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Strategy for the Screening of Tetrahydrobiopterin Deficiency among Hyperphenylalaninaemic Patients: 15-Years Experience

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Summary: Tetrahydrobiopterin deficiency in hyperphenylalaninaemic babies has to be rapidly recognized since the disease requires a specific treatment. Based on 15 years experience, we report on the evolution of a strategy for the detection of such patients. A total of 913 hyperphenylalaninaemic patients have been studied and 15 tetrahydrobiopterin deficiences have been detected or confirmed.

DHPR assay in dried blood samples and pteridine measurement in urine collected on filter paper combine convenient sampling and reliable tests for systematic investigation of hyperphenylalaninaemic patients for cofactor deficiency.

INTRODUCTION

The efficiency of early detection and treatment of phenylketonuria (PKU) in preventing the serious sequelae of the disease has been largely documented. In the last 25 years, most of the developed countries have set up programmes to screen for the disease in the neonatal period. However, 15 years ago it was reported that some hyperphenylalaninaemic patients suffer from a different disease (Smith, 1974; Bartholomé, 1974) caused by deficient synthesis or regeneration of tetrahydrobiopterin, the cofactor of phenylalanine- tyrosine- and tryptophan-hydroxylase (Figure 1). Because these diseases (McKusick 26163, 26164) require a treatment different from that of PKU (McKusick 26160), efforts have been made to identify tetrahydrobiopterin deficiency in early life (Curtius *et al.*, 1979; Danks and Cotton, 1980; Kaufman, 1980; Dhondt, 1984).

Based on 15 years experience of differential diagnosis of such variants, we report here the changes in our strategy for the detection of tetrahydrobiopterin deficiency and the results obtained on a prospective and retrospective basis in hyperphenylalaninaemic patients, in order to estimate the incidence of the disease and its variants and to define the best and least invasive strategy.

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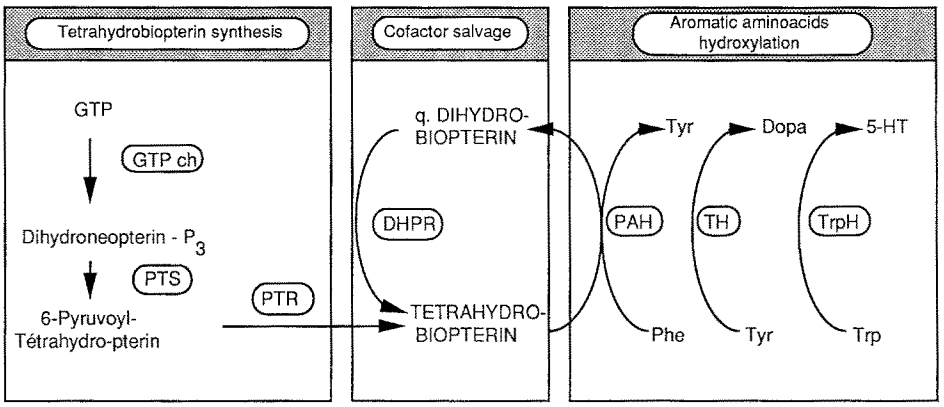

Figure 1 Enzymatic pathways involved in the synthesis and utilization of tetrahydrobiopterin. GTPch: GTP cyclohydrolase; PTS: pyruvoyl-tetrahydropterin synthase; PTR: pyruvoyl-tetrahydropterin reductase; DHPR: dihydropteridine reductase; PAH: phenylalanine hydroxylase; TH: tyrosine hydroxylase; TrpH: tryptophan hydroxylase

MATERIAL AND METHODS

Methods: Hepatic phenylalanine hydroxylase (PAH, EC 1.14.16.1) and dihydropteridine reductase (DHPR, EC 1.6.99.7) activities were measured according to methods previously described (Dhondt and Farriaux, 1981). Urinary pteridines were determined by HPLC chromatography (Dhondt *et al.*, 1981). When dried urine samples were used, a part of the filter paper soaked with urine (approximately 2 × 4 cm) was eluted in 5 ml of 0.05 mol/L HCl for 1 h at room temperature and then analysed as previously described (Dhondt *et al.*, 1981). DHPR in dried blood samples was measured by the method of Arai and colleagues (1982) with minor modifications, in particular the expression of activity per mg of haemoglobin.

Patients: A total of 913 patients (born in France, Belgium, Portugal and Czechoslovakia) have been investigated. Prospective screening concerned 440 hyperphenylalaninaemic babies on whom a systematic investigation of tetrahydrobiopterin metabolism was performed in the neonatal period. Retrospective investigations were performed in a cohort of 473 older hyperphenylalaninaemic or phenylketonuric patients. On some occasions, these investigations were performed because of the discovery of abnormal neurological symptoms or evidence of slow psychomotor development. For most of the other cases analyses were performed in order to establish the incidence of misdiagnosed patients.

RESULTS

Between 1972 and 1984, liver enzyme activities were measured in 61 hyperphenylalaninaemic patients (Figure 2) classified according to their dietary phenylalanine tolerance (Dhondt *et al.*, 1983). Although most typical PKU patients have PAH levels lower than 5% of the normal mean (72.8 nmol of tyrosine formed/h/mg protein

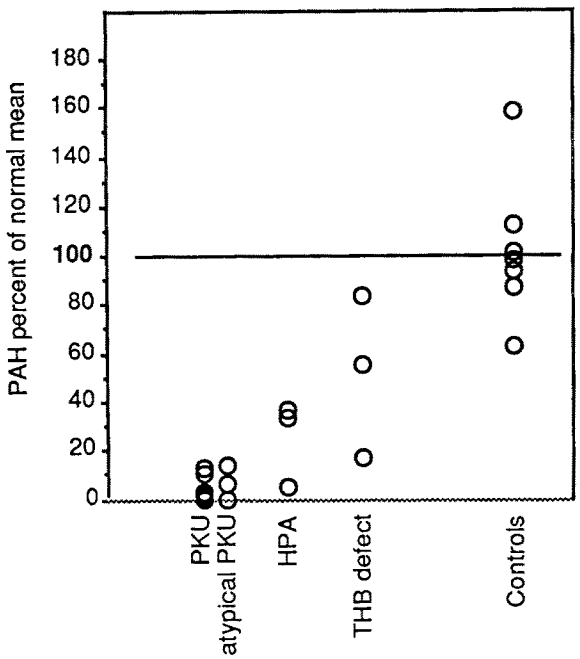

Figure 2 Hepatic phenylalanine hydroxylase (PAH) in controls and patients with hyperphenylalaninaemia

(Dhondt and Farriaux, 1981)), two have a significant residual activity (10 and 12%) despite having phenylalanine tolerances lower than 400 mg/d. Three patients with tetrahydrobiopterin deficiency, initially detected by urine pteridine measurement, were investigated. In one (patient AA, Table 1) a reduction in PAH activity (17%) was observed. No defect in DHPR activity was found during that period.

Our experience of pteridine determination in urine began in 1980; 907 hyperphenylalaninaemic patients have been checked using this procedure. Using frozen urine samples, the method failed to recognize two siblings with DHPR deficiency (patients AD and SD, Table 1). They presented a moderate elevation of blood phenylalanine at birth and the urine pteridine profile was within the normal limits for age. Tetrahydrobiopterin estimation, by differential oxidation procedure (Milstien *et al.*, 1980) was less than 10% of total biopterin, but at that time inadequate storage of the urine samples was suspected rather than a real lack of tetrahydrobiopterin, since the neopterin/biopterin (N/B), ratio was normal. The final diagnosis was established by liver enzyme measurement (B. Leroux, personal communication).

With dried urine samples, the distributions of values in either controls, PKU or known tetrahydrobiopterin deficient patients were similar to those obtained with frozen urine (Figure 3). In particular, a decrease in N/B ratio versus age and a positive correlation between biopterin excretion and blood phenylalanine levels in PKU patients was found as previously reported (Dhondt *et al.*, 1986a).

DHPR assay in dried blood samples was introduced to avoid missing DHPR deficiency by urinary pteridine measurement. No difference between controls and

Table 1 Patients with tetrahydrobiopterin deficiency

	Patient	*Origin*	*Age*	*Neonatal blood Phe* (mg/dl)	*Neopterin* (nmol/mmol	*Biopterin* creatinine[a])
PTS deficiency	AA	caucasian	5 mon	20	26 468	< 50
	NP	caucasian	7 1/2 mon	35	7581	97
	DK	caucasian	13 y	4–6	12 248	140
	OM	caucasian	3 weeks	10	25 872	< 50
	DK	Algerian	2 mon	8	38 436	39
(Atypical form)	TH	caucasian	2 mon	20	10 650	71
	TS	caucasian	1 mon	7	16 040	170
	KN	Algerian	1 mon	6	20 160	91
DHPR deficiency	AD	African	15 days	8	9610	4307
	SD	African	21 days	7	15 564	4571
	ID	African	5 days	9	10 237	4521
GTP-cyclohydrolase deficiency	JWS	La Réunion	1 mon	13	215	70
	WS	La Réunion	1 day[b]	5	256	56
			5 mon	15	124	41
Primapterinuria	PP	caucasian	3 weeks	positive 21 (3 weeks)	18 670	868
	EM	Algerian	2 mon	< 3[c]	34 807	1902

[a] See reference (Dhondt *et al.*, 1989) for normal values according to age
[b] Antenatal diagnosis (Dhondt *et al.*, 1990)
[c] Blood phenylalanine controlled because a brother had presented mild hyperphenylalaninaemia in the neonatal period

hyperphenylalaninaemic patients and no variation of activity vs. blood phenylalanine levels were observed. However, a significant negative correlation of enzyme activity with age was observed: DHPR (nmol ferrocytochrome C/min/mg haemoglobin) = -0.14 log [age(days)] + 3.48, $r = 0.447$, $n = 300$. Zero activity in samples from three known DHPR deficient patients confirmed the efficiency of the method. One patient, a sibling of a known DHPR deficient patient, was confirmed to have DHPR deficiency (patient ID). 18 out of 550 (3.3%) hyperphenylalaninaemic patients had DHPR values in the heterozygote range ($\leq$ 50% of normal mean value for age). Six families were investigated; the results are presented in Figure 4. In one family (Figure 4, family Sc.), because a girl had previously died with a clinical picture which might retrospectively have been due to 'malignant PKU', a sister presenting with severe retardation but without hyperphenylalaninaemia was investigated. The urinary profile of pteridines was normal but DHPR activity was 27% of the normal. Investigation of the whole family found a zero or nearly zero activity in the mother and a child, who are both clinically normal. Their urinary pteridine profiles were also normal.

A total of 15 tetrahydrobiopterin deficient patients were studied (Table 1). Among the 913 hyperphenylalaninaemic patients, 546 were from three different areas of Europe: the North of France (145), Czechoslovakia (349) and Portugal (52). This epidemiological survey resulted from the collaboration of six screening centres

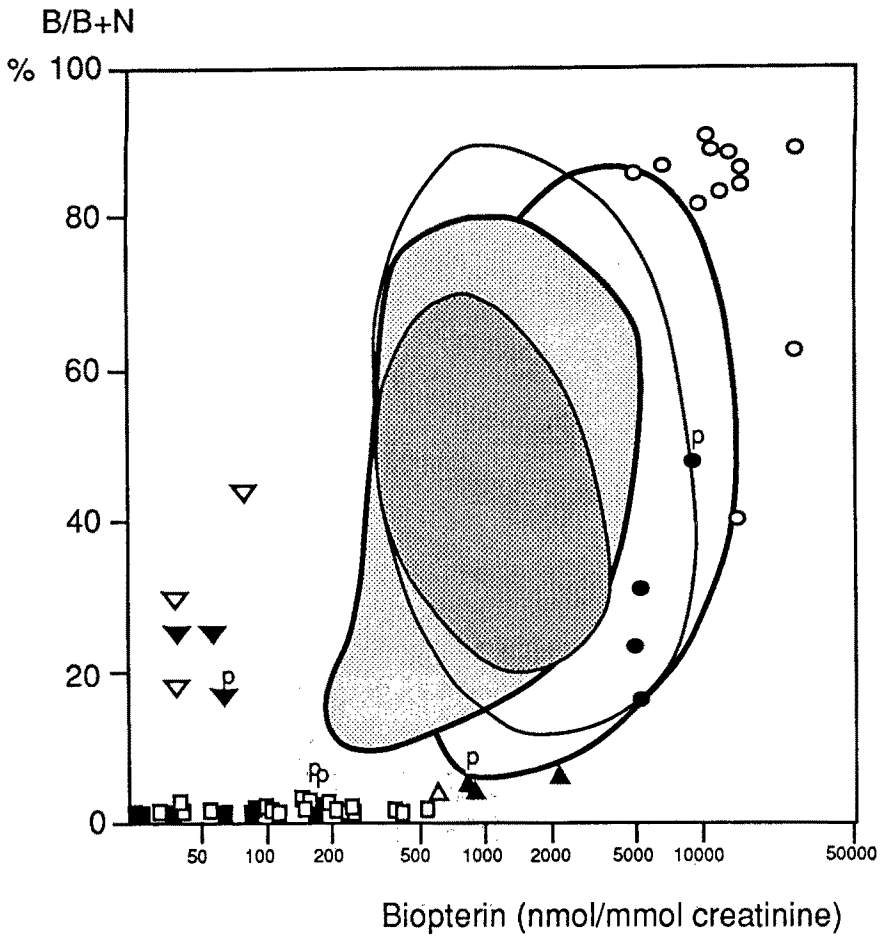

Figure 3 Distribution of percentage of biopterin versus biopterin excretion
Area of values from normal subjects ($n = 569$) and from PKU patients, with liquid urine samples
Area of values from normal subjects ($n = 34$) and from PKU patients, with urine samples collected on filter paper
▼ ▽ GTP cyclohydrolase deficiency
■ □ PTS deficiency
● ○ DHPR deficiency
▲ △ Primapterinuria
Closed symbols represent data of patients described in Table 1, open symbols data from the literature
[p] Indexed symbols represent results obtained with urine collected on filter paper
B: Biopterin; N: Neopterin

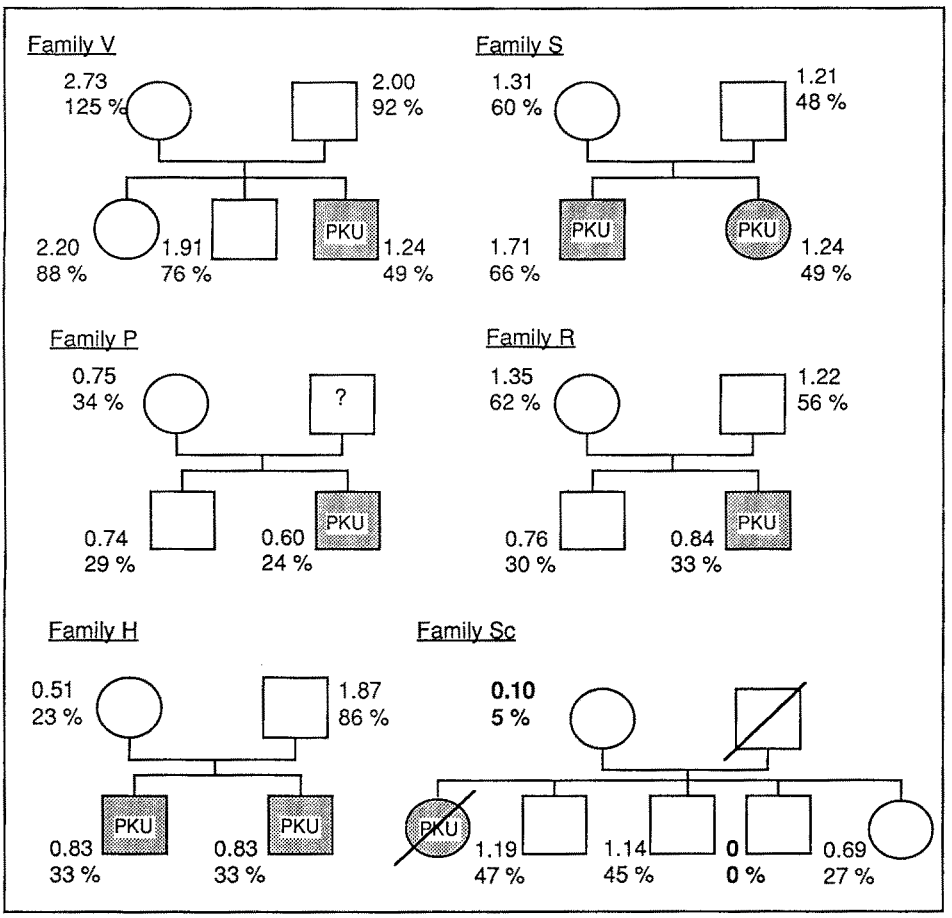

Figure 4 Pedigree and DHRP activity in six families where children were found to have reduced blood DHPR activity.
DHPR activities are expressed in nmol ferrocytochrome C/min/mg haemoglobin and in percentage of mean normal activity for age, at the side of the individual's symbol in the pedigree

(Lille, Prague, Brno, Bratislava, Porto, Lisbon). The percentage of investigated or reinvestigated hyperphenylalaninaemic patients varied from 90 to 95%. Out of these 546 cases, only one patient (DK) was already known to have a tetrahydrobiopterin synthesis deficiency; no other cases were detected.

DISCUSSION

Since the first description of tetrahydrobiopterin deficiency, the number of cases has grown. So far three defects have been identified: GTP cyclohydrolase (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTS) and dihydropteridine reductase deficiences,

which are responsible for hyperphenylalaninaemia with progressive neurological disorders (Scriver *et al.*, 1989). Because of their particular prognosis and their specific treatment, it was important to set up methods for systematic investigation of tetrahydrobiopterin metabolism in hyperphenylalaninaemic babies. In the late 1970s, the only way to identify tetrahydrobiopterin deficient patients was to verify that the activity of hepatic PAH apoenzyme was normal. The tetrahydrobiopterin loading test was then introduced. The test is based on the capacity of tetrahydrobiopterin to restore the *in vivo* PAH activity which results in a rapid decrease in the blood phenylalanine levels in tetrahydrobiopterin deficient patients. In the early 1980s, pteridine measurement in biological fluids was introduced as a way to detect and characterize the enzyme defect.

Systematic liver biopsy was abandoned because it was found to be too invasive and poorly informative for PKU and HPA evaluation (Dhondt *et al.*, 1983; 1986b). In addition, the anecdote of a reduction in *in vitro* phenylalanine hydroxylase activity in a case of PTS deficiency, which was attributed to a lack of *in vivo* stability of the apoenzyme in the absence of its cofactor (S. Kaufman, personal communication), demonstrated the risk of misdiagnosis.

The introduction of urinary pteridine measurement was theoretically aimed to explore all known defects in tetrahydrobiopterin metabolism. It rapidly appeared that important age-dependent changes in urinary excretion of pteridines occur in childhood, mainly in the neonatal period (Dhondt *et al.*, 1986a). In addition, in PKU subjects, pteridine excretion depends on the blood phenylalanine level at the time of urine collection. While these variations do not alter the diagnostic power in typical forms of tetrahydrobiopterin deficiency, they are to be taken into account in the recognition of some mild variants. The estimation of the percentage of biopterin as tetrahydrobiopterin has been proposed as a way to recognize DHPR deficiency (Milstien *et al.*, 1980). In our hands, the differential oxidation method found the percentage of tetrahydrobiopterin to be lower than 20% in a great number of urine samples obtained during the neonatal period. This observation was attributed to the lability of tetrahydrobiopterin and inappropriate storage conditions. However, with such an explanation in mind and because the N/B ratio was normal, two cases of DHPR deficiency were missed despite a low percentage of tetrahydrobiopterin. Such a false negative interpretation of the N/B ratio in the neonatal period, also pointed out by Matalon (1984), led us to reconsider our strategy by including systematic measurement of DHPR in red blood cells according to the method of Arai and colleagues (1982). Minor adaptation of the method was carried out to increase its sensitivity, and results were expressed per mg of haemoglobin to overcome differences in haematocrit between newborns and older subjects. However, a progressive decrease in DHPR activity with age was observed, confirming results recently reported by Surplice and colleagues (1990). 3% of PKU patients were found to have DHPR activity in the heterozygote range, a figure also reported by Sahota and colleagues (1985) in a survey of normal neonates. In 4 out of 5 families presently studied (Figure 4), the pedigree agrees with a heterozygote trait, but these low DHPR values can also belong to the lowest part of a normal distribution. Shaota and colleagues (1986) and Armstrong and colleagues (1986) have previously suggested that a low DHPR

activity would predispose towards mental retardation. However, we did not find a correlation between DHPR activity and IQ in the PKU patients presently investigated (results not shown). Although blood DHPR measurement seems to be efficient for screening DHPR deficiency, the finding in a family of subjects with zero activity represents to our knowledge the first report of a 'false positive' result of enzymatic test. Unfortunately, this family refused further investigations aimed at defining the reason for this finding.

The last modification in the strategy was the use of dried urine samples for the pteridine measurement. For a long time, pteridines in body fluids were believed to be not stable enough to allow a mode of storage other than acidification or ascorbate protection and immediate freezing. Narisawa and colleagues (1983) first showed that urine collection on filter paper can be a suitable method and Naylor and collegues (1989) recently reported on its routine use. Our results were expressed relative to creatinine excretion to obtain a precise estimation of urine levels of biopterin, since the N/B ratio can easily be altered in the neonatal period or by intercurrent immunological diseases (Dhondt *et al.*, 1989). The results obtained from normal, PKU, and patients with PTS deficiency were in the same range as those observed with frozen urine.

A total of 15 patients have been studied during the period of the present study (Table 1). Among the eight cases with a urinary profile of PTS deficiency, three were subsequently classified as an atypical form of the disease, two in regard to the absence of the appearance of neurological signs after treatment withdrawal, and one ('peripheral' form) in regard to a normal profile of pteridines and neurotransmitter catabolites in CSF. Two other cases were found to have a 'primapterinuria', a new form of biopterin deficiency (Blau *et al.*, 1988; Dhondt *et al.*, 1988) characterized by the presence of 7-biopterin (primapterin) which is excreted in equal amounts to biopterin. Although the hyperphenylalaninaemia was moderate, the clinical survey showed that the patient was liable to episodic elevation of blood phenylalanine (Dhondt *et al.*, 1988).

The incidence of tetrahydrobiopterin deficiency appears to be very low in caucasian populations, in contrast to the higher incidence previously reported in the Chinese (Hsiao *et al.*, 1990) or Arabic populations (Ozand *et al.*, 1989) for PTS deficiency and in the Italian population for DHPR deficiency (Ponzone *et al.*, 1984). However, a precise estimation of the incidence of these diseases is difficult because in most samples a bias was introduced by investigation of patients having clinical problems. On the other hand, it has been speculated that some transient or moderate hyperphenylalaninaemia could be due to milder forms of cofactor deficiency. The only way to get precise information on the incidence of these diseases was to reinvestigate systematically the hyperphenylalaninaemic subjects. The incidence of tetrahydrobiopterin deficiency could be estimated as between 1/546 (0.2%), if only the 'epidemiological' survey is considered, and 15/913 (1.6%), if the whole group of patients is taken into account. This latter value may be either underestimated (two cases of DHPR deficiency exist in Portugal (Tavares de Almeida *et al.*, 1990), and one PTS deficiency in Czechoslovakia (Niederwieser *et al.*, 1987)), or overestimated since eight patients are of non-caucasian origin (three related DHPR deficient patients

are African, two related patients with GTP-cyclohydrolase deficiency are from La Réunion Island (a genetic isolate), and two cases with PTS deficiency and one of primapterinuria are from Algeria).

CONCLUSION

The strategy used to recognize tetrahydrobiopterin deficiency in the cohort of hyperphenylalaninaemic patients must commend itself by its convenience and simplicity. The measurement of pteridines in urine collected on filter paper discriminates tetrahydrobiopterin synthesis deficiency (GTP cyclohydrolase and PTS deficiency) from normal and PKU children. The direct DHPR assay on dried blood samples allows recognition of DHPR deficiency.

Both these assays can be proposed for easy use on all babies with hyperphenylalaninaemia screened in the neonatal period, whatever their blood phenylalanine level. In the case of abnormal or borderline results, conventional sampling remains indicated before the performing of more invasive investigations to prove and characterize a defect in tetrahydrobiopterin metabolism. In addition, this strategy appears to be well adapted to epidemiological study.

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J. Inher. Metab. Dis. 14 (1991) 127

BOOK REVIEW

Inborn Metabolic Disease: Diagnosis and Treatment. Edited by J. Fernandes, J.-M. Saudubray and K. Tada. Springer Verlag, Berlin, 1990, ISBN 3-540-50951-8, 730 pp., DM298.

Many medical texts are constructed wrongly for diagnostic use because you must known the diagnosis in a problem patient before you can use the text book. This book was designed to provide diagnostic and then therapeutic help in conditions which are usually too rare for medical apprenticeship to cover.

The contents include: Part I, a clinical approach, by Saudubray which is again refreshing. Part II, diagnostic procedures, starts well but is short and difficult to link to subsequent chapters.

Parts III–XV are standard chapters on listed conditions by a wide range of experts. A Section XVI on new trends in treatment is followed by a Section XVII on prenatal diagnosis and another XVIII on neuropsychiatric and psychosocial issues. The short subject index requires a definitive diagnosis for use. I found incidence/prevalence figures and diagnostic schemes difficult to locate despite their practical values. Only some authors have marshalled evidence on presentation clinically and biochemically. Such lapses are understandable by the frequent failure of the scientific literature to provide data on clinical and laboratory presentation and key steps in making diagnoses.

This journal and the SSIEM Council are trying to stimulate discussion on a minimum data set for a possible register on inherited metabolic diseases which is mentioned in the preface to this book. There is a difficult job selecting diagnostically *important* data from other data but such diagnostic data could be provided in order to generate a factual basis for schemes of diagnosis, especially those suitable for primary care physicians and non-specialized laboratory workers.

This edition will be used by specialists; it should be available to them for reference.

R. A. Harkness
J. W. T. Seakins