice those seen among the normal controls. Also in 1956, Hsia and Driscoll described observations made on heterozygous carriers of P.K.U. without the use of a load. Plasma levels of phenylalanine were determined after an overnight fast in 37 parents of known P.K.U. homozygotes and 34 normal adult controls. The plasma levels of the two groups overlapped considerably but the difference between the means was significant. The authors suggested that the data presented added confirmatory evidence for the detection of heterozygous carriers of P.K.U. and suggested that the phenylalanine loading merely brought out the difference between the two groups more strikingly. As previously stated, Knox and Messinger (1958) also demonstrated that persons heterozygous for P.K.U. had elevated basal levels of phenylalanine in their bloodstreams but used this as an argument to discredit the theory of "molecular disease" as postulated by Hsia et al. as being operative in P.K.U. The crux of their reasoning was based on the demonstration that serum phenylalanine levels in heterozygotes were 1.5 to 2.5 times normal when both heterozygote and normal levels were measured under the same conditions of fasting or phenylalanine loading. The telling sentences have also been quoted in the chapter dealing with Biochemistry and Metabolic Aspects. It would appear that, with /

016

the passing of time, Hsia has laid less emphasis on the "molecular disease" postulate. Be that as it may, phenylalanine loading tests are accepted as being more reliable in the detection of the heterozygote state than estimation of the fasting serum phenylalanine levels by themselves. Renwick et al. (1960) introduced a refinement in the loading technique by applying a correction factor for the dose of phenylalanine administered and utilised, as a base-line evaluation point, the serum phenylalanine level 4 hours after ingestion. This particular approach was further investigated by Wang et al. (1961) who used the weighted logarithms of fasting serum levels of phenylalanine as a discriminant measure and estimated the levels after loading at 1.5, 2 and 3 hours. They concluded that estimation of the serum phenylalanine levels at 1 and 4 hours did not increase the discriminant power of the measurements. By their method they were able to distinguish the heterozygote state with a classification error of 4 per cent.

It may be that the most precise method so-far adopted in the detection of the heterozygote is that first advocated by Hsia (1958) and subsequently refined by Jervis (1960) and Anderson et al. (1962). In this method, which depends for its efficacy on the concept that it is possible to determine the activity of phenylalanine hydroxylase in any individual by measuring the amount of tyrosine, the product of the reaction catalysed by the enzyme, it has been shown that homozygotes for the disease show little or no rise in serum tyrosine levels after the administration of a loading dose of phenylalanine. On the other hand, normal individuals display a rise which is approximately three or four times as high. Heterozygotes for the disease display a curve intermediate in configuration between the "plateau" curve of the homozygote and the "pinnacle" curve of the /

normal person. It has been demonstrated that there is no degree of overlapping in the levels displayed by the various groups.

Anderson et al. (1962) stated "the rate of tyrosine formation within a two-hour period after such loading appears to be a critical method of measurement of partial gene-enzyme deficiency".

Although it was stated by Woolf (1963) that the demonstration of urinary orthohydroxyphenylacetic acid was diagnostic for P.K.U., studies conducted by Cullen and Knox (1958) showed that equivalent loading with phenylalanine produced equivalent excretion of the acid in normal persons, heterozygotes for the disease and similar homozygotes. From this they concluded that the method was not suitable for such heterozygote detection.

Hsia et al. (1961) investigated the excretion of 5-hydroxy-indoleacetic acid after phenylalanine loading. It was demonstrated that heterozygotes for the disease excreted a smaller amount of this acid than a group of normal controls. They postulated that the depression in the synthesis of 5-hydroxyindole compounds by the heterozygotes might well result from inhibition of 5-hydroxytryptophan carboxylase by the presence of increased levels of phenylalanine and its metabolites in such persons.

As was suggested in the chapter dealing with Metabolic Aspects, the increased excretion of urinary phenylethylamine after the administration of mono-amine-oxidase inhibitors to homozygotes for the disease should be worthy of investigation in respect of heterozygote detection. Perusal of the literature has failed to show publication of results concerned with such studies.

At the present, it would appear that the most widely-used method of heterozygote detection is that of phenylalanine loading followed by subsequent estimation of the serum phenylalanine level./

Whether or not this method is really as accurate as the method of detection which depends upon the measurement of serum tyrosine levels is debatable. Nevertheless, with the development of modern methods, it should not prove unduly difficult to determine the serum levels of the two substances after such phenylalanine loading. The combined use of the methods of Wang et al. (1961) and Jervis (1960) would appear to be the method of choice. Whether the combined method will be superseded by the estimation of the urinary phenylethylamine after the administration of phenylalanine must await reports on the accuracy of this method in the detection of the heterozygote state.

Irrespective of which substance or substances may subsequently be measured, almost invariably the ingestion of phenylalanine causes the development of extreme nausea and vertigo in the subjects. Even after the disappearance of those symptoms there may be the objective impression of alcohol-induced intoxication; needless to say this stage is followed by that of the conventional "hangover".

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04.

"Guthrie Microbial Inhibition Assay".

This particular test-method is the most-recently introduced of the microbiological methods. (Guthrie and Tieckelman, 1960; Guthrie, 1961, 1962). Basically, the method depends on "biochemical antagonism" between two substances. It was shown by Woods (1940) that the mode of action of sulphanilamide in vivo was based upon competition with para-aminobenzoic acid. This latter substance was shown to be a vitamin necessary for the synthesis of a second vitamin, folic acid. The similar structures of sulphanilamide ( $p\text{-H}_2\text{N}-\text{C}_6\text{H}_4\text{-SO}_2\text{NH}_2$ ) and para-aminobenzoic acid ( $p\text{-H}_2\text{N}-\text{C}_6\text{H}_4\text{-COOH}$ ), immediately suggested that the action of sulphanilamide was due to competition for an enzyme-centre specific for this molecular structure. Since that discovery, it has become clear that metabolic pathways in living cells involve multiple steps, with possibilities for such molecular antagonisms arising at every step. In this sense, sulphanilamide was shown to be an "antimetabolite".

Subsequent to the presentation by Darwin of his views on the origin of species, genetic and biochemical studies conducted particularly in the last 50 years have re-emphasised and documented his central idea of the common origin of all living things. Genetic studies have shown that all living cells have a common apparatus for controlling inheritance through deoxyribonucleic acid. Biochemical studies have shown that similar metabolic pathways exist for the acquisition of energy in all living cells and that this energy is used to drive the biochemical reactions for the synthesis of living materials along such similar metabolic pathways. It is known that molecular changes in cells occur in very small steps involving relatively small expenditure or uptake of energy and /

small changes in molecular structure, so that the intermediates, because of this similarity between them, can compete with each other for the common enzyme-centres in a similar manner to that which sulphanilamide competes with para-aminobenzoic acid. This can be shown in numerous biological systems and Bonner (1946) showed that in a mutant of the mould, *Neurospora*, a double amino acid requirement resulted from a single genetic block in biosynthesis. The double requirement shown to be due to an "internal inhibition" of the second amino acid caused by the accumulation of the keto-acid precursor of the first, which then entered into competition for an enzyme which carried out the biosynthesis of the second amino acid from its keto-acid precursor. It was thus shown by Bonner that there can be "molecular antagonism" between two naturally-occurring metabolites, as well as between a naturally-occurring and a synthetic "antimetabolite". Hitchings et al. (1948) showed that a synthetic pyrimidine, 5-nitrouracil, produced a growth-inhibition of Lactobacillus casei which could be relieved by the addition of another pyrimidine, 5-bromouracil, to the medium. They demonstrated that "molecular antagonism" can exist between two "unnatural" compounds, one behaving as an inhibitor and the other as an agent relieving the inhibition (a "pseudo-metabolite"). Guthrie and Tieckelman (1960) were the first to utilise this considerable summation of knowledge in the development of microbial assays for the purpose of delineating biochemical differences associated with human disease. For their purposes and with especial reference to the detection of phenylalanine in P.K.U., they chose Bacillus subtilis, ATCC 6051 as the micro-organism and beta-2-thienylalanine as its antimetabolite. The fully-developed technique, as devised by Guthrie and Susi (1963), will now be detailed. /

100

PRINCIPLE. The inhibition of growth of Bacillus subtilis, ATCC 6051, by beta-2-thienylalanine in a minimal culture medium, is prevented by phenylalanine, phenylpyruvic acid and phenyllactic acid. This finding has permitted the development of a convenient agar diffusion microbial assay for P.K.U., employing small filter paper discs, impregnated with blood, placed upon the surface of the agar culture medium.

PROCEDURE. A. Preparation of Specimens.

1. A small amount of fresh blood obtained by heel puncture is applied immediately to a piece of thick, very absorbent filter paper. Generally Schleicher and Schuell No. 903 is employed in U.S.A. but in all investigations conducted for the purpose of this thesis, 31 ET Whatman has been employed. This particular British paper matches very closely the characteristics of its American counterpart. The blood spot, when air dried, should be at least  $\frac{3}{8}$ " in diameter (but not more than  $\frac{1}{2}$ " ) and close enough to the edge of the paper to facilitate punching out the disc. The paper is so absorbent that even very viscous blood from a young infant spreads through, so that the appearance of the blood spot is similar on both sides of the paper. These conditions must be met in order to obtain a uniform blood sample by means of the paper punch.

2. Before assay, the individual filter papers are numbered in series with pencil, placed on pieces of metal screening or wire test tube racks and autoclaved at 15 lbs. pressure for 3 minutes with dry steam. This prevents the subsequent diffusion of blood pigments from the paper discs into the agar during the incubation period with consequent masking of possible growth zones. Prolonged autoclaving will destroy the phenylalanine. /

3. A disc,  $\frac{1}{8}$ " in diameter, is then punched from the centre of the blood spot.

4. It is convenient to place the discs as they are punched in rows on a clean piece of paper, in the same sequence in which they will be placed on the agar dish for the assay. The discs are placed within numbered squares printed on the paper. An alternative method consists in using a "PERSPEX" plate with rows of depressions incorporated in it, placing each disc as it is punched in its corresponding depression in the "PERSPEX" plate.

B. Preparation of the Assay Medium.

The formula for this is a modification of Demain's medium (1958).

|                                      | <u>Grammes/Litre.</u> | <u>Grammes/Litre.</u> |
|--------------------------------------|-----------------------|-----------------------|
| Dextrose                             | 10.0                  |                       |
| K <sub>2</sub> HPO <sub>4</sub>      | 30.0                  |                       |
| KH <sub>2</sub> PO <sub>4</sub>      | 10.0                  |                       |
| NH <sub>4</sub> C1                   | 5.0                   |                       |
| NH <sub>4</sub> NO <sub>3</sub>      | 1.0                   |                       |
| Na <sub>2</sub> SO <sub>4</sub>      | 1.0                   |                       |
| Glutamic Acid                        | 1.0                   |                       |
| Asparagine                           | 1.0                   |                       |
| L-alanine                            | 0.5                   |                       |
| <u>Salts solution (10 ml.)</u>       |                       |                       |
| MgSO <sub>4</sub> .7H <sub>2</sub> O |                       | 10.0                  |
| MnCl <sub>2</sub> .4H <sub>2</sub> O |                       | 1.0                   |
| FeCl <sub>3</sub> .6H <sub>2</sub> O |                       | 1.0                   |
| CaCl <sub>2</sub>                    |                       | 0.5                   |

The constituents in the amounts shown in Table 4. are dissolved in 900 ml. water (distilled). The pH will be 6.8 to 7.0. A 90 ml. /

volume of the solution is dispensed in each of ten 8-ounce prescription bottles and sterilised by autoclaving. The 10 per cent solution of dextrose is sterilised separately and used in 10 ml. supplements in each bottle of the medium. Similar bottles containing 100 ml. of 3 per cent agar are prepared and sterilised. For use, the dextrose solution is added to the sterile medium and 100 ml. of the resultant mixture is added to 100 ml. of agar, previously melted and then cooled to 55 degrees Centigrade in a water bath.

### C. Preparation of the Inoculum.

The use of a dried spore powder as the inoculum is very convenient and helps to ensure uniformity of results. This is prepared as follows:- bottles or Petri dishes containing a potato infusion agar medium (e.g., Difco Bacto B 51) are prepared. The agar surface is inoculated heavily with a cell suspension of Bacillus subtilis ATCC 6051 from overnight cultures grown on Difco Heart Infusion agar slopes. During incubation for one week at 30 degrees Centigrade, the growth is examined microscopically at intervals for the presence of spores. When present in suitable amount, these are washed off the agar into 0.85 per cent sodium chloride solution and subsequently washed three times in similar sodium chloride solution by centrifuging at 11,000 r.p.m. Final suspension is made in distilled water at an optical density of 0.9, measured at 550 millimicrons wave length in a colorimeter; 0.3 ml. of this suspension is dispensed in each of a large number of screw-capped bijou bottles, dried on a shaking machine at 60 degrees Centigrade and stored at 2 to 5 degrees Centigrade with the cap tightly closed. In use, 1 or 2 ml. of Demain's medium is added to the bottle, the dried powder scraped off the sides of the bijou /

bottle with an orange stick and the suspension decanted into 200 ml. of the culture medium.

D. Beta-2-Thienylalanine.

This compound (California Biochemical Corporation, Calbiochem Catalogue No.5901) is made up in a 0.01M solution and 0.3 ml. is pipetted into each of a number of screw-capped bijou bottles, dried and stored at room temperature till needed. Personal preference - without loss of growth-inhibitory effects being shown in nine months of storage - is for the inhibitor to be made up in 5 ml. amounts and kept in the frozen state. In use, after thawing at room temperature, 0.3 ml. is pipetted into 200 ml. of Demain's medium with aseptic precautions. This provides a final concentration of  $1.5 \times 10^{-5}$  of the inhibitor.

E. Preparation of Controls.

Outdated blood is obtained from the blood bank and assayed for phenylalanine content by the LaDu, McCaman and Robins or any other accurate and readily available procedure. L-phenylalanine is then added to a series of aliquots of blood to make concentrations of 2, 4, 6, 8, 10, 12 and 20 mg. per 100 ml. With a sterile pipette, the blood is spotted on the Whatman filter paper, to make spots of between  $\frac{3}{8}$ " and  $\frac{1}{2}$ " in diameter. After drying, these control spots are kept in a dessicator at 2 to 5 degrees Centigrade. The controls are autoclaved simultaneously with the "unknowns" before the discs are punched from each spot.

F. Procedure for Assay.

After combining the culture medium, agar, beta-2-thienylalanine and the spore powder, all are thoroughly mixed by pouring back and forth and finally pouring into a suitable flat dish. Such a dish is 8" X 12" Pyrex baking dish or a transparent styrene plastic tray./

**PKU BLOOD TEST**

Laboratory **PKU** No **5062**

Write in pencil

Patient's Name: BABY JONES

Address 33 OAK AVE.

Ward 25

Hospital STOBHILL

Date of Specimen 17.10.65

Date first milk feeding 12.10.65

Bottle  Breast  Both

Date of Birth 11.10.65

FILL ALL CIRCLES WITH BLOOD



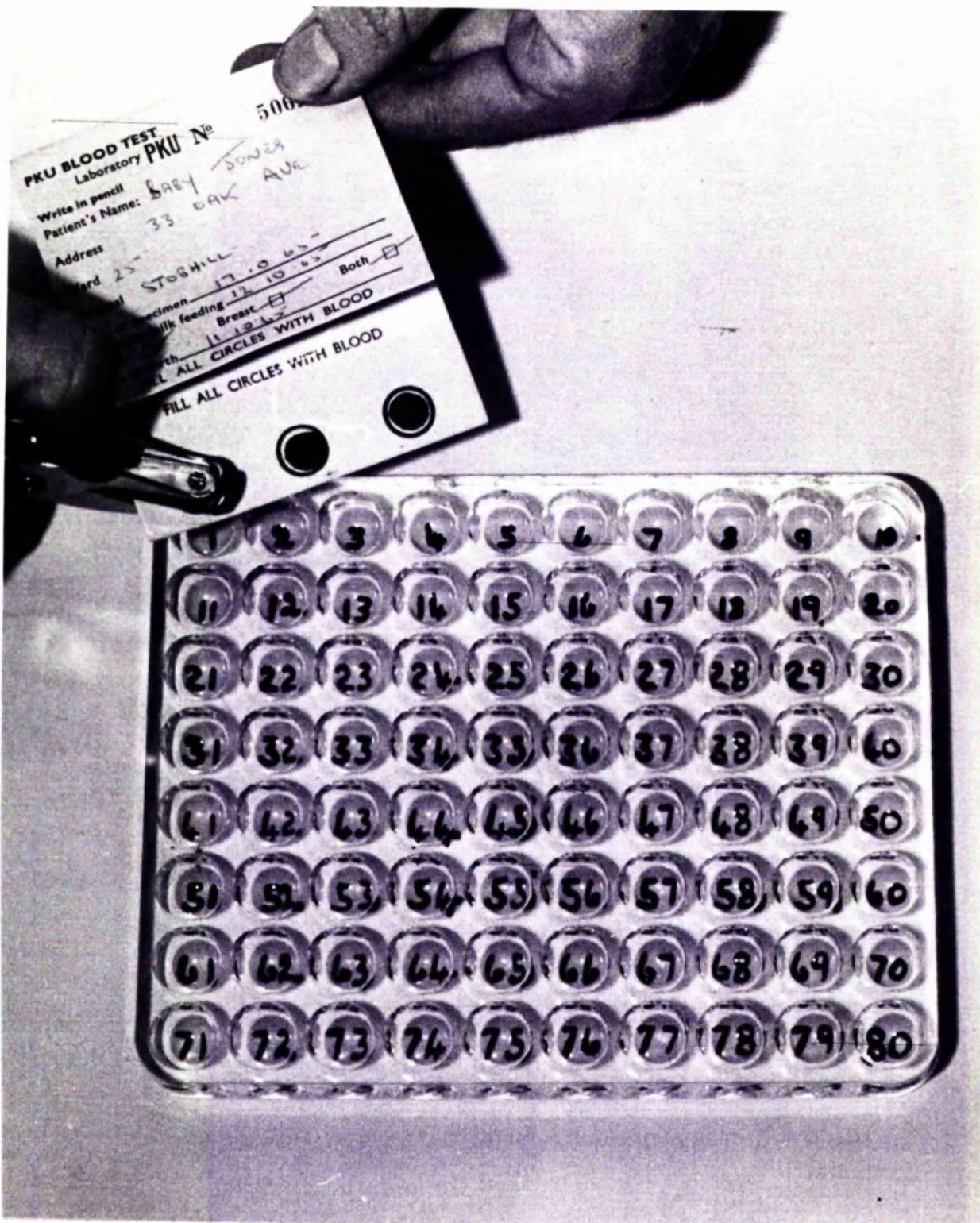


Plate 3.

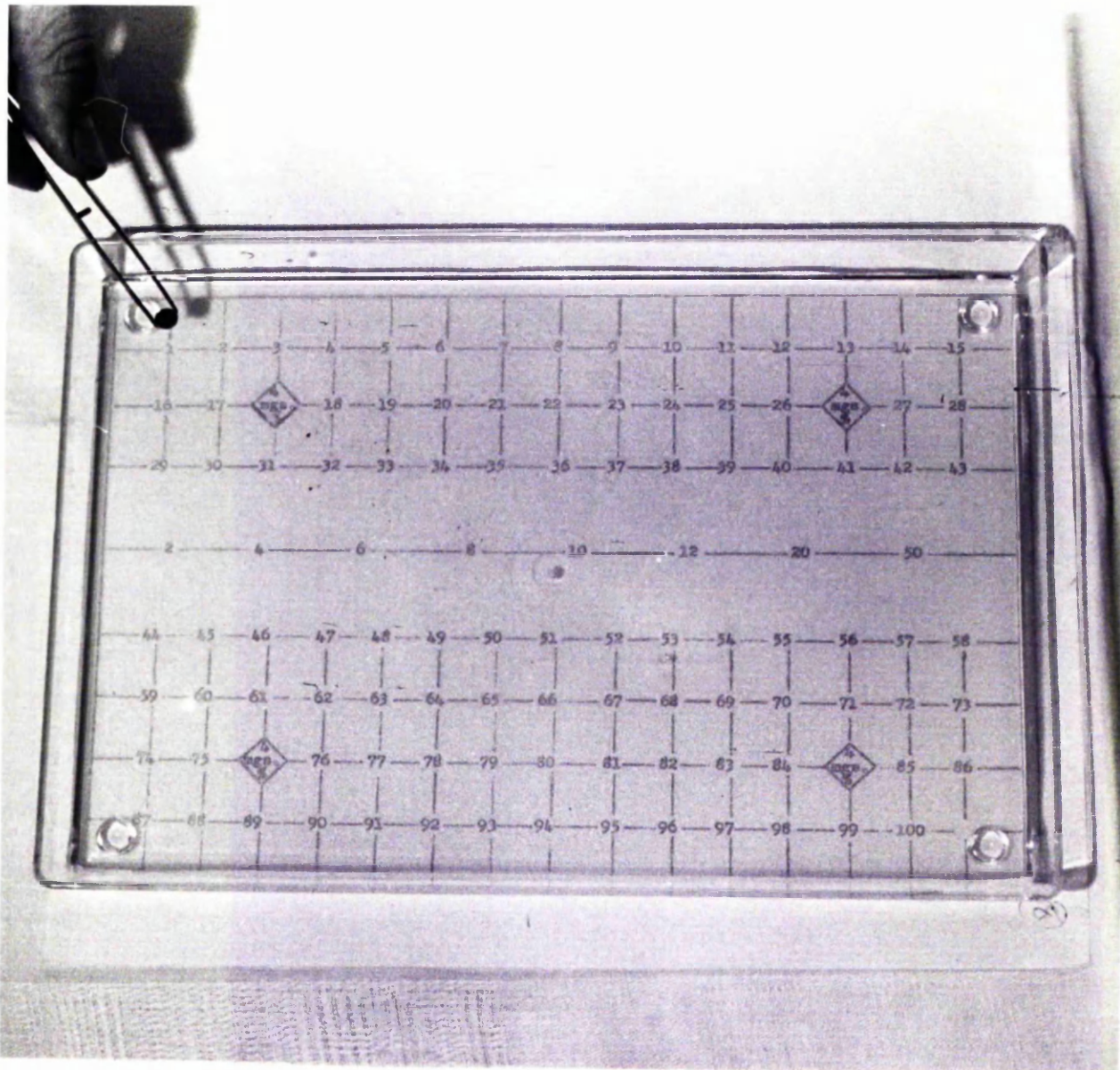


Plate 4.

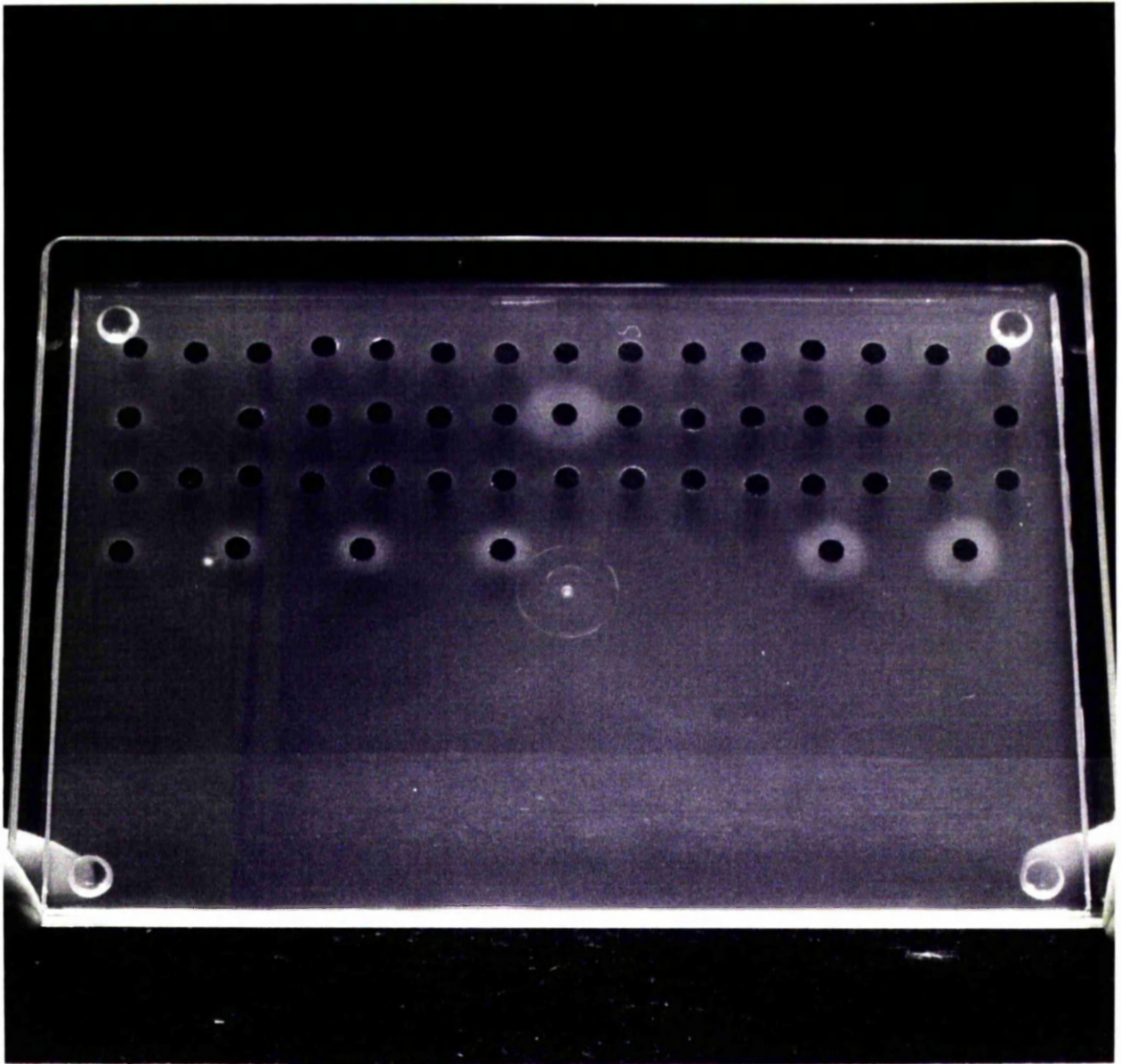


Plate 5.

It is essential that the top of the bench be perfectly flat to ensure an agar layer of uniform thickness. After the agar has set, the tray is placed over a paper pattern sheet with a printed grid containing an appropriate number of intersecting lines (50 to 100), and a suitable space approximately 1" wide, with positions marked for the control discs. With forceps, the control discs and the discs from the "unknowns" are placed in position. The agar tray is then placed in an incubator at 35 to 37 degrees Centigrade, overnight, for examination the following morning. In practice, the tray is kept at room temperature till 5 p.m. before placing in the incubator. In the incubator it is advisable to invert the tray lest condensation develop and cause masking of the results by distortion of the surface growth of the germinating spores of Bacillus subtilis or by causing wave patterns to develop on the surface of the agar. After 24 hours incubation, increasing background growth in the agar interferes with interpretation of results. The complete sequence is presented in pictorial form in plates 2 to 5. It will be seen from plate 5 that there is bacterial growth around the control discs and one other. Such discs are those which contain phenylalanine and thus neutralise the growth-inhibitory effect of the beta-2-thienyl-alanine. The diameter of the growth zone around a disc, compared with the diameter of the growth zones around the control discs, gives an estimate of the phenylalanine concentration of the blood. In the initial papers on the implications raised by the technique it was suggested that the critical level - above which retesting by the inhibition assay and re-examination by a biochemical method was mandatory - was 6 mg. per 100 ml. In consequence of a subsequently-proven P.K.U. who showed an initial level of 4 to 6 mg. per 100 ml. blood at 4 days, Guthrie (1964) recommends that all cases which /

initially show a blood phenylalanine level in excess of 4 mg. per 100 ml. should be retested by his method one week later. On such retesting, if the phenylalanine level has risen, biochemical evaluation must be undertaken. 4 mg. per 100 ml. is thus the "presumptive positive" level.

The Guthrie technique can be performed within a few days of birth utilising blood obtained by heel-prick. It can give an indication, at such an early age, if an infant is a possible sufferer from P.K.U., whereas, as already noted, ferric chloride urinalysis may not become positive till the infant is 4 to 6 weeks of age. It is comparatively inexpensive to perform on a large scale and one technician can set up 200 to 300 tests daily without being harassed. In consequence of its semi-quantitative nature, it can be readily utilised to follow the success of dietary therapy without having recourse, as would be the case in infants, to frequent fontanelle puncture with its attendant hazards.

Objections which have been raised to the Guthrie technique will be dealt with in the chapter dealing with "Discussion".

The bacterial "Inhibition Assay" already discussed, although efficient in the detection of P.K.U., requires confirmation before a "presumptive positive" case can be properly called "truly" positive. As previously stated, confirmatory evidence of a biochemical nature is mandatory, preferably conducted by an independent laboratory. It may be that access to such a biochemical laboratory is not easy. In such cases, and also for the further evaluation of the initial results by the laboratory making the preliminary microbiological diagnosis of the condition, the methods now to be detailed were formulated by Guthrie and Susi (1963) and modified by Efron et al. (1964). /

The procedure involves ascending overnight paper chromatography followed by development with ninhydrin reagent and further "inhibition assay" of paper strips cut from the chromatogram prior to development. A sheet of the Whatman filter paper has a pencil line ruled  $\frac{7}{8}$ " from, and parallel to, the bottom edge. Commencing at  $1\frac{1}{2}$ " from one edge, and at intervals of  $1\frac{1}{2}$ " thereafter,  $\frac{1}{4}$ " holes are punched in the sheet along the line. This line serves as the "origin" of the chromatogram in the calculation of the Rf. Into the holes punched in the sheet,  $\frac{1}{4}$ " discs punched from dried blood spots made on the Whatman filter paper are carefully manipulated. In this manipulation, it is essential that no finger contamination of the sheet be made. In addition to the  $\frac{1}{4}$ " discs of dried blood for purposes of confirmation, a  $\frac{1}{4}$ " disc of similar filter paper impregnated with 0.01 ml. of a M/100 L-phenylalanine solution is also inserted to act as a control. The filter paper is now used for ascending chromatography in the usual manner with, as solvent, n-butanol:acetic acid:water/100:3.3:100. After overnight exposure to the action of the solvent, the chromatogram is dried and inspected in a dark room with an appropriate ultraviolet light to make certain that the materials eluted from the blood spots have migrated vertically and in parallel lines. All fluorescing migratory substances are outlined in pencil. The control L-phenylalanine and any similar phenylalanine present in any of the blood spots can be seen (but not by everyone) as ultraviolet-absorbing spots at an Rf of approximately 0.3. This Rf can be calculated after marking the "front" of the solvent i.e., the height to which it has reached, calculating the ratio of:- phenylalanine migration/height of "front". /

The sheet of filter paper is now placed on a clean sheet of paper on a soft pine board and two lines are drawn in pencil  $2\frac{1}{2}$  mm. on each side of the centre of each "lane" and the full length, from 10 mm. below the origin to 10 mm. above the "front", making certain that the areas outlined in pencil under the ultraviolet light are included. Using these lines as a guide, a filter paper strip, 5 mm. in width is cut from each lane with a scalpel blade and set aside for "inhibition assay". The paper chromatogram from which the strip has been cut is now treated with ninhydrin solution (0.3 per cent indanetrione hydrate in 95 per cent ethanol) in the usual manner. This may be either by "dipping", i.e., by passing the chromatogram through a trough containing the solution, or by spraying the solution from an atomiser. The expected pinkish-blue spot will be seen in the control lane at an Rf of approximately 0.3 (coinciding with the pencilled spot already outlined under the ultraviolet light), with the 5 mm. central spot deleted from the spot as part of the previously cut-out strip. In the lanes from the dried blood discs, a similar ninhydrin reaction at the same Rf is sought for as an indication of the presence of an increased amount of phenylalanine. It is always advisable to include a dried spot of normal non-P.K.U. blood in one of the lanes. An "inhibition assay" agar test tray is now prepared in the manner previously described in this chapter. Using fine forceps, the filter paper strips cut from the chromatogram sheet are placed on the agar surface, parallel to each other and at least 1" apart. After overnight incubation in the usual manner, a large oval zone of growth is found, symmetrically surrounding the L-phenylalanine spot, as previously located under the ultraviolet light and also by the ninhydrin development of the chromatogram. These findings /

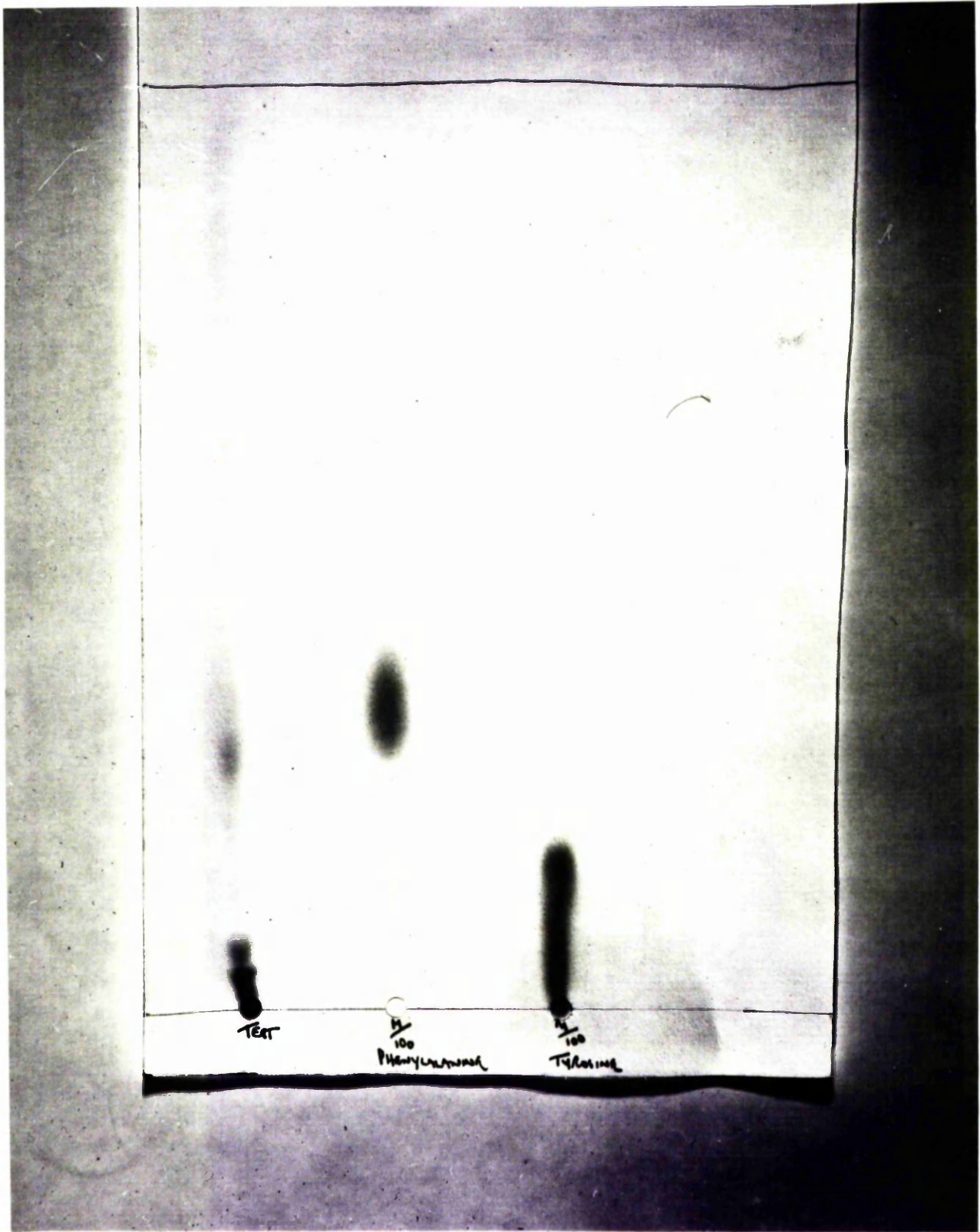


Plate 6.

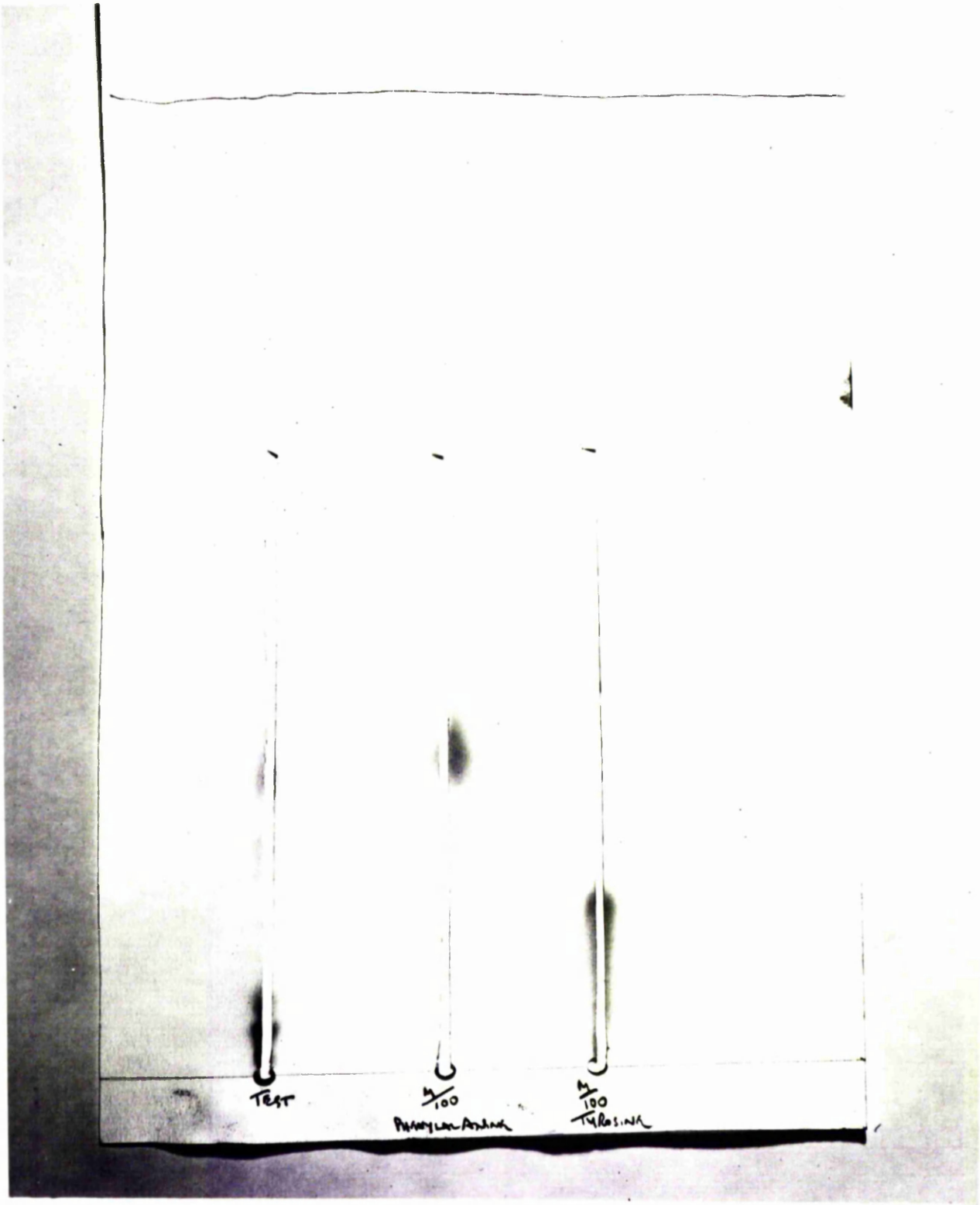


Plate 7.

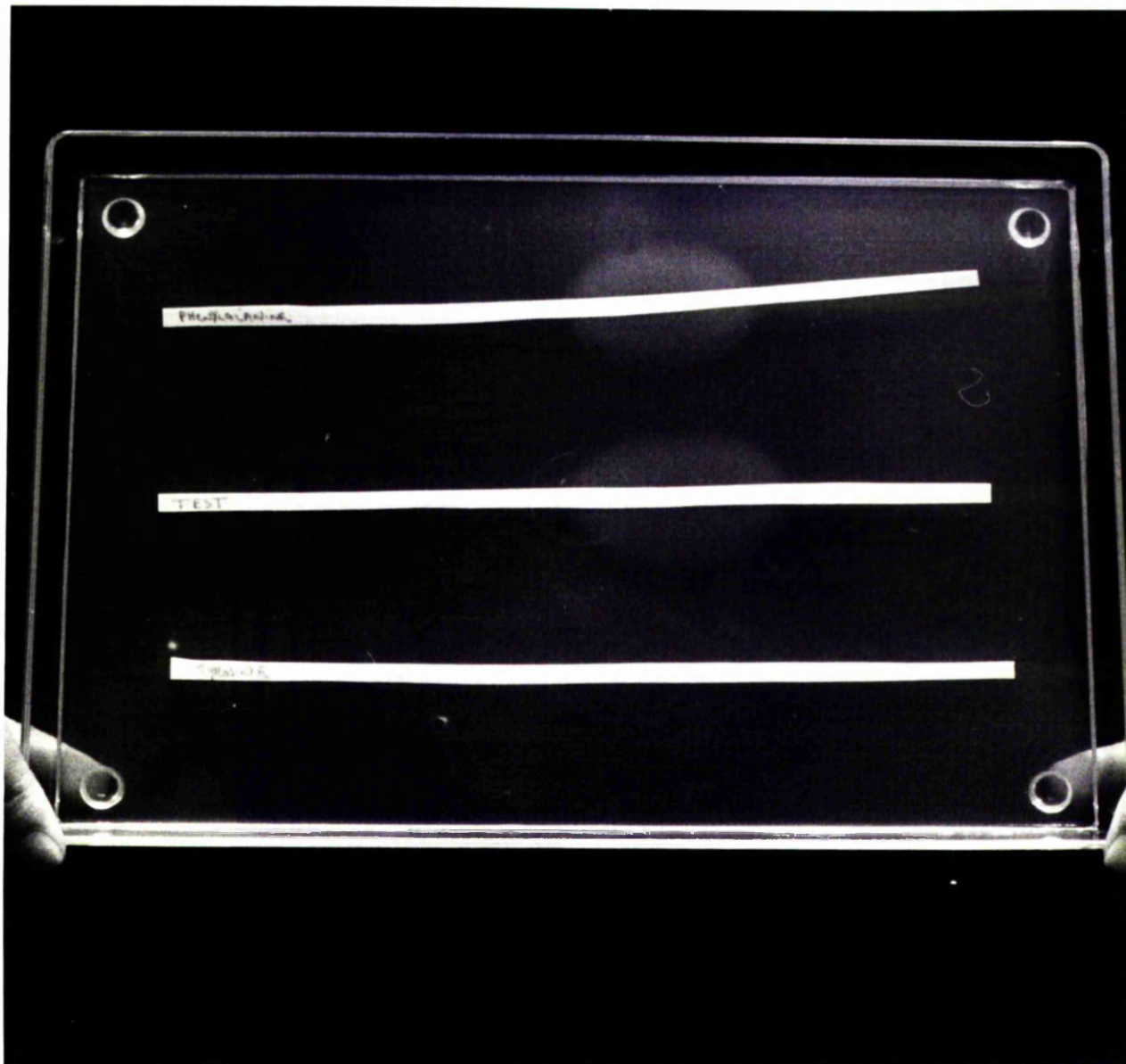


Plate 8.

provide unequivocal identification for the active substance in the dried blood spot or spots under investigation as being L-phenylalanine. This evidence is unambiguous, being derived, as it is, from three sources:-

- A. the partition coefficient or Rf (physical property),
- B. the ninhydrin development test (chemical property),
- C. the specific response of the "inhibition assay" (biological property).

These three tests have all been performed not only on the same specimen, but actually on the SAME SUBSTANCE as isolated in a specific location on the paper chromatogram.

Confirmation of a "presumptive positive" provided by the above methods will, when taken in conjunction with the pure biochemical method performed by an independent laboratory, be accepted as indisputable evidence of the existence of a "truly" positive case of P.K.U. (Plates 6, 7 and 8 illustrate part of the technique.)

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25.

Addendum.

This addendum gives full details for the preparation of the control discs and their actual value (expressed as mg. phenylalanine per cent) compared with their nominal value.

It is essential to commence preparation of stock solutions with blood which has been carefully evaluated by the IADu or the McCaman and Robins methods (outlined in the chapter dealing with Methods of Diagnosis) and found to possess a basal phenylalanine level of 2 mg. per 100 ml. On obtaining outdated blood from the blood bank which fulfils this specification, subsequent procedure is as follows.

L-phenylalanine is placed in a volumetric flask, the previously-evaluated whole blood is added and the mixture warmed in a water bath to allow of the development of a proper solution. Using the table below, the whole blood is diluted to the desired level. It has been found that this solution will keep quite well for a few weeks if kept in a refrigerator. When spotting the controls on the filter paper of the requisite quality, it is desirable that the spots should be of approximately the same diameter as those outlined on the collection cards. One disc is punched from the middle of each spot after the spotted filter paper has been autoclaved for 5 minutes at 15 lbs. pressure. If kept in a dessicator inside the refrigerator, such control discs can be kept for an indefinite period.

INSTRUCTIONS FOR MAKING STOCK SOLUTION.

100 ml. 100 mg.% stock = 98 mg. L-phenylalanine + 100 ml. 2 mg.%  
whole blood.

50 ml. 100 mg.% stock = 49 mg. L-phenylalanine + 50 ml. 2 mg.%  
whole blood. /

25 ml. 100 mg.% stock = 24.5 mg. L-phenylalanine + 25 ml. 2 mg.  
whole blood.

Reference to table 5 shows the respective amounts of 100 mg.% stock and 2 mg.% whole blood to be added together to make control disc mixtures of the nominal value stated. Table 6 shows the nominal value of such control disc mixtures compared with their actual value.

| Nominal value of control discs (mg.%). | 100 mg.% stock solution | 2 mg.% whole blood. |
|--|-------------------------|---------------------|
| 2                                      | ---                     | 10.0 ml.            |
| 4                                      | 0.2 ml.                 | 9.8 ml.             |
| 6                                      | 0.4 ml.                 | 9.6 ml.             |
| 8                                      | 0.6 ml.                 | 9.4 ml.             |
| 10                                     | 0.8 ml.                 | 9.2 ml.             |
| 12                                     | 1.0 ml.                 | 9.0 ml.             |
| 20                                     | 1.8 ml.                 | 8.2 ml.             |
| 30                                     | 2.8 ml.                 | 7.2 ml.             |
| 40                                     | 3.8 ml.                 | 6.2 ml.             |
| 50                                     | 4.8 ml.                 | 5.2 ml.             |
| 60                                     | 5.8 ml.                 | 4.2 ml.             |
| 70                                     | 6.8 ml.                 | 3.2 ml.             |
| 80                                     | 7.8 ml.                 | 2.2 ml.             |
| 90                                     | 8.8 ml.                 | 1.2 ml.             |
| 100                                    | 9.8 ml.                 | 0.2 ml.             |

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Table 5.

| Nominal value of control<br>discs (mg.%) | Actual value of control<br>discs (mg.%) |
|--|---|
| 2  | 2.0                                     |
| 4  | 3.96                                    |
| 6  | 5.92                                    |
| 8  | 7.88                                    |
| 10                                       | 9.84                                    |
| 12                                       | 11.80                                   |
| 20                                       | 19.67                                   |
| 30                                       | 29.44                                   |
| 40                                       | 39.21                                   |
| 50                                       | 49.02                                   |
| 60                                       | 58.84                                   |
| 70                                       | 68.61                                   |
| 80                                       | 78.44                                   |
| 90                                       | 88.21                                   |
| 100                                      | 98.01                                   |

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Table 6.

## Heredity and Prevention.

Penrose (1935) stated that phenylketonuria made itself manifest by the inheritance, on the part of the victim, of an autosomal recessive gene. Since this statement, there have been no descriptions of phenocopies and there has been no general disagreement with regard to the theory of monomeric recessivity as being operative in the inheritance of the disease. Nevertheless, when the studies of Jervis (1939) and Cohen and Kozinn (1949) are examined alongside those of Hsia et al. (1956) and Knox and Messinger (1958), there are seen to be possibilities of alternative modes of inheritance. Examination of this aspect of the subject must be undertaken in conjunction with discussion of the degree of absoluteness of the heterozygote state.

Jervis's study included two cases of affected children with one affected parent and that of Cohen and Kozinn one such case. In such instances two alternative methods of transmission offer themselves; the first - which has been shown to exist and to which further reference will be made in the chapter on Discussion - the mating of a homozygote with a heterozygote; the second - at present purely hypothetical - by the operation of a dominant mode of inheritance. In the first method, all the offspring will be either homozygous or heterozygous for P.K.U. in consequence of their possessing either a double or single dose of the responsible gene. The second hypothetical method of inheritance could be compatible with mutation at another locus which possesses influence on the genetic code responsible for the metabolism of phenylalanine, and also with genic interaction. In this latter type of case, the same locus may be concerned in both dominant and recessive P.K.U., even although the phenotypic manifestation must be dependent upon the /

action of modifying genes. Following this, such modifying genes must exert a suppressant effect on the gene assumed to be specific for P.K.U., whether this gene be present in single or double dose. Although it is possible that retardation in parents and sibs of P.K.U. patients may be coincidental, the possibility also exists of such persons being sufferers from P.K.U., being mentally retarded in consequence, but having the gene assumed to be specific for P.K.U. becoming suppressed in consequence of mutation at another locus having taken place together with concomitant genic interaction. With the advent of more precise methods of measuring levels of blood phenylalanine suitable for large-scale screenings, investigation of retarded parents and sibs of P.K.U. patients may prove eventually whether such a hypothetical method of inheritance does, in fact, exist.

Hsia et al. (1956) studied the levels of plasma phenylalanine, after giving loading doses of phenylalanine, in parents and sibs of known P.K.U. patients and compared such levels with those in control subjects. The parents and sibs of the P.K.U. patients were assumed to be heterozygotes for P.K.U. in consequence of the relationship; the controls were persons who had no consanguineous relationship with P.K.U. patients and were not suspected of being heterozygotes. The investigation disclosed a considerable degree of overlap in levels of plasma phenylalanine between the heterozygotes and the normal controls at various intervals after the administration of phenylalanine; although this overlap was pronounced in individuals, there was a statistically significant difference in the mean of the levels between the two groups. Hsia et al. state that "the results in a single individual must be interpreted with caution, (but) the abnormal values found in a group of known heterozygotes establish /

the decreased ability to metabolise phenylalanine as the phenotypic expression of a single P.K.U. gene". Be that as it may, there must nevertheless be doubt with regard to the validity of the baseline from which this investigation was conducted. In such circumstances it cannot be assumed that all sibs of known P.K.U. homozygotes will themselves be heterozygotes. In the event of such sibs not being heterozygous for the disease, it remains a possibility that this fact is partly responsible for the degree of overlap which was demonstrated in individuals. Conversely it is also a possibility that heterozygotes for the disease were inadvertently included in the control group. If the random heterozygote frequency be assumed to be in the region of 1 in 50 and the non-P.K.U. gene-carrier frequency to be 1 in 4 in a typical Mendelian inheritance-pattern family, it is obvious that the results in respect of one group investigated would not nullify the results obtained from the other group. With approximately equal numbers of presumed heterozygotes and presumed non-P.K.U. gene-carriers being investigated, there will be a 1 in 50 chance of the presumed non-P.K.U. gene-carrier being heterozygous for the disease and a 1 in 4 chance of the presumed heterozygote being a non-P.K.U. gene-carrier. Thus there is a twelve-fold disparity in the validity of results obtained from load testing between such groups.

Knox and Messinger (1958) studied the basal levels in blood phenylalanine between heterozygotes and control subjects classified as non-heterozygotes. They demonstrated that heterozygotes for P.K.U. had elevated basal levels of phenylalanine and that such heterozygotes had the same degree of inefficiency in metabolising phenylalanine under both fasting and loading conditions. What is truly relevant to the matter under discussion is that in both the /

above investigations, persons who fell into a category between true homozygosity and true heterozygosity were demonstrated. Knox and Messinger noted this in particular, pointing to the case of the sister of a known P.K.U. patient who was of this type and, not altogether convincingly, suggested that this resulted from her age. There was a very similar case in the study of Hsia et al. (1956) but no direct comment was made as to its possible significance. There can be no doubt that such cases mentioned by both those authorities could fit the hypothetical inheritance-pattern mentioned earlier, where suppression of the complete manifestations of homozygous P.K.U. could be mediated by genic interaction. In pursuit of such a concept along a somewhat-different direction, it should be noted that Poisner (1960) was able to demonstrate that schizophrenic relatives of patients suffering from P.K.U. exhibited a higher serum phenylalanine than did schizophrenic relatives of non-P.K.U. control subjects. Larson (1964) instances the cases of 13 "schizophrenic" patients in Swedish mental hospitals who had relatives suffering from P.K.U. He was more than cautious in his conclusions regarding them, stating "these 13 cases come from 34 families where numerous members were exposed to diagnosis. True enough, some parents and sibs of patients were more than moderately eccentric, but only in three instances was a parent or sibling among the persons institutionalised for psychosis. The theoretical possibility that heterozygotes run an increased risk of developing mental disease may be left open; as a rule, however, the parents and well sibs of patients with P.K.U. remain well".

In many cases patients with P.K.U. will reach adult age without the introduction and maintenance of a phenylalanine-restricted diet. With the advent of antibiotic and other forms of /

therapy, such patients are not now as liable as formerly to die during the course of an intercurrent infection: indeed, survival to the senium is a distinct possibility for many of those cases. The patient who has the good fortune to have the disease diagnosed in earliest infancy and who thereafter receives properly controlled dietary therapy should be able to live a comparatively normal and self-supporting life. The patients in whom dietary treatment is not commenced at all, or in whom such treatment is not instituted till they are several years old, will not be able to lead such self-supporting lives except in exceptional cases.

Prevention of the disease depends mainly upon the non-procreation of two heterozygotes. Such a tenet necessitates several considerations for its full implementation, the principal being foreknowledge of the existence of the heterozygote state. At present there is no simple and satisfactory method of detection of such heterozygotes. Even if it were feasible to estimate the basal serum phenylalanine levels of all persons prior to marriage, the existence of the overlap between heterozygotes and normal non-P.K.U. gene-carrying individuals (estimated by Knox and Messinger (1958) to occur in 15 per cent of examined persons) is such that no discrete separation can be made with absolute certainty. In point of fact, what could be achieved by the adoption of such a method would be the ability to refer presumptive heterozygotes to one of several categories:- probable gene-carriers, probable non-carriers and "undecided". Such a method of categorisation has been suggested by Larson (1964). On the basis of monomeric recessivity mode of inheritance of the disease, it is certain that consanguineous marriages must increase the risk of propagation and perpetuation /

of P.K.U. Statistically, this risk is estimated by Larson (1964) to increase by a factor of 12.5 per cent in the case of first cousin marriages. In the event of heterozygotes mating fortuitously and their first child proving to be homozygous for P.K.U., the chances of their having a normal child in an additional three child family will be  $1 - 1/64$ , i.e. 98.4 per cent (Larson, 1964). However, it must be noted that two of three non-homozygote children will be heterozygotes.

As has been stated earlier, P.K.U. mothers may have offspring who are homozygous or heterozygous for the disease. With the development of neonatal screening programmes for the detection of the disease in its earliest stages, it becomes increasingly evident that the total population of phenylketonurics must increase in future generations unless steps are taken to limit their procreation. In the past, such elimination of genes for P.K.U. as occurred has been due to non-reproduction of homozygotes. In the future, the influences maintaining the gene in the population will be not only mutation and greater fertility of the heterozygotes but also direct transmission by successfully-treated homozygotes. The progeny from the mating of a homozygote and a non-P.K.U. individual will all be heterozygous for the disease although having normal phenotypes. One half of the progeny of a homozygote and a heterozygote will be homozygous for the disease and the other half will be heterozygous.

The cases of the homozygous female who marries either a heterozygote or a non-P.K.U. male are worthy of further consideration. Where such a situation arises, the matter of supreme importance is that pertaining to the mental state of the child after birth. If it be accepted that the children born to a homozygous and untreated mother are, in the main, mentally retarded, it would appear /

reasonable to suppose that such retardation results from exposure of the foetus in utero to an increased level of phenylalanine or its metabolites. Such being the case, it remains to be shown what effect there will be on the processes of neurological development in the foetus in the event of phenylalanine restriction, administration of serotonin and/or 5-hydroxytryptamine (dealt with in the section on Biochemical Aspects), or the giving of mono-amine-oxidase inhibitors being practised throughout pregnancy. One can postulate that results on the heterozygote foetus should be beneficial, in that the probable pathogenetic mechanism responsible for the development of the mental retardation will not have been operative, but that there should be little likelihood of beneficial results to be obtained in the case of the homozygote foetus, but the postulate with regard to the heterozygote foetus can only be verified or refuted by clinical trial.

The greater part of this thesis is devoted to the concept of phenylketonuria as a tangible problem to be tackled by all who come in contact with it; a problem in which the individual sufferer from the disease is the central figure. Be that as it may, no one directly concerned with the disease - whether it be in the early detection or in supervision of therapy designed to prevent mental retardation - can afford to remain aloof from consideration of the eugenic aspects. Our responsibility extends not only to the victim of the disease but to society as a whole.

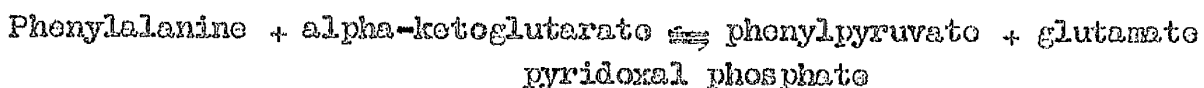
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104.

Treatment.

Undoubtedly the ideal form of treatment of phenylketonuria would be to replace the missing enzyme, phenylalanine hydroxylase. As such a replacement has not so-far proven possible, the next best form of therapy consists in the initiation and maintenance of a diet low in phenylalanine. Before giving consideration to such a form of treatment, other therapeutic measures which formerly have been utilised will be mentioned: these have consisted in the administration of amino acids, fructose, hormones, blood-transfusion and vitamins.

Wolf and Vulliamy (1951) administered glutamic acid to 2 patients suffering from P.K.U. but could not show that this had any effect on their intelligence. In 1956, Moister et al. showed that administration of single oral doses of L-glutamine, sodium L-glutamate, L-asparagine, glycine, sodium succinate or D-glucose, combined in a mixture, brought about a reduction in the level of urinary phenylpyruvic acid in 2 P.K.U. patients. It would appear that the rationale behind such a method of treatment is based upon the reversible reaction mentioned in the chapter on Biochemical and Metabolic Aspects, namely:-



No further studies along such lines were published till that of Bowman and King (1961) which dealt with the effects of glutamine and asparagine supplements given to 3 P.K.U. patients. The purpose of this study was, by increasing the available amino donor, to decrease the phenylpyruvic acid:phenylalanine ratio by means of transamination and to study the effects of this in those patients over a period of several months. The authors stated, that "the /

results contribute evidence of the hypothesis that (in P.K.U.) phenylalanine is converted to phenylpyruvic acid by means of a transamination process which may be reversed by increasing the available amino donor". Although it was stated that reduction of the phenylpyruvic acid:phenylalanine ratio was accompanied by "a slight though significant improvement in each subject's mental ability" it is obvious that further application of this technique in the treatment of infants diagnosed as suffering from P.K.U. prior to the development of mental retardation, must discount the existence of elevated blood levels of phenylalanine as being of a harmful nature.

The announcements by Jervis (1947) to the effect that the basic fault in metabolism in P.K.U. was a blockage in the conversion of phenylalanine to tyrosine, and in 1953 that the explanation for this lay in the demonstration of deficiency of the responsible enzyme in the livers of patients suffering from the disease, led to the investigation of the effects of the administration of tyrosine to such patients by Snyderman et al. (1955). This investigation showed that such administration had no effect on the intellectual functioning of such patients. Berry et al. (1958) showed that an increased intake of tyrosine in P.K.U. patients - admittedly when given in conjunction with a phenylalanine-low dietary - possibly led to diminished irritability in such patients. They suggested that, in such cases, tyrosine might take on the status of an "essential" amino acid and that the elevated levels of serum phenylalanine might result from tissue breakdown. Nevertheless, they did not advocate the administration of tyrosine unless given in conjunction with a phenylalanine-low diet. The deficient plasma levels of 5-hydroxytryptamine and urinary 5-indoleacetic acid, previously discussed in/

the chapter dealing with Biochemical and Metabolic Aspects, led to the investigation of the value of the systemic administration of 5-hydroxytryptophan by Pare et al. (1957:1958) and Kirman et al. (1957). Such investigations showed little promise and were not pursued to any great lengths. Kirman and Pare (1961) reported limited success in increasing plasma and C.S.F. levels of 5-hydroxytryptamine by administration of an amine-oxidase inhibitor and suggested that this form of treatment might have a part to play in the case diagnosed in early infancy.

Geisler and Stroder (1958) reported that large amounts of fructose, up to 6 g. per kg. body weight, raised phenylalanine tolerance and reduced the phenylketonuria present with a normal diet, when given to patients suffering from the disease. Perusal of the literature has failed to discover any subsequent publication confirming this finding. Geisler and Stroder's study had been preceded by the observation of Blainey and Gulliford (1956) to the effect that a high carbohydrate intake is an essential part of a phenylalanine-low dietary. When this particular form of therapy was initiated in two of their patients, phenylalanine content of the urine and plasma was increased when the carbohydrate in the diet was reduced. They considered this to be due to a specific effect of carbohydrate deficiency and <sup>not</sup> to a deficiency in calories. Notwithstanding this conclusion, they did not advocate the pursuance of a diet high in carbohydrate as being, per se, an effective method of treatment of the disease. The effect of blood-transfusion was studied by Mautner (1960); of pyridoxine by McGear and Tischler (1959) and of cortisone by Tholander (1958) and Fois (1960). In none of those studies was it possible to demonstrate any resultant beneficial effects on the disease. /

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It must nevertheless be pointed out that, in spite of the negative conclusions reached by McGear and Tischler (1959) regarding the efficacy of pyridoxine, recent work by Moncrieff (1965) has shown that when this vitamin is administered in doses of 50 mg. daily to children suffering from the disease and who are concomitantly receiving a phenylalanine-restricted dietary, there is a marked lowering of the blood levels of phenylalanine. It has been suggested by him that such administration will allow of an infant receiving a greater intake of phenylalanine than is normally possible without detrimental effects resulting therefrom.

Wolf and Vulliamy (1951) suggested that the mental and neurological effects of P.K.U. were due to phenylalanine intoxication and that a diet low in phenylalanine would be worthy of trial as a possible form of treatment; such a dietary to be based upon casein-acid hydrolysate. Bickel et al. (1953) reported on the administration of such a diet to a patient suffering from the disease. After preparation of the casein-acid-hydrolysate, treatment with activated acid-washed charcoal removed phenylalanine and tyrosine. Tryptophan, tyrosine and cystine were then added in amounts calculated to be slightly in excess of daily requirements. The preparation was then used on a P.K.U. girl aged two years who was an "idiot", unable to stand, walk or talk, who showed no interest in her food or environment and who spent her time groaning, crying and banging her head. Treatment was commenced in hospital and, during a four-week preliminary period when no phenylalanine was permitted, no definite clinical change other than loss of weight was observed, although the characteristic "mouse-like" odour was no longer appreciable. Significantly however, the levels of /

phenylalanine in the plasma and urine fell to normal, the excretion of phenylpyruvic acid ceased and the ferric chloride reaction became negative. Phenylalanine was then added in small amounts in the form of whole milk, a daily intake of 0.3 to 0.5 g. of phenylalanine being found sufficient for normal weight-gain with greatly improved biochemical findings. During the next few months of outpatient treatment a gradual improvement in her mental state took place. The child learned to crawl, to stand and to climb on chairs, her eyes became brighter, her hair grew darker and she no longer banged her head or cried continuously. When the amount of phenylalanine in the diet was increased to 5 g. daily, a definite deterioration in the child's condition occurred within a few days. The plasma phenylalanine and the urinary excretion of phenylalanine rose to high levels but fell whenever the extra phenylalanine was discontinued. Bickel et al. gave further details of the diet in 1954. In addition to the casein hydrolysate, gluten-free wheat flour and sugar were given ad lib., the phenylalanine supplement was supplied as milk and other supplements were cabbage, turnips, grated apple or banana and multi-vitamin drops. Such a diet provided 17 g. of protein, 0.3 g. of phenylalanine, 1.5 g. of L-tyrosine, 0.25 g. of L-tryptophan and 0.7 g. of L-cystine per diem. On this diet the blood levels of phenylalanine became normal, there was no excretion of phenylpyruvic acid in the urine and the child gained weight satisfactorily.

Wolf et al. (1955) reported their findings for two children, both aged 2 years and 8 months at the commencement of the treatment, who had been fed a phenylalanine-low diet for 9 to 10 months respectively. Marked intellectual improvement resulted and the E.E.G. became normal in one case in which it was previously abnormal.

A third child aged 5 years and 5 months was fed on this diet for a shorter period and the preliminary findings indicated that even at this age it appeared probable that the brain had suffered very little permanent damage. A mentally retarded child age 18 months who was having up to 4 major epileptic seizures and 10 to 20 petit mal attacks daily, was treated by Breude (1956) with a phenylalanine-restricted diet. At the commencement of the diet, urine chromatography showed a grossly excessive excretion of phenylalanine but the ferric chloride reaction became completely negative 5 days after treatment was commenced, petit mal attacks were greatly diminished in number and grand mal attacks stopped completely. The physical condition of the child improved and at the end of 6 months there was a marked improvement in the mental picture, the child having become bright, active, alert and interested in his environment. Also by the end of this period his hair had darkened considerably, a troublesome eczema had cleared, movements of the hands had become co-ordinated and repetitive movements of the thumbs and grinding of the teeth had ceased.

Woolf et al. (1958) described the progress of their three original treated cases together with seven additional new ones. The original cases had now been treated for a further 32 months and the additional cases for periods of 11 to 34 months. They also announced modification of the original diet adopted by Bickel et al. (1953) in which the main alterations were reduction in the intake of cow's milk with compensatory increase of casein hydrolysate, and the addition of DL-methionine. In the earlier cases the intake of phenylalanine, in the form of milk protein, was reduced until phenylpyruvic acid was no longer detectable in the urine. As the blood and urine phenylalanine levels were still abnormally high, /

the daily intake of cow's milk was reduced until those levels were the same as in normal children. DL-methionine was added to the diet (1 g. per day for children over one year) as an additional source of sulphur, since the process of hydrolysis and charcoal treatment depletes the casein of its sulphur-containing amino acids. The reason for the choice of methionine rather than cystine was in consequence of the fact that methionine can replace cystine but not the reverse. The diet was controlled in every instance by repeated biochemical estimations of the levels of phenylalanine and certain of its metabolites in the blood and urine. Paper chromatography was employed to ensure a proper concentration of phenylalanine compared with other amino acids. The advantage of this particular modification of the basic diet lay in the fact that any drop in blood phenylalanine level to below normal could be detected and corrected by increasing the intake of cow's milk, usually by 15 or 30 ml. per day. Too low a level was associated with an unsatisfactory rate of growth and too high a level slowed down the child's progress. The ages of the cases described, at the commencement of the dietary therapy, had been from 5 weeks to 5 years 5 months. In almost every case there was a sharp rise in the I.Q. and in some cases the rise was continuous, amounting to as much as 20 points in 2 years. It was pointed out that the effects of the diet were greater, the younger was the child and the less the degree of its mental deterioration as measured by the I.Q. It was also suggested by Woolf and his colleagues that a worthwhile improvement often resulted from dietary treatment in older children albeit though such improvement was only an increase in the mental rating from the level of "idiot" to that of "imbecile". As a rough guide, the authors considered treatment to be of value in the/

case of the child two years old or under with a D.Q. not less than 20; three years old with a D.Q. of 30 or over and four years old with a D.Q. of 40 or over. In border-line cases it was suggested that a six month's trial be advised, psychological testing being undertaken at the commencement, at three months and at the expiry of the probationary period, a decision then being taken as to the advisability of further dietary continuation.

Bickel and Grueter (1960) reviewed the position as they saw it. They reported on their personal experiences with 14 P.K.U. patients and compared these with comparable findings from reports on 79 cases treated by others. In 47 treated cases the data on mental development was compared with that from 19 untreated cases. Their findings, with regard to the series as a whole, showed that in the untreated group the development of intelligence continued to fall during the first 10 to 14 years of life, after which it remained approximately stationary. In those patients whose dietary treatment was commenced between the ages of 1 and 5, they found, as compared with untreated cases, an average increase in the I.Q. of 18 per cent. For 9 cases whose dietary treatment had been commenced before they had attained the age of 20 weeks, subsequent development was normal. After the age of 5, commencement of treatment conferred little benefit, but nevertheless such cases still showed an average increase in intelligence of nearly 6 per cent. On the basis of those findings, the authors believed justification existed for the continuation of such dietary measures up to the age of 10 - always stressing the vital importance of commencing treatment at the earliest possible age.

In 1960, Knox published "An Evaluation of the Treatment of Phenylketonuria with Diets Low in Phenylalanine". In this he /

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reviewed all cases of P.K.U. treated with a phenylalanine-low dietary and reported up to December, 1959. In this evaluation, all information available and relating to untreated P.K.U. patients was also noted. He was able to demonstrate that 44 patients over the age of 3 who received such dietary treatment showed no impressive change in mental or neurological status. With regard to 43 patients in whom treatment was commenced before the age of 3, findings were evaluated in detail. Twenty untreated cases with an I.Q. above 60 were identified. The treated group showed 18 times as many with an I.Q. above 60 and twice as many with a normal E.E.G. as the untreated group. Over a period under review, a minimal loss of nearly five points in I.Q. occurred in each 10 weeks by which treatment was delayed. The phenylalanine-low dietary, when commenced in the early weeks of life and maintained thereafter, was shown to be effective in preventing mental retardation and neurologic abnormalities.

It has been felt to be of importance to give more than outline-details of the earlier reports and reviews on the matter of the phenylalanine-low dietary. In the enthusiasm which greeted the initial reports by Bickel et al. (1953) and Woolf et al. (1955), many papers regarding the effects of the dietary on single cases were published by paediatricians but such individual detailing would be only a repetition of what has already been noted above.

It is uniformly agreed that the prognosis for the untreated child is poor on the extreme when directed specifically towards assessment of intellectual function. The earliest pronouncements on this matter were those made by Penrose (1946) who stated that 90 per cent would become either "idiots" or "inbeciles". Cowie (1951)/

found that normal or even near-normal mentality was rare in untreated P.K.U. and that only in 1 per cent did the I.Q. exceed 70. Surveys of large numbers of untreated P.K.U. patients, to determine the level of the I.Q., have been reported upon by Paine (1957 - 106 cases), Knox (1960 - 466 cases) and Partington (1962 - 75 cases). The levels for these three surveys are as follows:-

| I.Q.        | <u>Paine.</u><br>Percentage. | <u>Knox.</u><br>Percentage. | <u>Partington.</u><br>Percentage. | <u>Mean.</u><br>Percentage. |
|-------------|------------------------------|-----------------------------|-----------------------------------|-----------------------------|
| 0 - 20      | 70                           | 61.3                        | 64.4                              | 65                          |
| 21 - 40     | 23                           | 26.7                        | 23.3                              | 24                          |
| 41 - 60     | 5                            | 5.3                         | 9.7                               | 7                           |
| 61 - 80     | 1                            | 5.3                         | 1.9                               | 3                           |
| 81 and over | 1                            | 1.3                         | 0.6                               | 1                           |

It is thus evident from the above table that a marked degree of agreement exists between those three investigators with regard to the intelligence-levels obtaining in untreated P.K.U. One of the many unsolved problems relating to the disease is the occurrence of the 1 per cent who show atypical mental development. Particular instances of such cases have been detailed in the chapter dealing with psychological aspects of the condition. Discounting the hypothetical possibilities which come to mind, such as a partial deficiency in phenylalanine hydroxylation, development of the enzyme block after the period of earliest infancy, the possible existence of a third allele synthesising a protein with weak enzymatic properties, the inheritance of a modifying gene and the existence of an alternative metabolic pathway, a low renal threshold for phenylalanine and a naturally-occurring diet low in phenylalanine, it seems more than a possibility that such cases represent only the 90th. percentile of a distribution-curve of intelligence in P.K.U./

as Tischler et al. (1961) have suggested.

The earliest successful attempts at dietary treatment announced by Bickel et al. (1953) and Woolf et al. (1955) have been detailed earlier in this chapter. There can be no doubt that early initiation and maintenance of a phenylalanine-low diet is the cornerstone in treatment of the disease, in order that the onset of irreversible mental retardation be prevented. It is doubtful if anyone would disagree with this postulate. Nevertheless, certain basic requirements must be met, both with regard to the dietary principles in general and needs of the individual patient in particular. The following general principles are common to all P.K.U. dietary treatments:-

1. assurance of a protein-intake based on body-weight;
2. maintenance of blood-phenylalanine levels in the region of 3 to 6 mg. per 100 ml.

Although previously stated that mental retardation in P.K.U. may develop with a blood level of phenylalanine slightly in excess of 4 mg. per 100 ml., it should be noted that the homozygous infant will be able to tolerate levels of blood phenylalanine approximating to those of the heterozygous patients, i.e., 3 - 6 mg. per 100 ml. Most natural proteins contain 4 to 6 per cent phenylalanine (Armstrong and Tyler, 1955), thus making it impossible to prepare a well-balanced diet for the P.K.U. patient, while supplying all the necessary nutrients, from natural foods. The casein hydrolysate already mentioned is the basis of several proprietary foods which are of low phenylalanine content and are specially prepared for the P.K.U. dietary. Among such widely-used in Great Britain and the U.S.A. are ALBUMAID X.P. (Scientific Hospital Supplies, Ltd.), /

CYMOGRAN (Allen & Hanbury, Ltd.), LOFENALAC (Mead Johnson & Co. and B.D.H., Ltd.), MINAFEN (Trufood, Ltd.) and KETONIL (Merck, Sharp & Dohme, Ltd. In respect of the latter four, it may be stated

that their vileness of taste appears to be in inverse proportion to their phenylalanine-content. The composition of each of the foods listed is as follows:-

- ALBUMAID X P. - 370 calories per 100 g., contains no phenylalanine per 100 g. and contains no fat or carbohydrate;
- CYMOGRAN. - 400 calories per 100 g., contains less than 10 mg. phenylalanine per 100 g. and has a fat: protein : carbohydrate ratio of 9 : 29 : 38.5;
- LOFENALAC. - 450 calories per 100 g., contains 60 to 100mg. phenylalanine per 100 g. and has a fat : protein : carbohydrate ratio of 18 : 15 : 57;
- MINAFEN. - 550 calories per 100 g., contains less than 20 mg. phenylalanine per 100 g. and has a fat: protein : carbohydrate ratio of 31 : 17.5 : 48;
- KETONIL. - 250 calories per 100 g., contains not more than 100 mg. per 100 g. and contains no fat or carbohydrate. It is a "mixture of a phenylalanine deficient acid hydrolysate of casein fortified with glycine, L-tyrosine, DL-methionine, DL- tryptophan, L-histidine, minerals and choline chloride". Each kilogramme contains protein components - 625 g., water and inorganic salts - 243.38 g.
- Ketonil is not marketed in Great Britain and detailed information regarding it is difficult/

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to obtain. This product will not be dealt with subsequently.

From the above it can be seen that the dietary may follow one of two methods:- a. utilisation of ALBUMAID X.P. with complete supplementation of fat, carbohydrate, amino acids, etc., from natural sources, in a wide range of foods selected from tables supplied; which allow the provision of a known daily amount of phenylalanine to the child;

b. utilisation of one of the other phenylalanine-low dietary supplements with addition of modified "equivalent" foods (Lyman and Lyman, 1960) low in phenylalanine and allowing sufficient milk to maintain the proper phenylalanine level. With regard to the first method suggested, it should be noted that the taste of ALBUMAID X.P. is somewhat less nauseating than some of the other proprietary preparations and, prescribed on an age and weight basis, may prove to have some advantages insofar as feeding in the earliest months is concerned. In an interesting study by Wilson and Clayton (1962), it was pointed out that administration of unnatural mixtures, instanced by the other proprietary preparations mentioned above, may alter the intestinal flora of infants to whom they have been administered and thus lead to the non-production or destruction of essential nutrients and metabolites. This, in turn, could lead to the development of an amino acid imbalance and result in impaired growth of the child. Should such invariably prove to be the case, the giving of ALBUMAID X.P. might lead to less disturbance of intestinal bacterial flora and to consequent diminution in the risk of development of amino acid imbalance. It should be noted that ALBUMAID X.P. is prepared by hydrochloric acid hydrolysis of bovine blood serum. The other proprietary preparations are prepared by /

sulphuric acid or enzymatic hydrolysis of casein. In the latter type of diets, i.e. those utilising phenylalanine-low natural milk-source alternatives, - and those most generally used - the "equivalents" are such that 1 "equivalent" is equal to 15 mg. phenylalanine and extensive lists of such equivalents are supplied by the various manufacturers.

The actual protein requirements of infants and young children are still in doubt. The Committee on Nutrition of the British Medical Association, in 1950 suggested that for infants, children and adolescents, 14. per cent of the caloric intake should be provided by protein. The U.S. National Research Council Recommended Allowances for Early Infancy (1958) suggested that diets furnishing 1.6 g. of protein per lb. body-weight were the usual. However, since the protein for P.K.U. infants is generally supplied in the form of a casein hydrolysate, it may be necessary to give more protein than the normal amounts. It is thus desirable that P.K.U. patients have, from birth to the age of 3 months,  $1\frac{3}{4}$  to 2 g. per lb. body-weight; from 4 to 12 months,  $1\frac{1}{2}$  g. per lb. body-weight; from 1 to 3 years of age, 40 g. daily and from 4 to 7 years of age, 50 g. daily.

The phenylalanine requirements of the infant from birth to the age of 9 months appear to be in the region of 41 mg. per 1 lb. body-weight (Snydeman et al., 1955), but, as the same authors noted, requirements for normal children from the age of 9 months to 13 years have not been studied adequately. Centerwall et al. (1961) stated that there is a gradual decline in the requirements of the P.K.U. child for phenylalanine as age increases and suggested that a baseline in the commencement of treatment should provide for a maximum of 22 mg. per 1 lb. body-weight at the age of 7. It must be /

appreciated that those ranges are not absolute and are completely dependent on the individual tolerance of the child undergoing treatment and may require increase or decrease. Indeed, Paine and Hsia (1957) have shown that the phenylalanine-tolerance of one particular child of 6 months appeared to be 11 mg. per 1 lb. body-weight, a figure considerably lower than that set by Centerwall for a child of this age.

With regard to fat, at present there are no set requirements in the diet. Research has pointed to the fact that there may be better growth on fewer calories if some saturated fat is included in the diet (Holt, 1957). Casein hydrolysate contains only unsaturated fat and it is felt that this aspect of the dietary requires some attention. Blainey and Gulliford (1956), as earlier mentioned, drew attention to the fact that a dietary high in carbohydrate was essential in the treatment of P.K.U. when phenylalanine intake was reduced and proposed that 70 per cent of the total calories should be in the form of carbohydrate. The caloric requirements of the normal child, as suggested by the U.S. National Research Council Recommended Dietary Allowances, decrease from approximately 55 calories per 1 lb. body-weight at birth to approximately 40 calories per 1 lb. body-weight at 7 years of age. According to Blainey and Gulliford (1956) and Centerwall et al. (1961), it would appear that the child suffering from P.K.U. has need of a greater caloric intake, and they have suggested 60 to 65 calories per 1 lb. body-weight for the newborn P.K.U. infant with a gradual decrease to 40 to 50 calories per 1 lb. body-weight at the age of 7. Again it must be appreciated that the question of the proper caloric intake must proceed hand-in-hand with the growth and development of the individual child and that nothing will be gained by producing /

a marked degree of adiposity - as can readily happen. If the protein requirements of the P.K.U. patient are properly met by the adequate administration of a phenylalanine-low dietary supplement, there is no need for concern over the requirements of calcium. It is generally advocated that poly-vitamin-supplementation be prescribed in the infantile stage of development. As the child grows older, it may only be necessary to supplement the vitamin B complex.

In conjunction with phenylalanine-low dietary supplements, serving lists of foodstuffs have been evolved (Acosta and Centerwall, 1960; Acosta, 1964) which are of much more practical value than the already-mentioned "equivalents" of Lyman which were primarily designed for dieticians. Such lists are of particular value to parents, as opposed to dieticians, thereby enabling them to visualise the daily dietary requirements necessary for their particular child and take into consideration its own particular fads and fancies. The lists of Acosta and Centerwall are based on the food exchange lists for diabetics. In such lists, foods with similar nutrient values are placed in the same list although the amounts of food differ, in order that the nutrients may remain constant. Tables 7 to 9 (Acosta, 1964) illustrate the method employed in providing such information to parents who, after suitable instruction, are in the majority of cases able to cope adequately with dietary planning. Table 8 (Acosta, 1964) shows the phenylalanine, protein and calorie requirements recommended for various age groups and Table 9 (Acosta, 1964) illustrates the ages at which various foods and vitamins are introduced into the dietary. It is unfortunate that such extensive lists are intended primarily for the North American domestic market and certain items would not normally be readily available in Great/Britain.

Phenylalanine, Protein, and Caloric Content of Serving  
Lists Used on Restricted Phenylalanine Diets

|            | PHENYLALANINE | PROTEIN | CALORIES |
|------------|---------------|---------|----------|
|            | Mg.           | Gm.     |          |
| Vegetables | 15            | 0.3     | 5        |
| Fruits     | 15            | 0.2     | 80       |
| Breads     | 30            | 0.5     | 20       |
| Fats       | 5             | 0.1     | 45       |
| Desserts*  | 30**          | 2.0**   | 270**    |
| Free Foods | 0             | 0.0     | Varies   |

\*Special recipes must be used.

\*\*Averages from all dessert recipes.

Serving Lists for Phenylalanine Restricted Diet

VEGETABLES

Each serving as listed contains 15 milligrams of phenylalanine

Baby and Junior Vegetables

|  |         |
|--|---------|
| Beans, green, strained and junior. . . . .     | 2 Tbsp. |
| Beets, strained. . . . .                       | 2 Tbsp. |
| Carrots, strained and junior . . . . .         | 3 Tbsp. |
| Spinach, creamed, strained and junior. . . . . | 2 Tbsp. |
| Squash, winter, strained . . . . .             | 3 Tbsp. |
| junior . . . . .                               | 6 Tbsp. |
| Tomato juice . . . . .                         | 2 Tbsp. |
| Yam or sweet potato, strained. . . . .         | 2 Tbsp. |

Table Vegetables

|                                  |           |
|----------------------------------|-----------|
| Asparagus. . . . .               | 1 Stalk   |
| Beans, green, cooked . . . . .   | 3 Tbsp.   |
| Beets, cooked. . . . .           | 3 Tbsp.   |
| Cabbage, raw, shredded . . . . . | 4 Tbsp.   |
| Carrots, raw . . . . .           | 1/2 Large |
| canned. . . . .                  | 4 Tbsp.   |

Table 7.

|  |           |
|--|-----------|
| Cauliflower. . . . .                     | 1 Tbsp.   |
| Celery, raw (5 in. long stalks). . . . . | 2 Stalks  |
| Cucumber, raw. . . . .                   | 1/3 Med.  |
| Lettuce, head. . . . .                   | 2 Leaves  |
| Mushrooms, cooked. . . . .               | 1 Tbsp.   |
| Okra, pod, cooked. . . . .               | 1 Pod     |
| Onion, mature. . . . .                   | 1/4 Med.  |
| green . . . . .                          | 2 Med.    |
| Parsley. . . . .                         | 2 Sprigs  |
| Pumpkin, cooked. . . . .                 | 2 Tbsp.   |
| Radish, small, raw . . . . .             | 3 Small   |
| Spinach, cooked. . . . .                 | 1 Tbsp.   |
| Squash, winter, cooked . . . . .         | 2 Tbsp.   |
| summer, cooked . . . . .                 | 4 Tbsp.   |
| Zucchini, cooked . . . . .               | 1 Tbsp.   |
| Tomato, raw. . . . .                     | 1/4 Small |
| canned . . . . .                         | 2 Tbsp.   |
| juice . . . . .                          | 2 Tbsp.   |
| Turnip . . . . .                         | 4 Tbsp.   |

### FRUITS

Each serving as listed contains 15 milligrams of phenylalanine

#### Baby and Junior Fruits

|  |          |
|--|----------|
| Apricot-applesauce, strained and junior. . . . . | 10 Tbsp. |
| Banana . . . . .                                 | 4 Tbsp.  |
| Orange juice . . . . .                           | 3 Tbsp.  |
| Peaches, strained. . . . .                       | 5 Tbsp.  |
| junior. . . . .                                  | 7 Tbsp.  |
| Pears, strained and junior . . . . .             | 10 Tbsp. |
| Pear-pineapple, strained and junior. . . . .     | 7 Tbsp.  |
| Plums with tapioca, strained . . . . .           | 5 Tbsp.  |
| junior . . . . .                                 | 7 Tbsp.  |
| Prunes, strained . . . . .                       | 3 Tbsp.  |

#### Table Fruits

|   |           |
|---|-----------|
| Apple, raw . . . . .                    | 1 Small   |
| Apricots, canned . . . . .              | 2 Halves  |
| juice. . . . .                          | 1/4 Cup   |
| Avocado. . . . .                        | 2 Tbsp.   |
| Banana . . . . .                        | 4 Tbsp.   |
| Dates, dried . . . . .                  | 2         |
| Cantaloupe, small. . . . .              | 1/8 Melon |
| Fruit cocktail, canned . . . . .        | 2 Tbsp.   |
| Grapefruit, sections or juice . . . . . | 1/3 Cup   |
| Grapes, green, seedless. . . . .        | 20 Med.   |
| Guava, raw . . . . .                    | 1/3 Med.  |

|                                     |              |
|-------------------------------------|--------------|
| Grape juice . . . . .               | 1/3 Cup      |
| Lemon or lime juice . . . . .       | 3 Tbsp.      |
| frozen concentrate, mixed . . . . . | 1/2 Cup      |
| Mango . . . . .                     | 1/2 Small    |
| Orange, sections or juice . . . . . | 3 Tbsp.      |
| Papaya juice . . . . .              | 1/2 Cup      |
| Papaya, cubed . . . . .             | 1/4 Cup      |
| Peaches, raw . . . . .              | 2/3 Med.     |
| canned in syrup . . . . .           | 1-1/2 Halves |
| Pear, raw . . . . .                 | 1/3 Med.     |
| canned in syrup . . . . .           | 3 Halves     |
| Pineapple, raw . . . . .            | 1/3 Cup      |
| canned in syrup . . . . .           | 1-1/2 Sm. Sl |
| juice . . . . .                     | 1/2 Cup      |
| Plums, canned in syrup . . . . .    | 1 Med.       |
| Popsicle with fruit juice . . . . . | 1            |
| Prunes, cooked . . . . .            | 2 Med.       |
| juice . . . . .                     | 1/3 Cup      |
| Raisins . . . . .                   | 1 Tbsp.      |
| Strawberries . . . . .              | 3 Large      |
| Tangerine . . . . .                 | 2/3 Small    |
| Watermelon . . . . .                | 1/3 Cup      |

BREAD, CEREALS

Each serving as listed contains 30 milligrams of phenylalanine

Baby Foods

|  |         |
|--|---------|
| Barley cereal, Gerber's, dry . . . . . | 2 Tbsp. |
| Cereal food, Gerber's, dry . . . . .   | 2 Tbsp. |
| Mixed cereal, Pablum, dry . . . . .    | 3 Tbsp. |
| Oatmeal, Gerber's strained . . . . .   | 2 Tbsp. |
| Pablum, dry . . . . .                  | 2 Tbsp. |
| Rice Pablum . . . . .                  | 4 Tbsp. |

Table Foods

|                                   |         |
|-----------------------------------|---------|
| Biscuits* . . . . .               | 1 Small |
| Corn, cooked . . . . .            | 2 Tbsp. |
| Cornflakes . . . . .              | 1/3 Cup |
| Crackers, Barnum animal . . . . . | 6       |
| Crackers, graham . . . . .        | 1       |
| Crackers, soda . . . . .          | 1       |
| Crackers, saltines . . . . .      | 2       |
| Cream of Rice, cooked . . . . .   | 2 Tbsp. |
| Cream of Wheat, cooked . . . . .  | 2 Tbsp. |
| Hominy . . . . .                  | 3 Tbsp. |

\*Low phenylalanine recipes.

|                                     |                   |
|-------------------------------------|-------------------|
| Hominy grits, cooked. . . . .       | 2 Tbsp.           |
| Muffin, pineapple* . . . . .        |                   |
| Popcorn . . . . .                   | 1/3 c.,<br>popped |
| Potato, Irish . . . . .             | 2 Tbsp.           |
| Rice, cooked . . . . .              | 2 Tbsp.           |
| Rice Flakes, Quaker . . . . .       | 1/3 Cup           |
| Rice Krispies, Kelloggs . . . . .   | 1/3 Cup           |
| Rice, Puffed, Quaker. . . . .       | 1/3 Cup           |
| Sugar Crisps. . . . .               | 1/4 Cup           |
| Wheat, Puffed, Quaker . . . . .     | 1/3 Cup           |
| Yam, sweet potato, cooked . . . . . | 2 Tbsp.           |

FATS

Each serving as listed contains 5 milligrams of phenylalanine

|                       |         |
|-----------------------|---------|
| Cream, heavy. . . . . | 1 Tsp.  |
| Mayonnaise. . . . .   | 2 Tsp.  |
| Olives, ripe. . . . . | 1 Large |

DESSERTS

Each serving as listed contains 30 milligrams of phenylalanine

|                                  |                 |
|----------------------------------|-----------------|
| Cake* . . . . .                  | 1/12 of<br>cake |
| Cookies--Rice flour* . . . . .   |                 |
| Corn starch* . . . . .           |                 |
| Cookies, Arrowroot. . . . .      | 1-1/2           |
| Ice Cream--Chocolate* . . . . .  | 2/3 Cup         |
| Pineapple* . . . . .             | 2/3 Cup         |
| Strawberry* . . . . .            | 2/3 Cup         |
| Jello . . . . .                  | 1/3 Cup         |
| Puddings* . . . . .              | 1/2 Cup         |
| Sauce, Hershey. . . . .          | 2 Tbsp.         |
| Wafers, sugar, Nabisco . . . . . | 6               |

FREE FOODS

Each serving as listed contains little or no phenylalanine  
May be used as desired

|                                  |              |
|----------------------------------|--------------|
| Apple juice) If more than a cup, | Gingerbread* |
| Applesauce ) count as a fruit    | Gruva butter |
| Butter                           | Honey        |

\*Low phenylalanine recipes.

Candy  
  butterscotch  
  cream mints  
  fondant  
  gum drops  
  hard  
  jelly beans  
  lollipops  
Cornstarch  
Sauces\*  
  lemon  
  white

Jams, jellies, marmalades  
Margarine  
Molasses  
Oil  
Pepper  
Popsicle (with fruit flavoring only)  
Rich's Topping  
Salt  
  
Sugar, brown or white  
Syrups, corn, maple  
Tapioca

#### FOODS TO AVOID

Each serving is very high in phenylalanine  
May not be used except with physician's permission

Breads  
Cheese, all kinds  
Eggs  
Flour, all kinds  
Meat, poultry, fish

Legumes (dried peas, beans and seeds)  
Nuts  
Nut butters  
Milk (55 milligrams phenylalanine  
per ounce)

---

\*Low phenylalanine recipes.

Phenylalanine, Protein, and Calories  
Recommended for Various Age Groups

| AGE         | PHENYLALANINE             |                  | PROTEIN (25)               |                    | CALORIES/LB.          |       |
|-------------|---------------------------|------------------|----------------------------|--------------------|-----------------------|-------|
|             | Mg. per lb. (9)<br>Normal | PKU              | Gm. per lb. (25)<br>Normal | PKU                | Normal                | PKU   |
| 0-3 Months  | 1-9 Mo. (27)<br>41        | 20-22            | 2-6 Mo.<br>1.1-1.6         | 1 $\frac{1}{2}$ -2 | 2 Mo.<br>55           | 60-65 |
| 4-12 Months |                           | 18-20<br>11 (23) | 7-12 Mo.<br>.9-1.4         | 1 $\frac{1}{2}$    | 7-12 Mo.<br>45        | 55-60 |
| 1-3 Years   | *                         | 16-18            | 1.5<br>(40 gm. total)      | 40 gm. total       | 43-48<br>(1300 total) | 50-55 |
| 4-7 Years   | *                         | 10-16            | 1.3<br>(50 gm. total)      | 50 gm. total       | 42<br>(1700 total)    | 40-50 |

\*No research on requirements for this age.

Table 8.

TIMETABLE FOR SOLID FOODS

Ages at which various food and vitamins are introduced

| AGE          | Vitamin D | Vitamin C | Vitamin Orange Juice | Ripe Banana | Vegetables Sieved or Mashed | Fruit Puree | Special Cereal and Breads | Coarsely Chopped Foods | Special Desserts | Raw Fruit and Vegetables |
|--------------|-----------|-----------|----------------------|-------------|-----------------------------|-------------|---------------------------|------------------------|------------------|--------------------------|
| 2 Weeks      | Begin     |           |                      |             |                             |             |                           |                        |                  |                          |
| 3 Weeks      |           | Begin     |                      |             |                             |             |                           |                        |                  |                          |
| 1 Month      |           |           | Begin                |             |                             |             |                           |                        |                  |                          |
| 6-8 Weeks    |           |           |                      | Begin       |                             |             |                           |                        |                  |                          |
| 10-12 Weeks  |           |           |                      |             | Begin                       |             |                           |                        |                  |                          |
| 3 Months     |           |           |                      |             |                             | Begin       |                           |                        |                  |                          |
| 5-8 Months   |           |           |                      |             |                             |             | Begin                     |                        |                  |                          |
| 9 Months*    |           |           |                      |             |                             |             |                           | Begin                  |                  |                          |
| 10 Months    |           |           |                      |             |                             |             |                           |                        | Begin            |                          |
| 15-18 Months |           |           |                      |             |                             |             |                           |                        |                  | Begin                    |

\* Cup feeding should be introduced at 9-12 months of age.

Table 9.

That the administration of phenylalanine-low dietaries is not altogether straightforward may be illustrated by the reports of several investigators. Dodge et al. (1959) reported on the cases of two P.K.U. children who developed hypoglycaemia when treated with such a diet. In their cases, refusal to ingest an adequate amount of an unpalatable diet over a prolonged period resulted in the development of a state of undernutrition accompanied by fatty changes in the liver. With the development of consequent liver insufficiency, a short period of fasting led to the development of severe hypoglycaemia with convulsions and coma. Brimblecombe et al. (1961) pointed to the fact that an inadequate caloric intake, especially of carbohydrates, led to the development of a negative nitrogen balance with consequent elevation of blood phenylalanine levels. They also instanced the cases of two infants in whom restriction of phenylalanine had been possibly excessive and in whom such restriction had resulted in the development of subnormal phenylalanine levels with loss of weight, vomiting, listlessness and, in the case of one infant, the appearance of a severe eczematous rash which cleared rapidly on the addition of more phenylalanine to the diet. They concluded that, on purely empirical grounds, infants under the age of 12 months required a higher basic intake of phenylalanine than older children, possibly because of the rapid growth-rate of muscle at this age. Lewis (1960) drew attention to another unforeseen aspect of the problem when he detailed the case of a P.K.U. infant who had been diagnosed at the age of 18 days and placed on a phenylalanine-low dietary. At the age of 4 and one half months his blood phenylalanine level was quite satisfactory but his general condition was so poor that death seemed imminent if the dietary were to be continued. No other cause for /

the deterioration could be demonstrated and the dietary was discontinued. Recovery after the giving of a full-strength cow's milk preparation was immediate and dramatic; appetite improved, vomiting ceased and weight was rapidly gained. After clinical recovery he was again placed on a phenylalanine restricted dietary but very gradually indeed. On this re-introduction it was 6 weeks before the blood phenylalanine was allowed to return to a proper level. The child subsequently thrived well and, at the age of 17 months, psychological testing placed his development within normal limits for his age. Royston and Parry (1962) reported the case of an infant suffering from P.K.U. who developed a nutritional megaloblastic anaemia of infancy during the dietary treatment of the condition and who died at the age of 7 months. Woolf (1962) described the steps which led to the development of the phenylalanine - low dietary and stressed that, insofar as the treatment of the disease was concerned, P.K.U. must be regarded as a major problem of importance in amino acid imbalance. He too pointed out that phenylalanine is an essential amino acid and that if too little is administered the infant becomes ill and may die. He also stressed the point that phenylalanine requirements vary greatly from one infant to another but, especially in the first 18 months of life, vary considerably from time to time in the same infant. It was his view that there was a need for individual determination of phenylalanine requirements for each child suffering from the disease, such requirements to be brought continually under review. A dissenting opinion with regard to the worth of the conventional dietary therapy was voiced by Kirman (1961) who stated, that "dietetic treatment is tedious and difficult and - - - in view of lack of control, validation of results is still sub judice". His particular /

viewpoint was, that although the ideal form of treatment would be to replace the missing enzyme by the natural product or a synthetic analogue, such replacement was not possible at the present stage of our knowledge. He felt, nevertheless, that various alternative projects designed to produce desirable changes in the blood of P.K.U. patients, although utilising different approaches, were fundamentally sound.

The treatment of phenylketonuria should not be by individual to individual. Successful treatment of the disease requires teamwork of the first order on the part of the clinician, laboratory worker, health visitor, medical social worker, psychologist and dietician. In the proper handling of the patient suffering from the disease, all the above-mentioned will assume, from time to time, paramount importance. In very few children's diseases does the old adage of "treating the disease but forgetting the child" not operate: in P.K.U. it may operate more than in many others. It can never be tenable that this disease should be treated from a medical and nutritional standpoint alone. It must be appreciated that the introduction of the concept of a child requiring a special dietary, lest it become mentally retarded, has a profound psychological impact on the parents. From diagnosis of the condition onward, the family unit is involved. The act of feeding is the infant's earliest emotional experience and is very quickly centred upon the mother's love and care. Lack of understanding on the part of the mother will certainly lead rapidly to the development of insecurity in her relationship with her infant and this, in turn, will cause the development of maladjustment on the part of the child in its relations with society. To consider only one aspect, the normal processes whereby children attempt to exert power over /

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their parents by refusal to feed, in the case of an insecure P.K.U. child, will be liable to become accentuated by the development of knowledge on the child's part that it is "different" and that its dietary is already a source of anxiety for the parents. The emotional sequelae - to an already guilt-laden parent - must cause an increase in the parental anxieties and lead, in turn, to transference to the child of those feelings of inadequacy with resultant extreme aggravation of the existing tensions of the parent-child relationship. Again, parents who have fixed ideas with regard to infant feeding and whose views regarding such feeding reflect their own experiences and feelings towards food in general, are placed in an equally stressful situation when a phenylalanine-low diet is introduced. Such parents must be taught to strike a balance between overemphasis on the diet and their own over-solicitousness, must learn to withhold food from their child without feeling punitive and must not use the diet as a weapon in dealing with other aspects of the parent-child relationship. In general, should the parental education with regard to management of the child prove defective, the neurosis which develops must allow the phenylalanine-restricted dietary to become the dominant factor within the family, with the resultant possibility of the overall mental health of the family unit becoming adversely affected. Parents must therefore be supplied with as much information regarding the disease and its management as they can assimilate. They, in their turn, must continually strive to educate their own child in the reason for dietary restrictions being practised. Such education of the parents will allow of their gaining insight into their own attitudes to food and lead them to understand why it becomes of such vital importance to withhold certain foodstuffs /

from the child. Such a counsel of perfection is not easy to achieve.

When a phenylalanine-restricted dietary has been established, does it become possible ever to dispense with it? On this subject there is no universal agreement.

It has been suggested by Knox (1960) that therapy is only necessary during childhood, during the period of the development of the C.N.S. This suggestion was based retrospectively on the comparison of 44 P.K.U. patients over the age of three and who showed no impressive changes in mental or neurological status in consequence of such treatment, with 43 similar patients under the age of three in whom treatment produced obviously beneficial results. Centerwall et al. (1961) stated, that "the diet should probably be continued till the child is three years of age or older" and expected that it would require continuation in a modified form throughout the school years. Moncrieff and Wilkinson (1961) were reluctant to commit themselves on this matter although they stated, that "evidence on the matter is beginning to accumulate" without actually detailing this minutely. One of the earliest pronouncements was that made by Armstrong (1957) which stated that no deterioration occurred after the age of about six years. This statement is generally felt to be too dogmatic and not entirely supported by the observed facts. Horner et al. (1962) aligned themselves with those who suggested that dietary control could be relaxed at a comparatively early age, by publishing their findings in respect of three P.K.U. children who had been treated from earliest infancy. In each of these children, cessation of the diet was made when they reached the age of four and during the two and one half years, one and one half years and two months, respectively,

which had elapsed from cessation of the diet till publication of the report, no deterioration of significance was stated to have taken place in their mental development. Koch (1964) had an opportunity of subsequently examining two of the children and cautiously stated, that "discontinuance of dietary therapy may be more hazardous than previously thought". Bickel (1963) had already corroborated this suggestion by stating that dietary therapy should be continued at least till adolescence.

Although a general assessment of the conclusions of most authorities would appear to point to the fact that the greater part of the cerebral damage occurs in the first few months of life, that less is caused in the second and third years of life and that damage to the C.N.S. is possibly complete by the end of the fourth year, it is true to state that too few patients have been discovered in the earliest days of life and have had well-controlled treatment carried out for a sufficiently long period to allow of proper conclusions being drawn with regard to the safe age of termination of the dietary. There can be no denial of the fact that, in P.K.U., loss of intellectual capacity, as measured by the I.Q., once sustained can never be regained, where such loss results from the relaxation of strict dietary control. In this connection, one constructive suggestion deserving of further consideration is that made by Stern (1963) to the effect that signs of intoxication should be sought, rather than signs of brain damage, in the psychological assessment of P.K.U. children who have been allowed relaxation of the phenylalanine-low dietary. Such methods might be derived from those used in psychopharmacology and psychodietetics and would probably be applicable to children with a mental age of 5 or 6 upwards.

## RESULTS.

As was stated in the preamble to this thesis, surveys of neonates and early infants (infants within the first two months of life) born in Stobhill, Robroyston and Redlands Hospitals, Glasgow, were undertaken during the period of December, 1964, to November, 1965, inclusive.

A screening of pupils enrolled in the Special Schools and Occupational Centres in the City of Glasgow was also undertaken, as was periodic evaluation of the blood phenylalanine levels of known phenylketonurics receiving treatment, such patients primarily being cared for in the Royal Hospital for Sick Children, Glasgow.

In addition, a screening of patients in Lennox Castle Hospital, Lennoxtown, and the Royal Scottish National Institution, Larbert, was also performed. Both these institutions are hospitals for the mentally retarded.

Presentation of detailed results pertaining to each of the above surveys will be made and, in addition, incidental findings unconnected with such initial proposals will also be presented.

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Summary of Guthrie Test Results.

1. Neonatal and Early Infancy Survey.

|                       |        |
|-----------------------|--------|
| Cases Examined.       | 6,412. |
| Specimens Processed.  | 6,475. |
| Presumptive Positive. | 92.    |
| Confirmed Positive.   | 1.     |

2. School Health Survey.

|                            |        |
|----------------------------|--------|
| Pupils Examined.           | 2,115. |
| Specimens Processed.       | 2,121. |
| Presumptive Positive.      | 9.     |
| Confirmed Positive.        | 6.     |
| Previously Known Positive. | 2.     |
| Discovered Positive.       | 4.     |

3. Mental Defective Institutions.

|                            |        |
|----------------------------|--------|
| Cases Examined.            | 2,274. |
| Specimens Processed.       | 2,337. |
| Presumptive Positive.      | 73.    |
| Confirmed Positive.        | 30.    |
| Previously Known Positive. | 16.    |
| Discovered Positive.       | 14.    |

4. Known Cases for Assessment.

|  |      |
|--|------|
| Total Patients (including case discovered in neonatal survey). | 29.  |
| Specimens Processed.   | 180. |

5. Miscellaneous Sera Processed.

|                                      |      |
|--------------------------------------|------|
| Specimens Processed.                 | 425. |
| Confirmed Positive Cases Discovered. | 2.   |

\*\*\* END OF REPORT \*\*\*

|  |         |
|--|---------|
| Total Specimens Processed by Guthrie Method. | 11,538. |
|--|---------|

|   |     |
|---|-----|
| Confirmed Cases Discovered by Guthrie Method. | 21. |
|---|-----|

## Neonatal and Early Infancy Surveys.

### Stobhill and Robroyston Hospitals.

Both the above hospitals are administered by the same Board of Management and practice therein is more or less standard. Neonates are fed on half-cream National Dried Milk if not being breast-fed. In the case of first births, discharge of the mother and child generally takes place on the 8th. day and, for second and subsequent births, generally on the 7th. day. In spite of those generalisations earlier discharges are not infrequent. In the case of full-term, healthy infants, blood specimens for Guthrie testing were obtained on the day of discharge from hospital in the majority of occasions. In the case of neonates from whom no further specimens appeared necessary, i.e. where initial evaluation showed blood phenylalanine levels to be less than 4 mg. per 100 ml., the average period elapsing between birth and collection of the specimen for processing was 5.8 days.

During the period under review, 5,425 neonates and early infants were screened and, of those, 35 were shown to have initial blood phenylalanine levels of 4 mg. per 100 ml. or above. In those 35 cases follow-up was attempted, necessitating one or more letters to the General Practitioners responsible for 28 neonates discharged home. In the remaining 7 cases, follow-up specimens were obtained while the infants were still in hospital. From the 28 General Practitioners notified, 11 replies and repeat specimens of blood were obtained, a percentage reply of 39.3. Initial blood levels were as follows:- /

|                             |   |              |   |                         |
|-----------------------------|---|--------------|---|-------------------------|
| less than 4 mg. per 100 ml. | : | 5,390        | : | 99.36 per cent.         |
| 4 to 6 mg. per 100 ml.      | : | 16           | : | 0.29 per cent.          |
| 6 to 8 mg. per 100 ml.      | : | 17           | : | 0.31 per cent.          |
| 8 to 10 mg. per 100 ml.     | : | 2            | : | 0.04 per cent.          |
|                             |   | <u>5,425</u> |   | <u>100.00 per cent.</u> |

Follow-up levels were as follows:-

General Practitioner returns.

|                             |   |   |   |                |
|-----------------------------|---|---|---|----------------|
| less than 2 mg. per 100 ml. | : | 9 | : | 81.8 per cent. |
| 2 to 4 mg. per 100 ml.      | : | 2 | : | 18.2 per cent. |

Hospital returns.

|                             |   |   |   |                |
|-----------------------------|---|---|---|----------------|
| less than 2 mg. per 100 ml. | : | 4 | : | 57.1 per cent. |
| 2 to 4 mg. per 100 ml.      | : | 0 | : | 0 per cent.    |
| 4 to 6 mg. per 100 ml.      | : | 1 | : | 14.3 per cent. |
| 6 to 8 mg. per 100 ml.      | : | 1 | : | 14.3 per cent. |
| 10 to 12 mg. per 100 ml.    | : | 1 | : | 14.3 per cent. |

From the neonates and early infants screened, one proven positive case of phenylketonuria was discovered. This was of an infant who was admitted to Stobhill Hospital at the age of three weeks and will be discussed separately. In three of the cases followed-up in hospital it was demonstrated that elevation of the blood phenylalanine levels was accompanied by an elevation in the level of blood tyrosine. Table 10 illustrates these cases.

TABLE 10.

|                      | <u>Case 1.</u> | <u>Case 2.</u> | <u>Case 3.</u> |
|----------------------|----------------|----------------|----------------|
| Date of Birth        | 15.IV.'65      | 17.VIII.'65    | 23.X.'65       |
| Premature?           | Yes.           | No.            | No.            |
| Date of 1st.specimen | 13.V.'65       | 25.VIII.'65    | 28.X.'65       |
| Age at 1st.specimen  | 28 days.       | 8 days.        | 5 days.        |

|   |           |           |           |
|---|-----------|-----------|-----------|
| Initial phenylalanine estimation - mg./100 ml.    | 8 to 10.  | 4 to 6.   | 8 to 10.  |
| Subsequent phenylalanine estimation - mg./100 ml. | 4 to 6.   | 10 to 12. | 6 to 8.   |
| Tyrosine demonstrated                             | 20.V.'65. | 4.IX.'65. | 29.X.'65. |
| Tyrosine level - mg./100 ml.                      | 40.       | 38.       | 79.       |
| Final phenylalanine estimation - mg./100 ml.      | 2.        | 2.        | 2.        |

In each case, tyrosine levels were estimated by the LaDu biochemical method referred to in the section dealing with Diagnostic Methods. The tyrosine level in Case 1 had fallen to 1 mg. per 100 ml. in September, 1965 and this fall had been gradual throughout the intervening period, according to information received from the Biochemistry Laboratory. In Case 2, subsequent to the demonstration of the elevated tyrosine level, 100 mg. ascorbic acid was administered to the infant by mouth. Estimation of the tyrosine level on 5.IX.'65 showed it to be less than 1 mg. per 100 ml. In Case 3 the level of tyrosine in the blood had fallen to 70 mg. per 100 ml. on 5.XI.'65 without the administration of ascorbic acid. No opportunity has as yet arisen to allow of tyrosine reassessment in this particular case. In all three cases blood phenylalanine had fallen to normal levels within a period of 14 days from the date of the initial evaluation.

Where follow-up specimens were requested, the time elapsing between birth and the taking of the initial specimen had varied from 5 to 28 days. Of two premature infants, the initial specimens were collected at the ages of 10 and 28 days respectively. If those premature babies be included, the average period elapsing between birth and the collection of the initial specimen was 6.6 days. If the premature infants are excluded from the calculation this figure falls to 5.8 days, i.e. the same number of days as was found in the /

case of 99.36 per cent of the neonates from whom no repeat specimen appeared necessary.

Where the co-operation of the General Practitioner was secured, the time elapsing between the reading of the initial test and the reading of the second test varied between 12 and 23 days, with an average of 15.6 days. If the figure of 5.8 days is accepted as being not unreasonable, it is then apparent that - in the case of the General Practitioner patient - the average lapse of time between birth and the establishing of proof of the existence of a normal level of blood phenylalanine will be 21.4 days, if normal methods of correspondence are used.

#### Redlands Maternity Hospital.

This hospital is administered by a different Board of Management and the date of discharge is earlier. Mother and infant are normally discharged on the 5th. day but, again, earlier discharge is not infrequent. Feeding of the infants is similar to that adopted by Stobhill and Robroyston Hospitals. As in these two latter hospitals, full-term, healthy infants had specimen cards impregnated with blood on the day of discharge. Where it subsequently appeared unnecessary to obtain further specimens, i.e., where initial evaluation showed blood phenylalanine levels to be less than 4 mg. per 100 ml., the average period which had elapsed between birth and specimen-collection for initial Guthrie processing was 4.1 days.

During the period under review, 987 neonatal and early infancy screenings were performed and, of those, 53 were shown to have initial levels of blood phenylalanine of 4 mg. per 100 ml. or more. Again follow-up was attempted in every case where there was an /

initially raised level and, in this connection, 51 General

Practitioners were notified and their co-operation sought. From those 51, 21 replies and repeat specimens of blood were received, a percentage reply of 41. Three cases were followed-up in hospital.

Initial blood levels of all cases were as follows:-

|                             |            |                           |
|-----------------------------|------------|---------------------------|
| less than 4 mg. per 100 ml. | : 934      | : 94.63 per cent.         |
| 4 to 6 mg. per 100 ml.      | : 23       | : 2.33 per cent.          |
| 6 to 8 mg. per 100 ml.      | : 22       | : 2.23 per cent.          |
| 8 to 10 mg. per 100 ml.     | : 6        | : 0.61 per cent.          |
| 10 to 12 mg. per 100 ml.    | : 2        | : 0.2 per cent.           |
|                             | <u>987</u> | <u>: 100.00 per cent.</u> |

Follow-up levels were as follows:-

General Practitioner returns.

|                             |      |                |
|-----------------------------|------|----------------|
| less than 2 mg. per 100 ml. | : 15 | : 71 per cent. |
| 2 to 4 mg. per 100 ml.      | : 6  | : 29 per cent. |

Hospital returns.

|                          |     |
|--------------------------|-----|
| 4 to 6 mg. per 100 ml.   | : 1 |
| 6 to 8 mg. per 100 ml.   | : 1 |
| 10 to 12 mg. per 100 ml. | : 1 |

In two cases followed-up in hospital it was shown that there was an elevation of blood tyrosine above normal limits. Table 11 illustrates these cases.

TABLE 11.

|                        | <u>Case 1.</u> | <u>Case 2.</u> |
|------------------------|----------------|----------------|
| Date of Birth.         | 23.11.'65      | 3.V.'65.       |
| Premature?             | No.            | No.            |
| Date of 1st. specimen. | 28.11.'65.     | 8.V.'65.       |
| Age at 1st. specimen.  | 5 days.        | 5 days.        |

|   |                      |               |
|---|----------------------|---------------|
| Initial phenylalanine estimation - mg./100 ml.      | 10 to 12.            | 4 to 6        |
| Subsequent phenylalanine estimation - mg./100 ml.   | 6 to 8.              | 10 to 12.     |
| Tyrosine demonstrated                               | 16. <u>III</u> .'65. | 22.V.'65.     |
| Tyrosine level - mg./100 ml.                        | 48.                  | 47.           |
| Final phenylalanine level.<br>(4 weeks after birth) | 4 mg./100 ml.        | 4 mg./100 ml. |

It has not been possible to follow-up the above two cases subsequent to discharge from hospital, both having been adopted. The third case followed up in hospital showed an initial blood phenylalanine level of 10 to 12 mg. per 100 ml. from a specimen taken 5 days after birth. A specimen on the 7th. day showed that the level had fallen to 4 to 6 mg. per 100 ml. Subsequent processing of a specimen obtained 14. days after birth showed the blood phenylalanine to be 2 mg. per 100 ml. The level of blood phenylalanine in respect of a maternal specimen also obtained on the 14th. day following the baby's birth was shown also to be 2 mg. per 100 ml.

Where follow-up specimens were requested by normal processes of correspondence with the General Practitioner, and where such were forwarded, the time between birth and the taking of the initial specimen had varied from 2 to 10 days, with an average of 4.3 days. In such instances, the time elapsing between the reading of the initial test and the reading of the second test varied between 8 and 71 days, with an average of 23.2 days. It is thus apparent that the lapse of time between birth and the proving of the existence of a level of blood phenylalanine within normal limits is, in this series, 27.5 days on average. It is not without interest in this connection that one of the infants from whom a second specimen was requested through the General Practitioner was the newly-born sib of a known phenylketonuric. /

Other Neonatal Cases.

Several neonatal and early infancy screenings were performed on babies outwith the previously mentioned hospitals.

Bellshill Maternity Hospital forwarded specimens in respect of four cases in whom it was thought that there was a possibility of the disease being present. Three of them showed an initial level at the age of 2 weeks, of 6 mg. per 100 ml., falling to a level of 2 mg. per 100 ml. after a further week had elapsed. All three were premature infants. The fourth had shown a positive "PHENISTIX" test when such was performed by the General Practitioner. The age of this child was then 3 weeks. On immediate admission to hospital it had not been possible to duplicate this result. Guthrie estimation of blood phenylalanine showed a level of 3 mg. per 100 ml. and this was duplicated by the LaDu biochemical method. LaDu estimation of the blood tyrosine showed this to be 42 mg. per 100 ml. At the age of 7 weeks, Guthrie testing of the blood showed the phenylalanine level to be within normal limits (2 mg./100 ml.) and LaDu estimation of the blood tyrosine showed this to be also at a normal level, namely 1 mg. per 100 ml.

Specimens were also forwarded from Seafield Hospital, Ayr. These were three in number and, of those, one was the neonate sib of an already-known phenylketonuric. This infant, at the age of 6 days, showed a blood phenylalanine level of 6 mg. per 100 ml. On repeat evaluation at the age of 12 days, blood phenylalanine was shown to be at a level of 2 mg. per 100 ml. Of the other two neonatal specimens, one was from an infant at the age of 12 days who was for adoption. This infant was shown to have a blood phenylalanine level of 2 mg. per 100 ml. The other was from an infant who, at the age of 35 days, had had a doubtfully positive PHENISTIX test result. /

In this case a blood phenylalanine level of 2 mg. per 100 ml. was demonstrated.

-----

The proven positive case of neonatal phenylketonuria, to which reference has already been made, was admitted to the paediatric department of Stobhill Hospital at the age of three weeks.

This infant, the mulatto daughter of a prostitute, was admitted with the presumptive diagnosis of:- "failure to thrive; ? congenital syphilis". Urinary PHENISTIX testing on admission gave a negative result but initial evaluation of heel-prick blood at the age of three weeks showed the presence of a phenylalanine level in excess of 20 mg. per 100 ml. Evaluation after the lapse of four days - utilising higher controls - showed the level to be between 60 and 70 mg. per 100 ml. On the day in which this level was found, treatment with MINAFEN was commenced and two days later the blood phenylalanine level was shown to be 30 mg. per 100 ml. Four days later the blood phenylalanine level was estimated to be 4 mg. per 100 ml., and one week later 2 mg. per 100 ml. Three weeks after the demonstration of the level of 60 to 70 mg. per 100 ml., Guthrie evaluation showed the blood phenylalanine level still to be in the region of 2 mg. per 100 ml. or less. Concomitant LaDu biochemical estimation estimated the level of the serum phenylalanine to be 6.2 mg. per 100 ml. It was then discovered that LaDu biochemical estimations had been performed on three occasions in the preceding two weeks, utilising blood withdrawn at the same time as that used in the Guthrie testing. Each of the Guthrie estimations had shown that blood phenylalanine levels were in the region of 2 mg. per 100 ml. or less. The comparable LaDu estimations had suggested that the serum phenylalanine levels were 3.9 mg. per 100 ml.; /

156.

6.0 mg. per 100 ml. and 4.1 mg. per 100 ml. In consequence of the previous reliance which had been placed by the paediatricians on the biochemical method of estimation, it was decided by them that treatment of the infant would be governed by the biochemical method of phenylalanine evaluation. Specimens of blood were nevertheless still forwarded at irregular intervals for Guthrie evaluation. Within a period of four months, 15 specimens had been received and processed. In each case the level of blood phenylalanine was estimated to be in the region of 2 mg. per 100 ml. or less. During this period it was learned subsequently that the LaDu biochemical method had consistently suggested a serum phenylalanine level of between 4 and 6 mg. per 100 ml. On being requested to see the child - now at the age of nine months, in October, 1965, - she was found to be very hypotonic, lethargic and vomiting almost every feed. Her condition was very poor and she was losing weight rapidly. The suggestion was made that the condition resulted from the fact of her having received too small an intake of phenylalanine for the previous four months, and that dietary restriction would require to be foregone for a short time. On cessation of the phenylalanine-restricted dietary and its substitution with whole milk, the general condition improved dramatically; she became bright and alert, the hypotonia rapidly disappeared, vomiting ceased and she gained weight daily. After continuing this regime for a period of one week she developed chickenpox and was transferred to an isolation hospital. In consequence of a breakdown in communication, it was not appreciated by the staff of the isolation hospital that the free dietary regime was only of a very temporary nature. Eleven days after the previous estimation of the blood phenylalanine level a further specimen was obtained and processed. This specimen showed that /

the blood phenylalanine level was now 60 mg. per 100 ml., and that in respect of the urinary phenylalanine, 12 mg. per 100 ml.

Immediate steps were taken to resume the phenylalanine restriction and the blood level was gradually brought down to a Guthrie estimation of 2 to 4 mg. per 100 ml. over a period of three weeks. It was agreed by the paediatricians concerned, that if Guthrie blood phenylalanine estimations had been accepted by them as being a more accurate reflection of existing levels than those suggested by conventional biochemical methods, the condition of this infant would not have been allowed to progress to the grave state which existed prior to temporary cessation of the phenylalanine-restricted diet.

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School Health Service Survey.

In the latter part of May, 1965, a survey of the pupils enrolled in the Special Schools and Occupational Centres of the City of Glasgow was commenced with the co-operation of the Medical Officer of Health for the City of Glasgow and the Principal Medical Officer, School Health Service.

The pupils enrolled in 18 Special Schools numbered 2,620 and those in 11 Senior and Junior Occupational Centres, 442. Parental consent to the withdrawal of finger-stab blood was obtained in respect of 1,792 (68.4 per cent) of the Special School population. Similar parental consent was obtained in respect of 323 (73.1 per cent) of the Occupational Centre population. From a total of 2,115 pupils screened, 7 were discovered to be victims of the disease. Of the 7, two were previously-known cases; one a male aged 25 years attending a Senior Occupational Centre and not receiving treatment, the other attending the Royal Hospital for Sick Children, Glasgow, (Professor Hutchison), for dietary supervision. Of the remaining 5 cases, 4 were confirmed by the LaDu biochemical method in the Biochemistry Department of the above hospital. The last case to be discovered by the Guthrie method had not had independent laboratory confirmation of the diagnosis by the time of publication of these results. Two of the 4 confirmed cases were from one sibship. Particulars of the individual cases are detailed below.

Case 1. B/B.; male; known case in Senior Occupational Centre not receiving treatment; born in Poland 28.IV.'40; parents and grandparents born in Poland; blood phenylalanine level in excess of 20 mg. per 100 ml.

Case 2. A/B.; female; discovered case in Special School; born in Glasgow 17.VI.'55; not known where parents /

and grandparents were born; initial blood phenylalanine level 12 mg. per 100 ml. on 24.V.'65; LaDu serum level 26.4 mg. per 100 ml. on 26.VIII.'65; admitted to R.H.S.C. (Professor Hutchison) 13.VIII.'65 where serum phenylalanine levels fluctuated between 16 and 26 mg. per 100 ml. during period of observation; I.Q. (Wechsler) 64; E.E.G. showed no significant abnormality; youngest of three sibs; eldest sister was found to have attended Special School and her serum phenylalanine estimation (LaDu method) disclosed a level of 33 mg.per 100 ml., thus establishing the fact that she was also a homozygote phenylketonuric, whereas her own daughter showed a serum phenylalanine level of 3.4 mg. per 100 ml. and was heterozygotic for the disease; A/B. not put on dietary treatment; classified as mentally handicapped but educable on 6.X.'61; I.Q. (Terman Merrill, Form L) at that date, 63; walked and talked at usual age; mother's pregnancy and the birth of A/B. herself uneventful; classified again as mentally handicapped but educable, 3.VIII.'62; reading (Burt Scale) classified as for age 5; mental arithmetic at 6 year level.

Case 3. L/C.; male; discovered case attending Occupational Centre; born in Glasgow 23.VIII.'58; parents born in Glasgow; paternal grandfather born in Tyrone, Ulster, and paternal grandmother born in Glasgow; maternal grandfather born in Greenock and maternal grandmother born in Newcastle; blood phenylalanine /

level 4.VI.'65, slightly in excess of 20 mg. per 100 ml.; serum phenylalanine level (LaDu method) 26.VII.'65, 22.8 mg. per 100 ml.; classified as mentally handicapped but educable 2.X.'63; subsequently classified (January, 1964) as mentally handicapped, ineducable but trainable; said to be a thin, active boy with defective speech and a very difficult temper; attended R.H.S.C. from age of ten months with scleroderma but this has now cleared.

Case 4. K/G.; female; discovered case attending Special School; sib of Case 3; initial blood phenylalanine level 8 to 12 mg. per 100 ml.; blood level four weeks later, slightly in excess of 12 mg. per 100 ml. and biochemical evaluation at this date showed a serum phenylalanine level (LaDu) of 13.9 mg. per 100 ml.; E.E.G. normal; I.Q. 57 (Terman Merrill, Form L, February, 1962); no further particulars regarding this case.

Case 5. W/H.; male; known case attending Special School and also R.H.S.C.; blood phenylalanine level in present screening, 4 mg. per 100 ml.; no further investigation necessary although blood level was at the highest limit of normality.

Case 6. M/G.; female; discovered case attending Special School; born in Glasgow as were parents and grandparents; date of birth 20.X.'58; initial blood phenylalanine estimation in excess of 20 mg. per 100 ml.; second estimation of same specimen, but with higher controls, demonstrated a level of blood /

phenylalanine slightly above 40 mg. per 100 ml.; biochemical evaluation (LaDu) demonstrated a serum phenylalanine level of 41.6 mg. per 100 ml.; E.E.G. normal; classified mentally handicapped but educable in November, 1963; I.Q. (Terman Merrill, Form L) 48; enuretic; did not walk till two years and six months; did not talk till age four; no further particulars available.

Case 7. M/M.; female; discovered case (19.XI.'65) attending Special School; initial blood phenylalanine level 20 mg. per 100 ml.; not yet confirmed by independent laboratory although Guthrie confirmatory procedure is positive; presumptive positive case; classified as mentally handicapped but educable on 28.I.'64; I.Q. (Terman Merrill, Form L) 74; no further particulars available.

In this survey, two further cases who showed an initial level of 4 mg. per 100 ml. were discovered and further specimens requested and obtained for processing. In one, the subsequent blood phenylalanine level was less than 2 mg. per 100 ml. and in the other, 4 mg. per 100 ml. This latter case - from personal knowledge of the family - warrants further investigation in the future.

Known Cases Receiving Treatment.

Specimens for Guthrie evaluation of blood phenylalanine in respect of patients were received from Drs. Riley and Fox, Stobhill General Hospital; Dr. Abramson, Seafield Hospital, Ayr; Dr. McBean, Royal Hospital for Sick Children, Glasgow, on behalf of Professor Hutchison.

There were four known cases attending the Paediatric Department of Stobhill Hospital.

Case 1. A/L.; male; date of birth 30.1.'60; sib of Case 2; four Guthrie estimations performed between June, 1964 and January, 1965; levels varied from 20 to 2 mg. per 100 ml.; severely retarded; parents of poor mentality and dietary enforcement poor in consequence; third sib unaffected.

Case 2. H/L.; female; sib of Case 1; date of birth 17.11. '62; retarded; one Guthrie estimation performed with a level of blood phenylalanine of 6 to 8 mg. per 100 ml.; domiciliary management as for Case 1.; third sib unaffected.

Case 3. M/McS.; female; date of birth 29.VI.'63; severely retarded homozygote sib in Lennox Castle Institution; good dietary control; nine Guthrie estimations performed in 15 months; levels of blood phenylalanine consistently in the region of 2 mg. per 100 ml.; not obviously retarded.

Case 4. J/O'H.; male; date of birth 14.IX.'61; P.K.U. diagnosed October, 1962; LaDu estimation of blood phenylalanine in October, 1962, showed a level of 32 mg. per 100 ml.; phenylalanine restricted dietary /

instituted October, 1962, and PHENISTIX testing of urine consistently negative since then; Guthrie blood phenylalanine estimation in October, 1965, showed level of 4 to 6 mg. per 100 ml.; severely retarded.

Guthrie blood phenylalanine estimations were performed in respect of four known cases from Seafield Hospital, Ayr.

Case 1. had three estimations performed in 4 months; levels ranged from 12 to 20 mg. per 100 ml. with an average of 15 mg. per 100 ml.

Case 2. had two estimations performed in 8 months, each showing a level of 20 mg. per 100 ml.

Case 3. had five estimations performed in 11 months; levels ranged from 2 to 12 mg. per 100 ml. with an average of 7.2 mg. per 100 ml.

Case 4. had five estimations performed in 8 months with levels varying between 2 and 30 mg. per 100 ml. with an average of 10.8 mg. per 100 ml.

From the Royal Hospital for Sick Children, Glasgow, 125 Guthrie estimations were performed for twenty known patients. Within this group, three were no longer receiving dietary treatment and Guthrie blood phenylalanine estimations in those cases showed levels in excess of 20 mg. per 100 ml. Of those receiving dietary treatment, four were from two sibships and one had a known phenylketonuric elder sister from whom no specimen was received. One case was known also to have been screened in the School Health Service survey. Blood phenylalanine levels, as estimated by the Guthrie method, were demonstrated to lie between 1 mg. per 100 ml. and /

4.0 mg. per 100 ml., with an average level of 6.3 mg. per 100 ml. Five of the treated cases showed levels, on at least one occasion, of 15 mg. per 100 ml. or above, with a maximum in one patient of 4.0 mg. per 100 ml. If such patients be excluded from data assessment, the average blood phenylalanine level is shown to be 4.1 mg. per 100 ml. in respect of cases receiving dietary treatment. In one case, born on 11.XI.'64, Guthrie estimation showed the blood phenylalanine level at the age of eleven weeks to be 70 mg. per 100 ml. LaDu biochemical evaluation of the same specimens showed a level of serum phenylalanine of 70.2 mg. per 100 ml. The Guthrie level had fallen to 4 to 6 mg. per 100 ml. on 16.IV.'65 and the average level has been 3.2 mg. per 100 ml. since that date. Of the members of the two sibships, in each the elder had been diagnosed at a later age than the younger and thus dietary restriction had not been instituted in the neonatal period. The average Guthrie blood phenylalanine levels for the sibships are:-

| Sibship. | Elder.                           | Younger.                                 |
|----------|----------------------------------|--|
| 1.       | (4 9/12yrs.) 6.7 mg. per 100 ml. | (1 7/12yrs.) less than 2 mg. per 100 ml. |
| 2.       | (5 6/12yrs.) 5.2 mg. per 100 ml. | (2 3/12yrs.) 3 mg. per 100 ml.           |

## Mental Defective Institutions.

The institutions screened were Lennox Castle Institution, Lennoxtown, and the Royal Scottish National Institution, Larbert. In neither case would the screening of the institutionalised population have been possible without the enthusiastic co-operation of the respective Medical Superintendents, Drs. MacGillivray and Methven, and their Deputies, Drs. Campbell and Wright.

### Lennox Castle Institution.

In this and ancillary institutions, 1,134 patients were screened. All patients had had urinary PHENISTIX or ferric chloride testing performed at some stage of their residence in the institution. It was thus known to the clinicians responsible that there were 10 such positive urines among the patients - presumed not to result from the administration of drugs - and thus that there were 10 presumed phenylketonurics. Such knowledge was not imparted to the staff of the bacteriology department of Stobhill General Hospital, where Guthrie processing was performed. 1,134 patients screened, 43 (3.8 per cent) showed an initial blood phenylalanine level of between 4 and 8 mg. per 100 ml. On repeat examination it was shown that the level of the second specimen in those cases was within normal limits, i.e. between 2 and 4 mg. per 100 ml. A further 23 (2.0 per cent) showed a level of 12 mg. blood phenylalanine per 100 ml., or higher. Repeat examination in those cases demonstrated the existence of similar, or higher, levels of blood phenylalanine, thus confirming presumptive positive results and, in every case of this type, it was subsequently possible to demonstrate the presence of urinary ortho-hydroxy-phenylacetic acid. It was thus proven that all such cases were true phenylketonurics. /

In some instances it was also possible to obtain corroborative biochemical evaluation by the Ladu technique. No specimens were processed which showed blood phenylalanine levels between 8 and 12 mg. per 100 ml.

From the above figures, it is apparent that the presumptive positive cases for the population screened amounted to 5.8 per cent of the whole; the previously-estimated phenylketonuric population was 0.9 per cent of the whole, and the finally determined phenylketonuric population was 2.2 per cent of the whole. Although only 10 patients were known to have positive PHENISTIX or ferric chloride reactions in their urine, it is not without significance that every case detected by the Guthrie method subsequently was shown to exhibit a positive reaction to PHENISTIX test-strips.

With regard to the previously-known phenylketonurics in the institution, seven were males and three were females. The ages ranged from 4 to 35 and five exhibited the so-called "typical" appearance of the phenylketonuric or had done so in earlier years. Blood levels of phenylalanine varied from 12 to 50 mg. per 100 ml., showing a mean level of 25 mg. per 100 ml. Four were from two sibships and, including those four, seven were known to have homozygote sibs and four of the seven also to have non-homozygote sibs. Two of the remainder who did not have homozygote sibs had non-homozygote sibs, including one female patient who had an apparently normal dizygotic twin sister working as a shorthand-typist. Only one had a history of psychomotor epilepsy.

The particulars of the discovered cases were as follows:-

Case 1. M/A.; aged 41 on diagnosis; confirmatory blood phenylalanine level 50 mg. per 100 ml.; hair light brown, eyes blue, complexion fresh; I.Q. 20; three normal sibs: female /

Case 2. P/C.; aged 20 on diagnosis; confirmatory blood phenylalanine level 20 mg. per 100 ml.; "typical" appearance; severely retarded; paternal grandparents born in Ireland; maternal grandparents born in Greenock, Scotland; hypotonic; male.

Case 3. J/D.; aged 39 on diagnosis; confirmatory blood phenylalanine level 20 mg. per 100 ml.; formerly possessed "typical" appearance; severely retarded; epileptic, as are mother and maternal aunt; paternal grandparents born in rural Perthshire, Scotland; maternal grandfather born in Neilston, Scotland, and maternal grandmother in Haddington, Scotland; hypertonic; male.

Case 4. M/F.; aged 57 on diagnosis; confirmatory blood phenylalanine level 70 mg. per 100 ml.; hair black, eyes brown, complexion sallow; mental age 1 year and 6 months; had pulmonary tuberculosis and facial lupus; died in September, 1965; female.

Case 5. E/G.; aged 6 on diagnosis; confirmatory blood phenylalanine level 12 mg. per 100 ml.; no further particulars; female.

Case 6. A/H.; aged 49 on diagnosis; confirmatory blood phenylalanine level 20 mg. per 100 ml.; male; "typical" appearance; hypertonicity; severely retarded; no further particulars.

Case 7. A/J.; aged 24 on diagnosis; confirmatory blood phenylalanine level 20 mg. per 100 ml.; female; very severely retarded; "typical" appearance; epileptic; has retarded female sib; no further particulars./

Case 8. C/M.; aged 12 on diagnosis; confirmatory blood phenylalanine level 12 mg. per 100 ml.; female; no further particulars.

Case 9. K/McG.; aged 44 on diagnosis; confirmatory blood phenylalanine level 40 mg. per 100 ml.; female; widow with four heterozygote children; all grandparents Irish; mental age 6 years; red hair, eyes blue, fresh complexion; admitted to institution in consequence of child neglect subsequent to death of husband; I.Q. (Terman Merrill) 39; two brothers alive and working in Ireland from whom blood and urine specimens were obtained - blood phenylalanine levels shown to be in the region of 2 mg. per 100 ml. and no demonstration of urinary ortho-hydroxy-phenylacetic acid; four heterozygote daughters of Case 9 had blood and urine investigated - blood phenylalanine levels between 1 and 3 mg. per 100 ml.; no demonstration of ortho-hydroxy-phenylacetic acid in the urine of two but demonstrable in two.

Case 10. A/McG.; aged 24 on diagnosis; confirmatory blood phenylalanine level 20 mg. per 100 ml.; female; very severely retarded; epileptic; said to have had "typical" appearance in earlier years; all grandparents born in Greenock, Scotland; no further particulars.

Case 11. J/P.; aged 24 years on diagnosis; somewhat "typical" appearance; two sibs, one male, homozygote P.K.U. sib and one male, unaffected; no further particulars; confirmatory blood phenylalanine level 12 to 20 mg. /

per 100 ml.; concomitant LaDu evaluation showed a serum phenylalanine level of 15 mg. per 100 ml.

Case 12. S/P.; aged 36 on diagnosis; confirmatory blood phenylalanine level in excess of 40 mg. per 100 ml. (concomitant LaDu estimation demonstrated a serum phenylalanine level of 44.9 mg. per 100 ml.); one male, homozygote P.K.U. sib and one male, unaffected (Cases 11 and 12 from same sibship); "typical" appearance; docile and amenable, in contradiction to affected sib (Case 11) who is liable to wild outbursts of shouting and is easily excited and apprehensive; I.Q. (Cattell) 56; no further particulars.

Case 13. L/S.; aged 62 on diagnosis; confirmatory blood phenylalanine level in excess of 20 mg. per 100 ml.; female; hair fair, eyes brown; mental age of less than 5; has recurring dermatitis; epileptic till 1941; no further particulars.

Summarising the above results, it can be seen that five were males and eight were females. The ages at the time of diagnosis ranged from 6 to 62 with an average age of 34.4, and seven displayed, or had earlier displayed, "typical" or "somewhat typical" appearances of the phenylketonuric. Confirmatory blood levels of phenylalanine varied from 12 to 70 mg. per 100 ml., showing an average level of 27.7 mg. per 100 ml. Four were epileptic or had previously had a history of epilepsy. Seven were severely, or very severely, retarded. Two were from one sibship in which there was an unaffected sib and a further two cases had unaffected sibs; one case had a /

retarded sib. Where it proved possible to trace the forebears in four cases, six of the grandparents had been born in Ireland, six in Greenock and four in Scotland other than Greenock. One case had black hair and this, together with one other, had brown eyes.

Insofar as is possible, Table 12 compares the findings in respect of the previously-known and the Guthrie-discovered phenylkotonurics in Lennox Castle Institution.

| <u>Table 12.</u>  | <u>Previously known.</u> | <u>Guthrie-discovered.</u> |
|---|--------------------------|----------------------------|
| 1. Number of patients.                                    | 10.                      | 13.                        |
| 2. Males : Females  | 7 : 3                    | 5 : 8.                     |
| 3. Average age.   | 20.2 years.              | 34.4 years.                |
| 4. Average blood phenylalanine (mg. per 100 ml.).         | 25.                      | 28.                        |
| 5. Epileptic.   | 1.                       | 4.                         |
| 6. Sibships affected.                                     | 2.                       | 1.                         |
| 7. Patients with unaffected sibs.                         | 6.                       | 4.                         |
| 8. Patients with P.K.U. sibs.                             | 7.                       | 2.                         |
| 9. Patients with Irish and/or Greenock-born grandparents. | ?                        | 3.                         |

All specimens which showed a blood phenylalanine level of 12 mg. per 100 ml. or higher were subjected to the confirmatory Guthrie procedure for the demonstration of the presence of phenylalanine as detailed in the chapter dealing with the Guthrie Technique.

The Royal Scottish National Institution.

In this institution 1,140 patients were screened by the Guthrie method. All patients had had urinary PHENISTIX testing performed immediately prior to blood screening. Of the 1,140 patients, it was known to the clinicians that 6 were presumed phenylketonurics and that 17, in all, had shown a positive or doubtful positive reaction with PHENISTIX test-strips when the urines had been tested. This information was not made available to the processing laboratory in Stobhill General Hospital till evaluation of the phenylketonuric population had been made by it. Estimation of the blood phenylalanine levels by the Guthrie method corroborated the existence of the six previously known cases and discovered the existence of one further case. All seven patients - 0.6 per cent of the institution-alised population - had blood levels of phenylalanine of 12 mg. per 100 ml., or above. There were no patients who exhibited blood phenylalanine levels between 4 and 12 mg. per 100 ml. All patients who were shown on initial examination to have blood phenylalanine levels in excess of 12 mg. per 100 ml. had repeat processing of blood specimens performed which confirmed the continuing presence of similar or higher levels. It was not possible to obtain specimens of urine for the demonstration of the presence of ortho-hydroxy-phenylacetic acid. All specimens in which this level of blood phenylalanine of 12 mg. per 100 ml. or higher was demonstrated, were subjected to the confirmatory Guthrie procedure for the demonstration of phenylalanine as detailed in the chapter dealing with the Guthrie Technique. It is thus seen that the previously-estimated phenylketonuric population of the institution was 0.5 per cent of the whole and the true phenylketonuric population actually to be 0.6 per cent of the whole. Particulars of the previously-known /

phenylketonurias in the institution are as follows.

Case 1. T/B.; male; aged 32 at the time of survey; confirmatory blood phenylalanine level of 20 mg. per 100 ml.; "typical" appearance; severely retarded; chronic pulmonary tuberculosis; no sibs; both maternal grandparents born in Midlothian, Scotland; paternal grandfather born in Ayrshire, Scotland, and paternal grandmother born in the County of Inverness, Scotland.

Case 2. N/J.; male; aged 16 at time of survey; confirmatory blood phenylalanine level 20 mg. per 100 ml.; "typical" appearance; low grade hyperactive ament; receiving chlorpromazine and sodium amylobaritone; no sibs; maternal grandfather born in County Mayo, Ireland and maternal grandmother born in Uddingston, Scotland; paternal grandparents born in South Uist, Scotland.

Case 3. J/R.; male; aged 44 at time of survey; confirmatory blood phenylalanine level 50 mg. per 100 ml.; "typical" appearance; low grade ament; no information regarding birthplaces of grandparents.

Case 4. M/M.; female; aged 58 at time of survey; confirmatory blood phenylalanine 20 mg. per 100 ml.; low grade ament; fair hair and blue eyes but no hypertonicity; no sibs; maternal grandparents both born in Glenurquhart, Inverness, Scotland; both paternal grandparents born in Rogart, Sutherland, Scotland. /

Case 5. T/S.; female; aged 43 at time of survey; confirmatory blood phenylalanine level 40 mg. per 100 ml.; low grade ament; dark brown hair, blue-grey eyes and hypertonic limbs; paternal grandparents born in Shetland Isles, Scotland; maternal grandmother born in London of French extraction and maternal grandfather born in Wales.

Case 6. D/W.; male; aged 55 at time of survey; confirmatory blood phenylalanine level 50 mg. per 100 ml.; moderately retarded; fair hair, blue eyes but no hypertonicity of limbs; no sibs; all grandparents born in Greenock, Scotland.

No history of antecedent intermarriage could be obtained in respect of the families of any of the patients investigated.

The particulars of the discovered case are as follows.

M/McR.; female; aged 10 at time of survey; confirmatory blood phenylalanine level 20 mg. per 100 ml.; urine negative to PHENISTIX testing before and after blood testing; low grade ament; dark hair, brown eyes and hypertonic limbs; no sibs; all grandparents born in Stornoway, Lewis, Scotland; mother said to have been mentally retarded; no available history of antecedent family intermarriage.

Of the 17 PHENISTIX-positive urine tests, seven were found to be from patients receiving P.A.S. for treatment of pulmonary tuberculosis (but not Case 1), one was receiving soluble aspirin, one had chronic pancreatitis and was also receiving chlorpromazine, one was a diabetic and there is some doubt as to whether or not the eleventh had, in fact, given a positive PHENISTIX reaction. /

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All the six previously-known phenylketonurics exhibited a positive urine reaction with "PHENISTIX" although in two such cases, in consequence of their receiving chlorpromazine with sodium amylbarbitone and prochlorperazine respectively, the colour of the test-strip was not that truly associated with the presence of urinary phenylpyruvic acid. The discovered case, both before and after Guthrie testing, never displayed a positive urinary PHENISTIX reaction.

Summarising the results in respect of all the phenylketonurics in this institution, four were male and three female. The ages at the time of the survey ranged from 10 to 58 with an average age of 37, and three displayed the "typical" appearance of the phenylketonuria. Confirmatory levels of blood phenylalanine varied between 20 and 50 mg. per 100 ml., with an average level of 31 mg. per 100 ml.; none were stated to be epileptic; six were severely retarded; there were no sibships; of twenty-four grandparents traced in respect of six patients, six had been born in the Hebrides, Scotland, five in remote districts of the Highlands of Scotland, five in the West of Scotland, two in the Shetland Isles, Scotland, one in Ireland and five elsewhere. Table 13 compares the results in the two institutions.

TABLE 13.

|                                 | <u>Lennox Castle.</u> | <u>Larbert</u> |
|---------------------------------|-----------------------|----------------|
| 1. Number of patients screened. | 1,134.                | 1,140.         |
| 2. Number of tests performed.   | 1,200.                | 1,147.         |
| 3. Presumptive positive cases.  | 66.(5.8%).            | 7.(0.6%).      |
| 4. Confirmed positive cases.    | 23.(2.0%).            | 7.(0.6%).      |

|   |             |               |
|---|-------------|---------------|
| 5. Previously known positive.                         | 10. (0.9%). | 6. (0.5%).    |
| 6. Discovered positive.                               | 13. (1.1%). | 1. (0.1%).    |
| 7. Average age of patients.                           | 27.3 years. | 37 years.     |
| 8. Ratio of males/females.                            | 12/11.      | 4/3.          |
| 9. Average blood phenylalanine (mg. per 100 ml.).     | 27.         | 31.           |
| 10. Epileptic.  | 5.          | 0.            |
| 11. Affected sibships.                                | 3.          | 0.            |
| 12. <u>Birthplaces of grandparents.</u> (4 patients). |             | (6 patients). |
| West of Scotland.                                     | 6.          | 5.            |
| Ireland.  | 6.          | 1.            |
| Remote districts of Scottish Highlands.               | 2.          | 5.            |
| Shetland Isles, Scotland.                             | 0.          | 2.            |
| Outer Hebrides, Scotland.                             | 0.          | 6.            |
| Elsewhere than above.                                 | 2.          | 5.            |

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Miscellaneous Sera Processed.

At the beginning of November, 1965, it was decided as routine practice, to screen all the sera received in the Bacteriology Department, Stobhill General Hospital, to determine if there were adult patients presenting with other diseases, and in whom there was no suspicion on the part of the clinician concerned regarding the presence of occult phenylketonuria. The results to be detailed are of an interim nature and are representative only of the processing of sera received in two weeks. Such results are nevertheless so important that they justify inclusion in this thesis. Sera are forwarded for performance of the Wassermann Reaction and other test procedures designed to detect the presence of antibody to *Treponema pallidum*; for the performance of the VIDAL reaction to demonstrate the existence of typhoid, para-typhoid and abortus fevers; for the performance of the Paul-Bunnell test in suspected infectious mononucleosis; for the estimation of the Rose Waaler test in suspected rheumatoid disease and for the detection of thyroid and gastric antibodies in suspected auto-immune diseases.

In the period of two weeks, 425 sera were processed by the Guthrie technique and two subsequently-confirmed positive results were obtained. The first was in a woman for whom the Rose Waaler test had been requested in consequence of pain and swelling in the knees. Blood examination on two occasions disclosed a phenylalanine level of 20 mg. per 100 ml. Subsequent examination of the urine showed the presence of a positive PHENISTIX test and also the presence of ortho-hydroxy-phenylacetic acid. Biochemical evaluation, performed by the LaDu method, showed a serum level of 24.6 mg. phenylalanine per 100 ml. She was married and had two children who were stated to be fit and to show no signs of mental retardation. /

Investigation of the children and the husband has been arranged.

She appears to be of low normal intelligence and is quite capable of managing her house and her family. She has suffered from asthma from an early age and had "eczema" of the face and scalp till late adolescence. She is now 24 years old.

The other case is that of a 42 year old woman presenting with a mammary carcinoma, for whom thyroid-antibody testing was requested. She has been found, on two occasions, to exhibit blood phenylalanine levels of 30 mg. per 100 ml. Her urine gives a positive reaction with PHENISTIX test-strips and also contains ortho-hydroxy-phenyl-acetic acid. No further particulars are at present available. In both cases, the corroborative methods for the detection of blood phenylalanine detailed in the chapter dealing with the Guthrie technique have also been utilised.

It is intended to continue routine processing of all sera received.



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## DISCUSSION.

Discussion of this thesis will be presented under several headings:-

1. Preliminary evaluation of the Guthrie technique.
2. Requirements of a survey-method.
3. Detailed examination of the individual projects.
4. Final conclusions.

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## Preliminary Evaluation of the Guthrie Technique.

From the outset, it was appreciated that limits of accuracy to be obtained with utilisation of this technique could not be compared with those possible in the performance of biochemical estimation of serum phenylalanine, such as the LaDu enzymatic or the McCaman and Robins fluorimetric methods. In such biochemical estimations, it is possible to obtain accurate results to the second decimal place. The primary purpose of the Guthrie technique being to discover neonatal cases of phenylketonuria, it is not necessary to operate within such a fine tolerance. As a corollary to this, the results to be obtained by the method must be such that quick scanning of the prepared test-plate can readily detect discs of neonatal blood which show a level of 4 mg. phenylalanine per 100 ml. or above. In every test-plate which was prepared, control discs were affixed in the strengths and positions as advocated by Guthrie (1964). This involved the placing of a 4 mg. per 100 ml. control disc in each quadrant of the plate and the placing of a row of control discs, ranging from 2 to 20 mg. per 100 ml., longitudinally along the meridian of the plate. Only where a repeat evaluation of an initially high blood phenylalanine level in excess of 20 mg. per 100 ml. was being performed was there inclusion of control discs in the range of 20 to 100 mg. per 100 ml. Each plate was read by one person and checked by one other. The function of the second person was primarily to confirm the assessment, on the part of the initial reader, of blood phenylalanine levels of 4 mg. per 100 ml., or higher. The second person also quickly scanned the whole plate in order to ensure that no discs which showed the presence of a significant peripheral growth of Bacillus subtilis had escaped detection by the initial reader. /

Realising that the technique was primarily to be utilised in neonatal screening for the presence of the disease and that, in consequence, it would not often be necessary to incorporate the "high" control discs, i.e., those from 30 to 100 mg. per 100 ml., preliminary investigation of the diameter of the zones of peripheral growth of the Bacillus subtilis was made. 100 plates were serially prepared over a period of ten working days, each studded with the control discs in the ranges of 2 to 20 mg. per 100 ml., such discs occupying the positions which they would in the event of the plates also being studded with discs containing neonates' blood. Each plate was read after 16 hours incubation at 37 degrees Centigrade. Measurement of the growth zones was made diametrically through the discs and was effected by use of a transparent millimetre rule. Such measurement was made to the nearest millimetre and was made by two persons - those who would be involved in the reading of the project plates. In the observer check, it was found that there was a discrepancy in evaluation of blood phenylalanine levels of 2 per cent at the 2 mg. per 100 ml. level and 0.5 per cent at the 4 mg. per 100 ml. level. It was presumed that this discrepancy arose from the faintness of the peripheral growth at low levels. It was agreed before the neonatal survey was commenced that there would be, in consequence, at least one neonate disc in every two hundred which would be placed in the category of "4 to 6 mg. per 100 ml." when such was not the case. It was nevertheless felt that such an eventuality must be faced, but that it was preferable for an infant to be rechecked rather than allow an initially presumptive positive case to be placed in the category of "less than 4 mg. per 100 ml.". As a result of this previously-known error, it is estimated that approximately 32 out of the total of 39 neonates in the whole /

of the survey who fell into the "4 to 6 mg. per 100 ml." category were of this nature. Be that as it may, it will be subsequently shown that one cannot afford to ignore levels of 4 to 6 mg. per 100 ml. in respect of recheck.

There were no discrepancies in the evaluation of the higher levels of control discs between the two observers. In all plates prepared for the initial observer comparison, background growth of a very faint nature was noted. During the actual screening project this background growth was noted, on infrequent occasions, to be much more pronounced. On such occasions, reading of the test-plate was made difficult. It was discovered that this increase in density of background growth had resulted from temporary failure of the thermostat of the incubator in use, thus allowing increase in the ambient temperature to take place. Such increase in temperature over an unknown period of time was sufficient to nullify the inhibitory effect of the beta-2-thienylalanine incorporated in the modified Demain's medium. The introduction of a new incubator overcame this problem.

The mean diameters of the zones of growth for the discs of 2, 4, 6, 8, 10, 12 and 20 mg. phenylalanine per 100 ml., were 16, 18, 21, 23, 26, 28 and 32 millimetres respectively. In consequence of this differential, it was not expected that there would be any great difficulty in ensuring separation of the values of blood phenylalanine at the truly important levels, namely those less than 4 mg. per 100 ml., and those of 4 to 6 mg. per 100 ml., or higher. It was appreciated, however, that there would nevertheless still be the possibility already mentioned, of one disc in every two hundred being placed in the 4 to 6 mg. per 100 ml. category when its true level was less than 4 mg. per 100 ml. In the actual performance /

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of the neonatal survey, it was found that there was some degree of difficulty in correctly placing approximately one disc in every two hundred, either into the category of "less than 4 mg. per 100 ml." or "4 to 6 mg. per 100 ml.". As previously stated, such a disc was placed in the higher category. During the performance of the neonatal survey, it was noted that there was never less than 1 millimetre of difference in the diameters of the zones of growth around the control discs at the 2 and 4 mg. per 100 ml. levels.

An additional matter investigated was that of comparability of specimens of blood and serum. Guthrie testing was performed on ten discs containing whole blood and ten discs containing comparable sera. It was found that when such discs were processed in the usual manner there was no significant difference in the diameters of the zones of growth. When similar specimens were processed without preliminary autoclaving, in consequence of the diffusion of blood pigment from the blood-based discs it was almost impossible to obtain an evaluation in respect of them. There was an increase in the zone of growth in the case of the unautoclaved comparable sera. Whole blood, autoclaved serum and unautoclaved serum from one specimen from one person were processed and the results compared. The average diameter of ten growth zones from whole blood was 18 millimetres, equating with a blood phenylalanine level of 4 mg. per 100 ml. and was of similar diameter in the case of autoclaved serum. The average diameter of ten growth zones from unautoclaved serum was 21 millimetres, equating with a blood phenylalanine level of 6 mg. per 100 ml. This phenomenon was noted by Partington and Sinnot (1964) who could not explain it other than by the assumption of the possible presence of a growth-promoting factor in unautoclaved fresh serum, plasma and probably whole blood. In the /

limited investigation of the subject performed at this juncture, no further explanation can be adduced.

During the neonatal survey, although it has been stated in the chapter dealing with Results that 99.36 per cent of the neonates in Stobhill and Robroyston Hospitals showed initial blood levels of phenylalanine of less than 4 mg. per 100 ml., and of the Redlands Hospital cases 94.63 per cent also showed levels of less than 4 mg. per 100 ml., it should be noted that the majority of such neonates displayed levels approximating to the level of 2 mg. per 100 ml. In consequence of this, it was generally easy to see the discs which displayed levels in the region of 4 mg. per 100 ml., or higher.

Although it is more than likely that some of the neonates must have been receiving antibiotics prior to the blood sample being taken, there were never any discs from such neonates which did not show a halo of peripheral growth. In order to determine if previous administration of penicillin could influence the diameter of the zones of growth, blood was obtained for processing from adults known to be receiving large doses of penicillin parenterally. In ten such cases, "clear zones" of total bacterial inhibition around each disc were noted on completion of Guthrie processing. This inhibition was complete and was obviously similar to that described by Partington and Sinott (1964). It was concluded from this that in any neonate who had been receiving penicillin, blood levels had not been sufficient to cause the development of such "clear zones". In the event that previous administration of penicillin and other antibiotics may cause inhibition of the peripheral growth of the Bacillus subtilis even in the presence of a raised level of blood phenylalanine, it may be desirable in future to incorporate space on the top leaf of the Guthrie card for the inclusion of particulars/

with regard to the prior administration of such antibiotics.

It could be shown that, in order to obtain homogeneous background growth, absolute flatness of the bench on which the plates were being prepared was absolutely necessary. A slope of 2 millimetres in the long axis of the poured plate was sufficient to cause the development of an increased density in the background growth in the deeper part of the poured plate subsequent to incubation. This was accompanied by a corresponding diminution of density in the peripheral growth around the discs at the shallow end of the prepared plate.

Some difficulty was experienced at the commencement of the neonatal survey as a result of the forwarding of insufficient blood specimens. When it was realised that this might prove a problem, prepared specimen cards were forwarded to the persons responsible for the collection of the initial specimen and no further difficulty was experienced in this respect.

No other problems were encountered in pursuance of the laboratory aspects of the Guthrie test during the whole of the neonatal or other projects.

#### Critique of the Guthrie test.

Since the introduction of this test there have been, on occasion, severe criticisms directed against it. Such criticism stemmed primarily from the work of Scheel and Berry (1962) who investigated the efficacy of the Guthrie method after the initial announcement of the results obtained by Guthrie in the pilot test (1961) which had been conducted by him, but before the publication of the large scale test-results involving the screening of 400,000 neonates. Scheel and Berry's results were published after their investigation of less than 100 neonates. They concluded, that "the/

inhibition method, with the use of whole blood, is probably not sensitive enough for use as a test for detection of phenylketonuria in a nursery for newborn infants".

Apart from the fact that the screening involving the 400,000 neonates established the validity of the Guthrie test as a screening test for the detection of neonatal phenylketonuria, Partington and Sinnott (1964) corroborated its value. They found one "false positive" (sic) Guthrie test in 2,400 blood samples from newborn babies and none in a further 1,734 from other sources. MacCreedy (1963), after the Guthrie screening of 50,000 neonates, found that about 1 in 700 gave borderline results suggesting a blood phenylalanine level of between 6 and 12 mg. per 100 ml. Brandon and Ashley (1963), in a Guthrie screening of 11,556 samples of blood serum, reported an incidence of 1 in 1,650 of "false positives" (sic).

It is now generally accepted that the Guthrie test is a reliable procedure for screening for the presence of phenylketonuria in neonates.

In this connection, it should be mentioned that the introduction, by Guthrie, of this particular method for such neonatal phenylketonuria detection, aroused so much interest in the U.S.A. that, whereas in December, 1964, such testing was mandatory for neonates in four of the individual States of the U.S.A., in December, 1965, testing for the presence of phenylketonuria in neonates, either by the Guthrie or some other method, is now mandatory in twenty-five such States.

Where the term "false positive" has been used by various authors, it should be appreciated that such should properly be referred to as "presumptive positive" tests.

## Requirements of a Survey-Method.

In choosing a method of testing for phenylketonuria, several factors must be considered:-

1. the reliability of the test.
2. the ease of obtaining specimens.
3. the cost in man-hours, materials, etc.,
4. the best age at which to test.

### 1. The reliability of the test.

All tests used in population screening for phenylketonuria are primarily of a qualitative nature for the detection of single substances in urine or blood. Thus methods using ferric salts, such as PHENISTIX test-strips, test for the presence or absence of phenylpyruvic acid in urine, as does 2:4-dinitrophenylhydrazine. The Guthrie test and the spectrophotofluorimetric test to be mentioned subsequently, test for the presence of phenylalanine in blood and/or urine. Paper chromatography tests for the presence of ortho-hydroxy-phenylacetic acid, phenylalanine or phenylpyruvic acid in urine, phenylalanine or (rarely) phenylpyruvic acid in blood.

Although none of those tests gives the concentration of the substance detected in strictly numerical form, in a very real sense they are all quantitative as well as qualitative. A normal adult has about 1 to 2 mg. phenylalanine per 100 ml. blood and excretes in his urine about 2 mg. of phenylpyruvic acid and 2 mg. of ortho-hydroxy-phenylacetic acid daily (Woolf, 1965). A phenylketonuric adult has been shown in this thesis to have up to 70 mg. phenylalanine per 100 ml. blood and such a patient may excrete 2,000 mg. phenylpyruvic acid and 200 mg. ortho-hydroxy-phenylacetic acid daily (Woolf, 1965). Each of the tests has a threshold; PHENISTIX and /

other ferric salts give a visible green colour only if the concentration of phenylpyruvic acid in the urine is above 5 to 10 mg. per 100 ml. (personal observation); The Guthrie test gives a growth zone only if the concentration of phenylalanine on the affixed disc is above 1.0 mg. per 100 ml. (personal observation); paper chromatography for the presence of ortho-hydroxy-phenylacetic acid is of value only if the amount of the substrate is in excess of 0.2 microgrammes in the quantity being eluted (personal observation). Whatever test is used, one is really testing for an abnormally raised concentration of a normal constituent above the threshold for the test. The most important factor determining the reliability of a test for the detection of phenylketonuria is the proportion of phenylketonurics who, at the time of testing, have the appropriate concentration above the threshold for the test.

The existence has been well documented of adults and children who excrete, either constantly or intermittently, so little phenylpyruvic acid that it is below the level for PHENISTIX or ferric chloride testing, although far above the normal (Mabry, Nelson and Horner, 1962; Guthrie, 1964; Jervis and Grocott (quoted by Guthrie), 1964). The term "occult phenylketonuria" was invented for such cases which are only capable of detection by paper chromatography of the urine or Guthrie testing of blood. The proportion of such cases among adults and older children is unknown but one would not expect it to be negligible. It would appear - as stated in the chapter dealing with Biochemical Aspects - that it generally takes 4 to 6 weeks for the urine of a homozygote phenylketonuric to become positive for PHENISTIX or ferric chloride testing. Thus the majority of such homozygote cases pass through a stage of occult phenylketonuria and it is in this stage that detection is desirable./

The efficacy of methods for detection of the disease which involve interpretation of colour change, for example PHENISTIX and ferric chloride, depends on true biological variation and observer error. Such true biological variation and observer error account for the degree of unreliability associated with such tests. Biological variation may occur from individual to individual and from time to time in the same individual. To what extent relative blame can be apportioned between the two factors cannot be stated. Having personal experience of great individual variation in blood phenylalanine levels from time to time in the same person, one cannot be dogmatic in apportioning blame in conventional urine testing more to observer error than to true biological variation, if such individual biological blood variation is paralleled by individual urine variation in the excretion of phenylalanine metabolites.

## 2. Ease of obtaining specimens.

It appears impossible to obtain a liquid specimen of urine from more than about 25 per cent of neonates. Testing with PHENISTIX or ferric chloride on a wet napkin should be possible in almost every case but it may prove difficult to obtain a freshly wetted napkin in cases where there has been difficulty in the correct interpretation of the initial test. As stated in the chapter dealing with Methods of Detection and Diagnosis, false-negative tests may occur unless the urine specimen being examined has been freshly passed. The collection of a specimen of urine by placing filter paper in the napkin should prove easier than trying to obtain a freshly wetted napkin. Such filter paper can subsequently be dried and forwarded to the centre where detection of ortho-hydroxy-phenylacetic acid by paper chromatography is undertaken.

The Guthrie test requires blood obtained - in the case of /

neonates - from a heel stab. In older children and adults this stab can be performed on the ear or finger. In the case of neonates, collection can be performed by resident nursing staff, ward doctors or medical laboratory technicians, on infants who have been born in hospital. In the event of such screening being extended to cover domiciliary confinements, collection could readily be made on or about the sixth or seventh day of life by the midwife attending the confinement. Although it has been shown that, in respect of neonates born in Stobhill or Robroyston Hospitals, the average lapse of time between birth and the collection of all initial specimens is 6.2 days (counting the day of birth as day one) and for neonates born in Redlands Hospital the comparable figure to be 4.2 days, for ease of administration and without significant loss of value in such a screening programme, there is no reason why initial specimen collection should not be made by the domiciliary midwife on the seventh day for infants born at home, and the the health visitor at a similar age in the case of infants born in hospital and discharged home prior to the seventh day of life. Such a programme would necessitate close co-operation with medical officers of health and an understanding, on the part of the midwife or health visitor, of the important nature of what superficially appears a trivial procedure. In the event of repeat specimens proving necessary, such prior arrangement with the responsible medical officer of health would ensure collection of the specimen being obtained by the health visitor after the midwife had relinquished her statutory obligations. Health visitors would also be able to collect repeat specimens, where necessary, from infants who had been born in hospital. Personal experience in the preceding twelve months has shown that where co-operation with the general practitioner is /

sought, such co-operation is either not forthcoming or is very tardy.

The risk of infection of the heel stab is minimal if the necessary preliminary sterilising is properly performed but is nevertheless not to be discounted entirely. Where such infection develops, it is not to be expected that it will be more than the slightest possible.

### 3. Cost of screening.

Screening test for neonates can be divided into field tests, carried out entirely by the health visitor, and laboratory tests, where specimens of urine or blood are collected by the health visitor or some other person and are forwarded for processing to the laboratory.

The field tests in general use employ ferric salts, either as PHENISTIX test-strips used on a wet napkin, or as ferric chloride solution dropped on a wet napkin, dropped directly into a test-tube containing a specimen of the infant's urine or dropped on filter paper which has been impregnated with urine and subsequently dried. The PHENISTIX test and, to a lesser extent ferric chloride solution used on a wetted napkin, may require repeated visits by the health visitor before they can be carried out successfully with freshly-passed urine. This does not apply to the case of filter paper soaked in urine and then dried, but phenylpyruvic acid is sometimes unstable under those conditions, giving rise to the false-negative tests previously mentioned. The cost of ferric chloride in powder form suitable for the preparation of 5 or 10 per cent solutions is 9/6<sup>d.</sup> per kg. The cost of PHENISTIX test-strips, as supplied by the Department of Health, Scotland, on a contract-based price to hospitals in Scotland, is 4/10<sup>d.</sup> per 50 strips. PHENISTIX test-strips have ousted ferric chloride testing almost completely /

throughout Scotland.

Laboratory tests employ technicians who are in even shorter supply than health visitors, but one junior technician can readily set-up 100,000 tests per annum, both Guthrie and paper chromatography. If such laboratory methods of testing effect a significant saving of health visitors' time, this can be economically worthwhile quite apart from any improved reliability of the test used. The methods of collecting specimens for both Guthrie testing and paper chromatography are less wasteful of the time of health visitors than, for example, PHENISTIX testing and in both tests the laboratory procedures can be streamlined to permit a high rate of working. Both Guthrie testing and paper chromatography can detect other inborn errors of metabolism in addition to phenylketonuria. In the case of the Guthrie test, it is at present possible also to screen for galactosaemia, maple syrup urine disease and histidinaemia. Current research-investigation of the potential capabilities possessed by the basic Guthrie test is now being directed towards the perfection of a "multiple test" for a number of rare inherited conditions (Guthrie, 1965). Such conditions, associated with the presence of an elevated level of a blood constituent, are tyrosinaemia, hyperprolinaemia, hydroxyprolinaemia, citrullinuria, hyperlysinaemia, hyperglycinaemia, methioninaemia, homocystinuria, oasthouse urine disease, sarcosinaemia, valinaemia and congenital lysin intolerance. For the detection of such diseases a mixed inoculum, prepared from several aminoacid-requiring mutant auxotrophs, is used as a multiple screening test requiring but a single spot of blood from each neonate. Such a test is designed to show a positive response to a single blood disc from a neonate with any one of these presumably-rare conditions. Such a procedure will make a practical/

proposition of screening of all neonates for the presence of these inborn errors of metabolism, although each condition by itself is probably too rare to warrant separate procedures. As an extension of such a project, it is not impossible that use of mutant micro-organisms, each with a genetic block early on the biosynthetic chain, will expand the test response to include other possible human errors of an inborn nature not yet recognised.

In the case of paper chromatography, it is now also possible to test for the presence of galactosaemia, tyrosinosis, tyrosyluria, gargoylism, proteinuria, glucosuria, cystinuria and homocystinuria (Woolf, 1965).

A recently-described method of mass screening for phenylketonuria, using automatic spectrophotofluorimetry to estimate blood phenylalanine concentration (Hill, Sumner, Pender and Roszel, 1964), awaits evaluation in extended clinical trials. The initial cost of such an automated analyser will be prohibitive for the majority of laboratories.

Costing of the Guthrie method has been investigated in detail. The preparation of 100,000 test cards, suitably printed, is £40.0.0, inclusive. The cost of the materials necessary for the preparation of the media required for screening of 100,000 neonates annually is £33.0.0. and the yearly salary of a junior technician responsible for the preparation of the media and the setting-up of the tests - always under senior supervision - is (at present rates) £560.0.0, on average. Excluding the cost of the time of the health visitor, hospital doctor, etc., in the collection of the blood specimen, the cost of screening 100,000 neonates by the Guthrie method amounts to £1,030.0.0, or thereabouts. The individual laboratory cost of Guthrie screening of a single neonate is thus 2.47 pence. If the /

cost of necessary secretarial assistance is incorporated, employing a clerkess/typist, a further £500.0.0. or thereabouts must be added. The inclusive cost of Guthrie investigation of a single neonate is thus shown to be 3.67 pence. This compares favourably with the individual cost of a PHENISTIX test-strip, namely 1.16 pence, since it would appear not to be uncommon for the health visitor to use two or three test-strips in each of the two investigations generally performed by her on a neonate at the ages of 2 and 6 weeks. The cost of the PHENISTIX test-strips used by the health visitor on one neonate may thus be 4.64 to 6.96 pence or even higher.

#### 4. Age at which to test.

At birth, phenylketonurics are clinically and biochemically normal (see chapters on Methods of Diagnosis, and Biochemical Aspects), but the lack of phenylalanine hydroxylase causes a gradual accumulation of phenylalanine in the blood and tissues. The rate of accumulation of phenylalanine must depend on the dietary intake, rate of growth and renal loss of phenylalanine, all three very variable from infant to infant. The rates of formation of phenylalanine metabolites, among them phenylpyruvic acid and ortho-hydroxyphenylacetic acid, rise steeply along with the rise in phenylalanine blood levels but here again there must be considerable individual variability.

It can be seen, from reference to the chapter on Results, that there is a significant difference in the numbers of presumptive positive cases of phenylketonuria when neonates from Redlands Hospital are compared with those born in Stobhill and Robroyston Hospitals. It would appear that such a difference in numbers of presumptive positive cases in these hospitals (55 and 35 respectively) must be a direct result of the earlier date of discharge of the /

neonate from Redlands Hospital. This will be considered subsequently in detail but, at this stage, it must be pointed out that such a conclusion differs somewhat from that reached by Hsia, Berman and Slatis (1964), who state that, inter alia, values of serum phenylalanine are not appreciably affected by age, in the case of the case of neonate evaluated in the first four weeks of life. In view of personal findings in the period under review, it is felt that the preferred time for the collection of the initial specimen should be - as already stated - on the sixth or seventh day after birth (counting the day of birth as day one). If such an age for the collection of specimens is adopted, there would appear every possibility - as shown by the demonstration of normal blood phenylalanine levels where repeat specimens have been processed - that the numbers of presumptive positive cases will be greater than if the collection of the initial specimen were to be deferred to a later date. Such a state of affairs is nevertheless preferable to that which would occur in the event of the initial specimen being collected on or about the tenth to the fourteenth day, namely, the possibility of true cases of phenylketonuria escaping detection at the earliest possible date.

In the case of PHENISTIX or ferric chloride testing of urine, phenylpyruvic acid should be sought for on two occasions; at about the age of two weeks and then again at the age of six or eight weeks. Urine specimens for the detection of ortho-hydroxy-phenylacetic acid are best obtained at the age of two weeks (Woolf, 1965). It should be noted that, in all the above cases, the times of collection are as much related to the abilities of the different tests to detect a small rise in the concentration of the relevant substance as to the rates at which the different substances accumulate or are excreted./

A practical point in this connection is that the duties of the health visitor commence statutorily at the end of the lying-in period. In the majority of births, she will have seen the infant by the time it has reached the age of two weeks and, where follow-up specimens have been requested either for infants born in hospital and discharged home or else where the initial specimen may have been taken by the domiciliary midwife in the case of home confinements, it should be quite feasible for her to collect the repeat specimen without disruption of her daily duties.

One possible additional prerequisite for successful prosecution of mass neonatal screening might be the ready availability of reliable methods for the confirmation of initially presumptive positive results. In the case of the Guthrie method, such confirmatory methods are those detailed in the chapter dealing with the Guthrie Technique. Furthermore, any adequately-equipped biochemical laboratory will possess the necessary equipment for the determination of serum phenylalanine levels by the LaDu method already outlined in the chapter dealing with Methods of Diagnosis. In this connection, recent work has shown that this method of evaluation is not truly accurate, in the majority of estimations, unless the serum has received previous ultra-centrifugation (Wolf, 1965).

## Detailed Examination of the Individual Results.

### 1. Neonatal Survey.

In the case of the neonates screened in Stobhill and Robroyston Hospitals, the item of outstanding importance is that pertaining to the number of infants in respect of whom no processing of repeat specimens was necessary, 5,390 or 99.36 per cent of the total. Put in another way, in only 35 (0.64 per cent) was it necessary to repeat processing of blood in order to determine whether or not phenylketonuria might be present. A percentage magnitude of this order implies that, under similar conditions of sampling, in only 64 out of 10,000 neonates will further investigation be required in order to confirm or disprove the presumption that phenylketonuria exists. Although no truly positive cases of the disease were discovered in the purely neonate hospital-born infants, it cannot be said that there were no such cases born and fully investigated. Of the 35 infants from whom repeat specimens were requested, general practitioner co-operation provided only 11 repeat specimens of blood. The clinical picture of the other 17 infants, in respect of whom no repeat specimens were obtained, remains unknown.

In the case of the 18 neonates from whom repeat specimens were requested and received, 15 showed blood phenylalanine levels to be within normal limits at the second processing. Thus 5,405 (99.63 per cent) of all the neonates screened were shown to have blood phenylalanine levels within such normal limits and not to require still-further investigation.

With regard to the three infants in whom an increased level of tyrosine was demonstrated, prematurity was only apparent in one. It is also of interest to note that although the initial blood phenylalanine level was appreciably elevated above that accepted as the /

upper limit of normality (4 mg. per 100 ml.) in the case of two, in the third the initial level was only in the range of 4 to 6 mg. per 100 ml.

With regard to the Redlands Hospital neonatal screening results, it is immediately apparent that the number of presumptive positive cases is appreciably higher. In this hospital, it was necessary to request repeat specimens of blood from 53 infants (5.37 per cent of the total). In the case of the 24 repeat specimen obtained, 21 showed blood phenylalanine levels to be within normal limits, i.e. less than 4 mg. per 100 ml. Thus, of 987 neonates screened in this hospital, 955 (97.77 per cent) were shown to have blood phenylalanine levels within normal limits and not to require still-further investigation. The number of presumptive positive results would, in this hospital, appear to have resulted from the earlier discharge of the neonates and, in consequence, the earlier time of obtaining the initial blood specimen for processing. It must nevertheless be pointed out that no attempt was made to obtain specific information with regard to race, maternal age, parity, etc., in any of the hospitals where the neonatal surveys were conducted.

Neither of the Redlands Hospital infants who showed an increased level of blood tyrosine was thought to be premature. Although the initial blood phenylalanine level was appreciably raised in the case of one, in the other it was only slightly elevated. It may be significant that the final blood phenylalanine estimation performed in both those cases showed this level to be at the upper limit of normality (4mg. per 100 ml.) at the age of four weeks. Although the possibility exists that both such cases might be heterozygous for the disease, in consequence of their having been adopted it has not been possible to arrange further investigation. /

Numbers of specimens received from other hospitals for processing of neonatal specimens were too few to permit of any detailed significant conclusions. Nevertheless, the influence of prematurity on the development of the phenylalanine hydroxylase system is shown in respect of the three specimen received from premature infants from Bellshill Maternity Hospital and it would appear likely that the positive PHENISTIX test reported by the general practitioner in the case of the fourth infant had resulted from the presence of urinary para-hydroxy-phenylpyruvic acid. This substance was stated by Effron and MacCready (1965) to be present in the urine in approximately 1 in 160 full-term infants, to result from immaturity of the tyrosine degradative pathway and to give a positive urinary PHENISTIX reaction. This supposition is confirmed by the presence of a blood tyrosine level in this particular infant of 42 mg. per 100 ml. at the age of almost four weeks. The specimen in respect of the neonate sib of an already-known phenylketonuric received from Seafield Hospital, Ayr, was shown not to be that of a homozygote phenylketonuric.

The truly-confirmed neonatal case discovered and subsequently treated at Stobhill Hospital possesses several interesting features. In the first place it must be noted that few reports of true cases have been published in respect of negro or mulatto individuals. Guthrie (1964) specifically mentioned only one negro case of true phenylketonuria having been discovered in the Guthrie-test screening of 400,000 infants in which twenty-nine of the participating States in the U.S.A. submitted data. A personal communication by Guthrie (October, 1965) stated that, insofar as he was aware, no further negro cases had been discovered. Stadler, Meyer and Leland (1956) reported the case of a mulatto with a positive urinary test for /

phenylpyruvic acid and with mental deficiency. This patient, at the age of 26, strikingly demonstrated the blonde hair and blue eyes associated with the "typical" appearance of phenylketonuria. In their paper, Stadler, Meyer and Leland could report only one previous reference to the disease involving a member of the coloured races. This was with reference to "Phenylpyruvic Oligophrenia in Melanoderma" by Ferriera-Fernandes (1950).

The Stobhill Hospital patient has, at the age of 10 months (November, 1965) brown eyes and black hair. Evaluation of the mother's blood by Guthrie testing showed the existence of a phenylalanine level of 2 mg. per 100 ml., thus showing that she is not a homozygote phenylketonuric. It has not been possible to obtain any particulars relating to the father. The mother appears to be of Caucasian ethnic grouping and thus it is assumed that the father is Negroid.

The deterioration in the condition of this child in consequence of the administration of a diet too low in phenylalanine is paralleled remarkably by the case reported by Lewis (1960). Furthermore, the dramatic improvement in her clinical condition, when feeding with a full-cream dried cow's milk preparation was commenced, was exactly that noted by Lewis. The degree of damage to the brain which may have resulted from the initially very high phenylalanine blood level and the level which was reached in the unfortunate prolongation of the unrestricted dietary remains to be assessed. At the age of ten months (November, 1965) she would appear to be developing within normal limits for her age.

The vindication of the Guthrie method of blood phenylalanine estimation as compared with the conventional biochemical L<sub>α</sub>Du enzymatic method is, in the blood control of this particular patient/

quite evident. There is every reason to suppose that, had the Guthrie method of blood phenylalanine control been adopted as standard practice in the governing of phenylalanine intake, the unfortunate deterioration in her clinical condition would not have taken place. There is no practical difficulty, by utilising this method of assessment, in placing the values of blood phenylalanine anywhere between 2 and 6 mg. per 100 ml., with an accuracy of 1 mg. per 100 ml. From the disparity in estimations given by the Guthrie and the LaDu methods in the blood phenylalanine evaluation of this particular infant, it is to be expected that future dietary treatment of phenylketonurics admitted to Stobhill Hospital will be governed by the Guthrie method.

One final practical point emerging from the investigation of this particular infant was the discovery of minimal knowledge, on the part of the hospital dieticians, of dietary management of phenylketonuric infants and children. This has now been rectified.

#### Further conclusions and postulates regarding the neonatal results.

From the Stobhill and Robroyston Hospital neonatal screenings, it is to be expected that, in nationwide screening, 64 per 10,000 may exhibit a presumptive positive Guthrie test, in the event of the blood specimen being obtained on or about the sixth day of life. Of this figure, 50 may result from the observer-error of 0.5 per cent. Investigations now being carried out directed toward modification of the method detailed in this thesis suggest that, in future, it may be possible to eliminate this error completely. In this event, the number of presumptive positive test results would then approximate to a figure of 14 per 10,000. If the incidence of homozygous phenylketonuria be accepted as being in the region of 1 per 10,000 of all births in Scotland, this would necessitate still-further /

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investigation of fourteen neonates in order to discover one confirmed homozygote phenylketonuric.

In view of the poor response from the general practitioners from whom co-operation was requested, it is desirable that - in the case of hospital-born infants - repeat blood specimens be obtained by the health visitor. In the case of infants born at home, it is thought that the initial specimen should be taken by the domiciliary midwife and again the health visitor could obtain the repeat blood specimen where necessary.

It is felt that all cases which initially show a blood phenylalanine level of 4 mg. per 100 ml., or above, should have routine blood chromatography performed for the detection of tyrosine. This would be possible by utilisation of one of the unused blood spots on the Guthrie test-card. It will be of interest to determine what the incidence of elevated levels of blood tyrosine will prove to be among neonates and what, if any, may be the subsequent manifestations. By use of urine chromatography, Woolf (personal communication, 1965) has discovered that there is an incidence of 2 per cent tyrosyluria in neonates born in the area around Oxford, England. Hudson (1963) noted the possibility of abnormal metabolites of tyrosine being produced where blood tyrosine levels were maintained at a high level over a prolonged period and suggested that a form of brain damage, similar to that produced in phenylketonuria when untreated, may result.

The difference in the numbers of presumptive positive cases of phenylketonuria in neonates of Redlands Hospital when comparison is made with those in Stobhill and Robroyston Hospitals, would appear to be accounted for purely by reason of the blood specimen being obtained 1.5 days earlier. This difference in the time of taking of the /

initial blood specimen has led to an increase in the number of presumptive positive Guthrie tests of approximately 700 per cent, when the numbers of neonates were placed on a pro rata basis. This difference presupposes the existence of a falling gradient in blood phenylalanine between the periods of 4.3 and 5.8 days after birth and is at variance with the conclusion reached by Hsia, Berman and Slatis (1964) who stated, "one can - - - assume that the serum phenylalanine level is the same in all neonates (during the first thirty days of life)". The personally-obtained result is of the utmost significance in relation to the stabilising of the phenylalanine hydroxylase enzyme system and has a direct bearing on the suggested optimum time of collection of the initial blood specimen for Guthrie evaluation.

In connection with the development and the mode of action of the enzyme systems responsible for the proper metabolism of phenylalanine and tyrosine, much has been written (Woolf and Edmunds, 1950; Knox and LeMay-Knox, 1951; Kretchmer et al., 1956; Bloxam et al., 1960; Zannoni and LaDu, 1960). It is suggested that the cases in which excess of blood tyrosine was demonstrated resulted from the operation of the following mechanisms. Stobhill Case 1 (table 10). This was the only known premature infant. It is likely, in this case, that there was immaturity of the enzyme system converting parahydroxy-phenylpyruvic acid into homogentisic acid and such immaturity led to dietary phenylalanine and tyrosine acting as loading doses with resultant hydroxyphenyluria. Concomitant factors - postulated from the knowledge that there had been no supplementary administration of ascorbic acid in this case and that there had been a marked degree of neonatal jaundice - may have been the presence of liver damage and the lack of ascorbic acid (Knox and Le-May-Knox, 1951). /

In Stobhill Case 2 (table 10) there was said to be no prematurity.

In view of the dramatic fall in the blood tyrosine subsequent to the giving of oral ascorbic acid, this is doubtful. It would appear

that this was a case of induced tyrosinosis and hydroxyphenyluria of prematurity as instanced by Woolf and Edmunds (1950). Stobhill

Case 3 and Redlands Cases 1 and 2 (tables 10/11 ) were probably not

so simple of explanation. Stobhill case 3 was definitely a full-term infant of pure Pakistani blood. In this case it is probable

that the initial blood phenylalanine level was the result of the immaturity of the phenylalanine hydroxylase system and this

supposition is substantiated by its rise to still higher levels and its subsequent fall to normal. In this event, the rising level of

blood phenylalanine could act as a loading dose and, with concomitant malfunction of the phenylpyruvic acid/homogentisic acid enzyme

system, cause the development of induced tyrosinosis and hydroxyphenyluria. With the proper maturation of the phenylalanine

hydroxylase enzyme system, normal metabolism of phenylalanine would take place, the phenylalanine "load" would be removed and the blood

tyrosine would gradually return to normal. The two Redlands cases are probably of yet a different nature. In consequence of the final

blood phenylalanine level performed at the age of four weeks after birth showing a level of 4 mg. per 100 ml., there is a possibility

of both those cases being heterozygotic for phenylketonuria. Again it is regretted that no further contact with those infants has been

made. In such an event, it is likely that the mechanism postulated by Dancis and Bailis (1955) becomes operative and that the excess of

blood phenylalanine leads to inhibition of tyrosinase on tyrosine, with the amount of inhibition being directly related to the

competition of phenylalanine and tyrosine for the tyrosinase in consequence of their structural similarity.

School Health Service Survey.

From a Special School and Occupational Centre enrolled population of 3,062 pupils, 2,125 (69.07 per cent) were screened. Including previously-known phenylketonurics, the number of confirmed positive cases was discovered to be six, with every possibility that the last case to be discovered and at present only listed as presumptively positive, will also prove to be a confirmed positive case. The presumption is thus shown to exist that there are seven homozygote phenylketonurics in 69.07 per cent of the enrolled Special School and Occupational Centre populations. In this event, it is likely that the incidence of homozygous phenylketonuria in the whole of this school population is in the region of 1 per cent.

All of the children attending Special Schools and Occupational Centres have had urinary PHENISTIX testing performed at some stage in their school career. All of the cases discovered by the Guthrie testing proved subsequently to have positive urinary PHENISTIX tests.

It was perhaps unexpected that, of the total number of proven cases, four attended Special Schools and not Occupational Centres. The last, and as yet unconfirmed case, also attends a Special School. Thus five of the seven are deemed to be mentally handicapped but educable. Of the two who attend Occupational Centres and are thus mentally handicapped, ineducable but trainable, one had previously attended Special School. In this particular case it is not known whether there had been a deterioration in intellect between initial classifications or whether initial classification had not been quite accurate. It would appear more probable that the latter explanation is correct. Only one of the cases is receiving dietary therapy.

Since it has not been possible to ascertain what is the number

of mentally handicapped children who are examined by the medical officers of the School Health Service qualified in mental defective ascertainment in the City of Glasgow, it is impossible to state how many children are examined and found to be mentally handicapped, ineducable and untrainable. If, as stated by Paine (1957), Knox (1960) and Partington (1962), the average percentage of untreated phenylketonurics who have an I.Q. of 41 or above is ten, the corollary to this must be that 90 per cent of untreated phenylketonurics possess an I.Q. of 40 or below and thus be mentally handicapped, ineducable and untrainable. In this event, it is possible to assume tentatively that the population of phenylketonuric children in the City of Glasgow, not recognised as educable or trainable, may be in the region of sixty.

#### Known Cases Receiving Treatment.

Of the patients attending Stobhill Hospital, only one is not retarded. In this case, in consequence of the knowledge of an already-affected sib, diagnosis of the disease was effected in the early weeks of life and treatment immediately instituted. As a result of the severe degree of mental handicap shown by the other cases, there is not serious insistence on the maintenance of the phenylalanine-restricted dietary by the paediatricians. There is no personal knowledge of the patients in respect of whom specimens have been forwarded from Seafield Hospital but, from the average blood phenylalanine levels noted, there would appear to be every possibility of such patients being severely retarded, with the possible exception of Case 3. With regard to the Royal Hospital for Sick Children, almost 71 per cent of those receiving treatment show satisfactory average levels of blood phenylalanine. Provided that the disease /

in such patients has been diagnosed at an early age and treatment has been immediately commenced, one would expect that there would not be evidence of profound retardation. Unfortunately it has not proven possible to obtain information with regard to intelligence levels in this group of treated cases. It is known that not all cases being treated at this hospital are resident within the City of Glasgow. Again it has not been possible to ascertain what the proportion of such Glasgow children may be. Notwithstanding the lack of information, when the presumed numbers of phenylketonuric children resident within the City of Glasgow (postulated in the discussion of the School Health Service Survey) are considered, it is at once obvious that the majority of Glasgow phenylketonuric children are either in an undiagnosed condition or else have been diagnosed but not considered worthy of treatment. Only further investigation of the child population of the City of Glasgow considered mentally handicapped, ineducable and untrainable, can ascertain which of those two postulates is correct. The importance of early diagnosis of the disease and immediate introduction of the phenylalanine-restricted dietary is brought out in the comparison of the blood phenylalanine levels in the members of the two sibships. It is known that the degree of retardation in the cases of the younger members is minimal when compared with that of the older members.

#### Lennox Castle Institution.

The discovery, by the Guthrie method, of thirteen previously-unsuspected cases of the disease immediately vindicates its use as a screening test for an institutionalised population. Comparing the "already-known" and the Guthrie-discovered" cases, the increase in the proven incidence of the disease by 130 per cent is immediately/

obvious. It is also immediately obvious that the average age of the "Guthrie discovered" group is 14.2 years more than the average age for the "already known" group. In initial consideration of the "already known" group, it would appear that there is a disease-loading of males in respect of females, but consideration of the finally-proven numbers shows this not to be so. Two further inferences may be made by examination of the "already known" versus "Guthrie discovered" cases, the first, that where there are already-known sibs affected with phenylketonuria there is a greater possibility of the disease being recognised in any one individual and, secondly, that the existence of epileptiform seizures, or the history of such having previously been present, may lead to the assumption that mental retardation has resulted from the presence of this disorder in a severe form. The matter of birthplaces of grandparents will subsequently be discussed. It should also be stressed that the presence of the so-called "typical" appearance must be discounted as a diagnostic aid. Of the ten "already-known" phenylketonurics, five possessed or had possessed this appearance but in the "Guthrie-discovered" and hitherto-unsuspected group, seven fell into this category yet no significance had been attached to this supposed diagnostic aid. It is tempting to postulate that, in the investigation of the heterozygote children of Case 9, the demonstration of the urinary excretion of ortho-hydroxy-phenylacetic acid in two of the four sibs, even in the presence of an undoubtedly normal level of blood phenylalanine denotes the existence of the twilight zone between true homozygosity and true heterozygosity. This may, in further investigation, prove to be the case but it is nevertheless more than likely that this excretion is simply the phenotypic manifestation of the presence of a defective allele. /

The finally-proved incidence of homozygous phenylketonuria in this institution was 2.20 per cent.

The Royal Scottish National Institution.

It is immediately apparent that there is a significant difference in the incidence of the disease in the two institutions. The institutionalised population in the Royal Scottish National Institution is only 0.53 per cent greater than the comparable population of Lennox Castle, yet there are sixteen fewer homozygote phenylketonurics, a difference in the incidence of the disease in favour of Lennox Castle of 14 per 1,000. In addition, only one further case was discovered in the Royal Scottish National Institution by the Guthrie method, increasing the hitherto-existing incidence of homozygous phenylketonurics by 17 per cent as against 130 per cent in the case of Lennox Castle. There can be no comparison made between the six "already-known" and the one "Guthrie-discovered" case. In respect of this latter case, however, it should be noted that there was no excretion of PHENISTIX-detectable urinary phenylpyruvic acid before or after discovery, so that such excretion of phenylpyruvic acid must be less than 5 mg. per 100 ml. urine. It should be noted that this patient had dark hair and brown eyes.

The 17 PHENISTIX-positive urine tests are of interest. If screening of such an institution were to be performed initially with the use of the PHENISTIX test-method and there was no antecedent knowledge of individual urine test-reactions, had the patients been receiving comparable medication there is every possibility that two homozygote phenylketonurics would have been missed as a result of the differing and lasting colour-change produced on the test-strips by the drugs administered. There is also the certainty that the Guthrie-discovered case would also not have been detected by such

The difference in the average age of the "already-known" cases compared with the average age of the "already-known" cases in Lennox Castle, 42.0 years compared with 20.3 years, is possibly a reflection in the longer period of institutionalisation on the part of the Royal Scottish National Institution patients.

The finally-proven incidence of phenylketonuria in this institution was 0.6 per cent of the institutionalised population.

Two further matters of considerably importance in relation to the disease as it has been shown to present in the two institutions must be considered. These are, the probability of differing gene-frequency and the incidence of heterozygosity, both related subjects.

#### Differing gene-frequency.

Carter and Woolf (1961), as already mentioned in the chapter dealing with Genetics, showed that there was a possibility - on a genealogical basis - of the gene for phenylketonuria being about four times as common in the populations of the West of Scotland and Ireland, when comparison was made with the population of South-East England.

Only within the last three years have patients from the West of Scotland been admitted to the Royal Scottish National Institution, Larbert. Till then, the areas of referral of such patients were primarily the North, East and South-East of Scotland, and, to a lesser extent, England. In consequence of this, it can be assumed that the proportion of patients in this institution with antecedents in the West of Scotland must be very small in comparison with the remainder. Conversely, patients admitted to Lennox Castle Institution have always been admitted from the West and the South-West of Scotland, with but few exceptions. In consequence of this administrative arrangement, it is thus to be expected that patients in this /

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institution with their antecedents in the West of Scotland must greatly outnumber the remainder. With the population of Lennox Castle Institution differing from that of the Royal Scottish National Institution, Larbert, by only 0.53 per cent and with the previously mentioned factors operating in respect of both institutions, it is to be expected that the proportion of Lennox Castle patients with antecedents in the West of Scotland compared with the remainder of the population in this institution will be <sup>in</sup>inverse proportion to their corresponding groups in the Royal Scottish National Institution.

In the case of Lennox Castle Institution, the incidence of homozygous phenylketonuria has been shown to be 3.29 times that experienced in the Royal Scottish National Institution, Larbert. This increased incidence must be, prima facie, a direct reflection of the increased incidence of the homozygous disease in the population which is drawn primarily from the West and South-West of Scotland when compared with the incidence of the homozygous disease in the population drawn primarily from the remainder of Scotland. From this increased incidence there is thus presumptive evidence that there is at least a threefold increase in the gene-frequency for phenylketonuria in the West and South-West of Scotland compared with the rest of Scotland.

Reference to table 13 will show that, in the case of twenty-four grandparents of six homozygote phenylketonurics from the Royal Scottish National Institution, Larbet, eight were born in the Shetland Isles and the Outer Hebrides, Scotland, five were born in remote districts of the Scottish Highlands, five were born in the West of Scotland, three were born in parts of Scotland other than those already mentioned, one was born in Ireland and two in places other than those already mentioned. In respect of such phenyl-

ketonuric patients in this institution, the proportion of grandparents born in the West of Scotland compared with those born elsewhere, is thus  $5/19$  and of Irish-born grandparents compared with those born elsewhere,  $1/23$ . The joint ratio for this group is thus  $6/48$ , i.e.,  $1/3$ .

In respect of the homozygote phenylketonuric patients in Lennox Castle Institution, it was only possible to obtain data pertaining to the birthplaces of grandparents for four. For such patients, six of the grandparents had been born in the West of Scotland, six in Ireland, two in remote districts of the Scottish Highlands and two in places other than those already mentioned. The joint West of Scotland and Ireland ratio for birthplaces of grandparents compared with those born elsewhere is thus, for this group,  $12/4$ , i.e.,  $3/1$ .

From the above inverse proportionality of the ratios, it is therefore evident that the initial premise was correct and that the true increase in the incidence of gene-frequency for phenylketonuria in respect of patients in the West of Scotland is at least three times that for the remainder of Scotland and possibly even greater. It would also appear that the responsibility for this increase in incidence is shared by the West of Scotland and the Irish antecedents, but with data only obtainable for approximately 17 per cent of the homozygote phenylketonuric population it is not possible to draw valid conclusions from this small sample which would be representative of the whole phenylketonuric population. It is hoped that it will eventually prove possible to determine the birthplaces of all the homozygote phenylketonurics in Lennox Castle Institution and thus fully clarify the matter. On a purely factual basis, however, it has been shown that in the case of twenty-four /

grandparents of six homozygote phenylketonurics from the Royal Scottish National Institution, Larbert, nineteen had been born in parts of the British Isles in which Viking influence had been paramount for many centuries or else in parts where the possibility of intermarriage has existed till comparatively recent times. It has not been possible to determine to what extent one such factor is operative more than another. It may be that in such areas of former Viking conquest, opportunities for intermarriage have been greater than in other parts of Scotland as a result of their degree of insularity. Should this be so, the apparent influence of Viking heritage would be discounted and the increased incidence of the disease be purely a function of more frequent consanguineous matings.

#### Incidence of heterozygosity.

In comparing the results of Guthrie testing in the two institutions, it is striking that 3.8 per cent (forty-three patients) of the screening tests performed in Lennox Castle Institution showed initial blood phenylalanine levels of between 4 and 8 mg. per 100 ml., whereas no patients in the Royal Scottish National Institution displayed this finding. All initially presumptive - and finally confirmed - positive cases of homozygous phenylketonuria in the Royal Scottish National Institution showed initial blood levels of phenylalanine to be 12 mg. per 100 ml. or higher. There were no obvious differences in the populations of the two institutions to account for the presence of this group displaying slight elevation of blood phenylalanine above normal levels in one, and its absence in the other. In the case of Lennox Castle group, it was not known how soon after the ingestion of food such specimens had been taken and repeat examination was undertaken with specimens having been obtained in a fasting state. /

On such repeat examination, none of the specimens from this group of 3.8 per cent showed fasting blood levels of phenylalanine to be in excess of 4 mg. per 100 ml. Within this group, 46.5 per cent (twenty patients) showed blood phenylalanine levels to be 2 mg. per 100 ml., or less. The remainder, 53.5 per cent (twenty-three patients), showed blood phenylalanine levels of between 2 and 4 mg. per 100 ml. Of this particular group, 52.1 per cent (twelve patients) had levels of blood phenylalanine between 3 and 4 mg. per 100 ml.

It would appear that an unknown proportion of the cases within this group of 3.8 per cent of the total institutionalised population are heterozygous for phenylketonuria and that the greater proportion of the group representing 1.06 per cent of the total institutionalised population - the last group of twelve patients with fasting blood phenylalanine levels between 3 and 4 mg. per 100 ml. - will be heterozygotes. In view of the difficulties of phenylalanine load-testing severely retarded individuals it has not been possible to pursue individual studies in those patients. It is not suggested that this whole group is retarded and institutionalised in consequence of its being afflicted with heterozygous phenylketonuria but that the proportion of presumed heterozygotes within this group would again be an indication of the increased gene-frequency of the disease in a population whose roots are primarily fixed in the West of Scotland and Ireland.

#### Miscellaneous sera.

With regard to the processing of such sera, it has been realised from the commencement of the routine screening that the numbers to have been processed by the time of completion of this thesis would be too few to allow of positive, valid conclusions

The discovery, nevertheless, of two confirmed positive homozygote phenylketonurics in a screening of 425 presumably negative sera, is so unexpected that, although providing the stimulus for continued routine processing of such sera, it also justifies such continuation for an indefinite period. When it is considered that there was no previous suspicion, on the part of the clinicians responsible for the forwarding of the sera, of there being any homozygote phenylketonurics among their patients, one may be forgiven for wondering what the finally-established incidence for homozygous and heterozygous phenylketonuria will prove to be in the Glasgow and West of Scotland area.

Although no formal intelligence testing was performed in the case of the patient interviewed, independent opinions from several sources agreed that her mentality was no worse than that of many patients in the wards of a general hospital.

At this juncture - and immediately before forwarding this thesis for binding - it was learned that the younger of her two children, aged four months, was attending a paediatric department of another Glasgow hospital in consequence of microcephaly. Steps were immediately taken to contact the paediatrician concerned with a view to obtaining Guthrie test cards impregnated with blood from both children. It is not possible, at this stage, to state whether or not the younger child is microcephalic as a result of concomitant homozygous phenylketonuria or whether it is heterozygous - as one would expect it to be - and the occurrence of the microcephaly is in the nature of a random happening. It is intended that the father will be investigated by phenylalanine load-testing to determine if he should be a heterozygote for the disease.

It would be inappropriate not to mention the courtesy of /

Dr. James Farquhar, Senior Lecturer in Paediatrics, Department of Child Health, University of Edinburgh, for allowing me the privilege of seeing a homozygote phenylketonuric child of one year and five months (November, 1965) who was found, at the age of nine months, to be suffering from the disease. Although treatment was instituted as soon as the diagnosis had been made, this child is now retarded to some extent. Guthrie testing of the child's blood had shown that the level of phenylalanine (October, 1965) was 4 to 6 mg. per 100 ml. At this date, blood from the mother (aged twenty-two) was forwarded to me for phenylalanine estimation and was discovered to be between 20 and 30 mg. per 100 ml. The mother is thus a homozygote phenylketonuric, unsuspected and untreated, apparently of low, normal intelligence. The father, aged twenty-five, shows a blood phenylalanine level of 2 mg. per 100 ml. and his intelligence is not high. Phenylalanine load testing of the father is being arranged, since there is prima facie evidence that he is heterozygote for the disease. In any event, this would appear to be the first occasion in which an untreated phenylketonuric mother is supervising the dietary treatment of her affected child.

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## FINAL CONCLUSIONS.

In the Preamble to this thesis, it was stated that an attempt would be made to provide answers to four questions, of an indirect nature, which were posed in respect of the Guthrie technique for the detection of phenylketonuria.

Firstly, is utilisation of the Guthrie technique feasible in the detection of neonatal phenylketonuria? It is submitted that the results obtained in respect of the neonatal screening programme provide an affirmative answer to this question.

Secondly, can utilisation of the Guthrie technique discover any hitherto-unsuspected cases of the disease? Again the submission is made that the results in respect of the mental defective institutions have provided a dogmatic and positive answer to this question.

Thirdly, does the Guthrie technique possess any significant advantages over conventional methods of detection of phenylketonuria by urinalysis? In terms of numbers of specimens which can be processed, lessened costs, greatly increased accuracy and ease of performance, these advantages have been shown to exist.

Lastly, is there any frequency-variation in the incidence of phenylketonuria in Scotland? It is submitted that the interpretation of the results in respect of the screening of patients in the mental institutions suggests this to be the case.

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